

In vitro studies on the differentiation potential of human periodontal ligament-derived progenitor cells for developing functional periodontal ligament 3D cell culture models: A systematic review of methods and approaches

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Abstract

Background: The successful translation of periodontal regeneration therapies from laboratory to clinic relies on physiologically relevant preclinical models. 3D periodontal ligament (PDL) cell culture is a transformative technology in preclinical dental research, which serves as a link between laboratory experiments and human trials. PDL plays a central role in tooth anchorage, alveolar bone stability, and orthodontic tooth movement (OTM) due to the self-renewal ability of PDL-derived progenitor cells. Its regenerative capacity is often compromised by periodontitis or mechanical stress. PDL-derived progenitor cells are characterized by their self-renewal and multilineage differentiation. They are the primary drivers of tissue repair. However, the complexity of the PDL role in tooth regeneration necessitates advanced 3D modelling to accurately simulate in vivo responses.

This review aims to summarize all previously described 3D PDL cell culture models and systematically analyze them with respect to their structure, cell composition, expression of PDL marker genes, and applications.

Methods: PubMed, Scopus, Web of Science (WoS) and Google Scholar databases were searched for studies published from 2000 to 2026 by two researchers. Study selection, data extraction, and risk of bias assessment were performed independently by three reviewers following PICO criteria. Only studies published in English were included.

Results: Fifty-eight in vitro studies of initially 1095 papers met our inclusion criteria and were included in this systematic review. These in vitro studies demonstrated that PDL-derived progenitor cells can differentiate into specialized dental cells within different 3D cell models, as verified by lineages specific markers and histological examination of their structure and mineralization. The use of 3D cell models in various functional applications was also analyzed.

Conclusions: Under certain conditions, 3D PDL cell models can mimic native PDL tissue, making them a promising option for regenerative dentistry and for studying OTM, bone remodeling, and other functional aspects. However, the review results confirmed substantial heterogeneity in experimental conditions for 3D PDL cell culture manufacturing, complicating the comparability of PDL models and rendering a quantitative analysis impossible. More studies on this subject, along with consistency in experimental approaches, are needed to ensure comparability and quality control.

1. Introduction

The periodontal ligament (PDL) is a specialized, highly organized connective tissue that serves as the critical interface between the dental root and the alveolar bone (1). Its complex structure ensures the teeth are firmly anchored in the jawbone, limiting their range of movement. Beyond its role as a physical anchor, the PDL serves as a source of progenitor cells exhibiting mesenchymal stem cell characteristics and regenerative capacity, which help maintain tooth vitality and respond to mechanical stimuli (2–4). Periodontal diseases and traumatic injuries often lead to irreversible PDL destruction and tooth loss (1).

Conventional surgery usually results in tissue reattachment via a long junctional epithelium, which does not mean “true” regeneration, because a new tissue fails to recapitulate the intricate, oriented fiber architecture necessary for functional restoration (5). Over the last decade, the field of regenerative dentistry has advanced significantly. Many approaches for PDL regeneration have been developed and tested *in vivo* on animal models (6–11). New clinical studies on patients were performed (12–17). Although there remains a fundamental question about how to regenerate fully functional periodontal tissue, this question requires extensive study. The implementation of 3D cell cultures has enhanced research in this field. Experimental models, in which PDLF cells were grown and examined in 3D, have already shown promising results (18).

The predominant cell type in the PDL is fibroblasts (PDLF). They are a heterogeneous population with different phenotypes, spatial locations, and functional roles (19, 20). Among them are PDL-derived progenitor cells, which belong to the mesenchymal cell family and are commonly referred to as periodontal ligament stem cells (PDLSC) or PDL progenitor cells (21–24). These cells are essential for PDL regeneration (23–27). They contribute to PDL regeneration by producing growth factors and cytokines that can influence the behaviour of other cell types such as osteoblasts and osteoclasts, ultimately affecting bone formation and resorption (2, 4). PDL-derived progenitor cells have the potential to differentiate into various cell types and synthesize collagen fibers, thus contributing to the reorganization of the surrounding tissue (28).

3D cell culture models can be broadly classified into scaffold-based and scaffold-free, each with distinct advantages and applications. Scaffold-based models use materials such as hydrogels or other matrices for cell embedding, whereas scaffold-free models rely on cell aggregation and self-assembly. Specific models, such as spheroids, organoids, and microfluidic systems, are commonly used within these categories (29, 30). In our review, we adhere to the 3D cell culture nomenclature provided by the authors of the original studies.

Compared with conventional 2D cell cultures, 3D cell culture models offer an advanced means of recapitulating the physiological conditions of tissues/organs. Due to the spatial organization of cells within a 3D structure, interactions between cells and their extracellular matrix (ECM) can be represented more physiologically. It is also possible to study the co-culture of 3D cellular models with different cell types (31). The differentiation potential of PDL-derived progenitor cells, which is of great importance for regenerating the periodontium, has been extensively investigated in numerous studies in various 3D cell culture models. In addition, various growth factors, biomaterials, and mechanical stimuli have influenced the differentiation behavior of PDL-derived progenitor cells in these models (32). The potential of PDL cell organoids to benefit the regeneration of the surrounding tissue can, therefore, be influenced by various factors. This provides opportunities to uncover mechanistic insights and opens promising avenues for developing novel therapies for periodontal regeneration.

To provide an overview of research developments uncovering the differentiation potential of PDL-derived progenitor cells in 3D cell culture models, which might significantly contribute to periodontal

regeneration and to the application of these cellular models in clinical practice, we present a systematic review of the existing literature and summarize findings on 3D PDL modeling.

To identify applicable 3D models for orthodontic tooth movement (OTM), periodontal disease, and bone remodeling investigations, this review focuses on the primary comparison and evaluation criteria for 3D cell culture models developed from human progenitor PDL cells, including structural tissue evaluation using microscopic and histological methods, as well as the expression of PDL gene and protein markers.

2. Methods

2.1. *Focused question.*

The focused question for our research is: how do various 3D scaffolding and culture techniques compare in their ability to promote the multilineage differentiation potential of human PDL-derived progenitor cells when developing biomimetic periodontal ligament models?

The study was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. To identify the scope of this review, we defined the following PICOS:

(P) Participants. Human PDL-derived progenitor cells.

(I) Intervention. 3D cell culture techniques.

(C) Comparison. We compare criteria for 3D PDL cell models.

(O) Outcomes. 3D cellular models were developed to mimic a tissue of interest. However, they may not completely recapitulate the structure and physiology of the corresponding tissue. 3D models, which are built of cells derived from a specific tissue, ideally should resemble native tissue in structure and protein expression profile according to the 3D model assessment criteria. Thus, as outcomes, we set a resemblance to native PDL tissue assessed by (1) microscopic and histological methods and (2) expression of gene and/or protein PDL tissue markers.

(S) Study type: *In vitro* experiments.

2.2. *Database search strategy.*

Two independent authors (OV and IP) searched the PubMed, Scopus, Web of Science (WoS), and Google Scholar databases for articles. The search was conducted using the keywords listed in Table 1, combined with the Boolean operators AND, OR, NOT, or | sign. The restriction was set to Title/Abstract (PubMed), Title/Abstract/Keywords (Scopus), Topic (WoS), and full text (Google Scholar). The publication date filter was set to include papers published from 2000 to 2026 for each search round. All found literature sources were collected to form a Reference Management Software EndNote 21 library. Duplicates were excluded. Papers not relevant to the topic of this systematic review, papers published

not in English, or with no full text available, were also excluded from further assessment. Seven papers were added using hand-mode search.

2.3. Study selection and data extraction strategy.

During the initial screening step, papers were subjected to abstract screening, and off-topic articles were filtered out. The studies collected after the screening step were subjected to detailed analysis. In this step, we applied assessment criteria. Criteria for inclusion of papers into further analysis were: (1in) *in vitro* studies on 3D model constructs made of human PDL progenitor cells built on a scaffold-based system; or (2in) *in vitro* studies on 3D model constructs made of human PDL progenitor cells built with a scaffold-free system; and always (3in) experimental peer-reviewed articles. Exclusion criteria were: (1ex) studies without precise structural analysis (e.g., transmission electron microscopy (TEM) or scanning electron microscopy (SEM) or histological staining (e.g., hematoxylin and eosin (H&E), Masson's trichrome, Richardson or Picrosirius Red staining for collagen, Periodic Acid Schiff (PAS) staining for polysaccharides); (2ex) studies without protein or transcript markers assays (polymerase chain reaction (PCR), immunofluorescence (IF), immunohistochemistry (IHC), enzyme linked immunosorbent assay (ELISA), western blot (WB), or analytical kits for specific proteins); (3ex) other than periodontal types of differentiation (neuronal, adipose, chondrogenic etc.); (4ex) studies on oral cancer 3D models; (5ex) *in vivo* studies; (6ex) studies on non-human PDL cells.

Three independent reviewers then evaluated the full texts of the selected publications to confirm they matched the eligibility criteria. Individual outputs were recorded in the summary table (Table 2). All references were further grouped by the 3D culture model type. Different approaches to 3D culture were characterized: self-assembling, cell micro-mass culture, cell sheets, bioprinting, and scaffold-based cultures. Data of structural characterization, either by histological staining (H&E, Masson's trichrome, Richardson or Picrosirius Red staining for collagen, PAS staining for polysaccharides) or spectroscopic analysis, were analyzed and extracted into a summary Table (Tab. 2). The culture duration and differentiation medium used in each study were determined. Further, marker expression of specific transcripts/proteins in 3D models was characterized. For this purpose, data from IF, IHC, PCR, ELISA, or WB, reported in selected papers, were analyzed. If any selected paper contained, besides *in vitro* studies, a part with *in vivo* studies, this part was not included in any of the analyses mentioned above. Furthermore, papers were analyzed with respect to the functional characterization of 3D constructs. They were grouped into separate topics: (1) mechanical loading that mimics tooth movement, (2) immune response to oral infections, (3) effects of hypoxia, and (4) involvement of PDL cells in vascularization processes. Quantitative analysis was not performed in this review due to the wide variety of conditions used to produce 3D cell constructs, which makes quantitative comparison infeasible.

2.4. Outcome measures.

The primary outcomes were (1) structural recapitulation of the native PDL tissue that was assayed by means of histological staining (Masson Goldner, H&E, Picrosirius Red staining, etc.) and/or spectroscopic studies, as well as (2) expression of protein and/or transcript markers inherent for PDL

tissue that was assayed by means of any of the following techniques: IF, IHC, PCR, ELISA, WB, or kits for specific protein detection.

2.5 Risk of Bias Assessment.

A risk of bias assessment was conducted to ensure the credibility of papers selected for the review. There are no approved tools for evaluating studies based on 3D cell culture technologies. Bias assessment tools designed for *in vivo* or *in vitro* studies, such as the National Toxicology Program OHAT risk of bias tool for human and animal studies (33), are not fully applicable to our review, given its aim and framework. Thus, we used a quality assessment tool, adapted from the CONSORT guidelines for *in vitro* experiments, improved and implemented by Ramamoorthi et al. and AlFatlawi et al. (34, 35).

The evaluation was based on a question grading scheme (Table 3) that covers the main parts of articles and addresses reproducibility and validity. The risk of bias assessment was conducted only for the sections of each study that were relevant to the review. In studies that included additional experiments not eligible for inclusion, the risk of bias assessment for these experiments was not considered.

3. Results

3.1 Study selection and characterization.

A list of 1095 papers was formed by an initial search of four databases by two researchers through an initial search of four databases: PubMed (n = 56), Web of Science (n = 352), Scopus (n = 173), and Google Scholar (n = 507), and seven papers were found through a hand-search. Figure 1 shows the PRISMA flowchart for the selection process. 332 papers appeared in more than one database. One version was selected for each of these duplicated references, while the other(s) were counted as duplicates and excluded. Furthermore, after screening titles and abstracts, 371 research papers were found out of the scope of the review (*in vivo* studies, studies done on other than PDL cells, or studies done on PDL cells but with other than the periodontal type of differentiation (neuronal, epithelial, or chondrogenic, etc.) and these papers were excluded as well. 392 papers were subjected to detailed full-text analysis, after which 334 papers were excluded, and 58 papers passed all eligibility criteria and were further considered in this systematic review.

3.2 Quality of studies.

We conducted a risk-of-bias assessment to verify the credibility of the studies included in the review. Before analysis, all references that met the eligibility criteria were checked according to the checklist provided in Table 3. In the analysis of methods, only 11 references showed no risk of bias (36–46). 39 articles showed a medium risk of bias because of minor imperfections (e.g., not concise title, not clearly stated objectives etc.), and 10 articles showed high risk of bias in the methodological part because of shortcomings in statistical data processing or the failure to provide detailed and precise descriptions of research methods (47–56). 19 papers did not provide a statement of conflicts of interest (48, 50, 54, 55,

57–71), and 2 papers (49, 71) were published in a journal with an impact factor of less than 1. The results of the risk-of-bias assessment are provided in Table 4.

3.3 Characterization of 3D PDL cell culture models.

101 3D PDL-derived cell models were described in the 58 papers included in the analysis. Sixteen papers employed scaffold-free 3D cell culture models (43, 48, 50, 53, 54, 58, 64, 65, 70–77). In 3 studies (54, 62, 74), 3D PDL cell culture models were grown in a bioreactor. Vurat et al. (78) printed PDL spheroids using a pneumatic extrusion bioprinter Incredible+ (Cellink). In another study (51), 3D PDL cell constructs were grown in culture chambers designed to mimic OTM. In the study by Ono et. al. (70), bioprinted tubular structures were fabricated from spheroids using a 3D printer with hydroxyapatite (HA) or titanium particles incorporated. Sano et al. used a net mould device (NM14-2, TissueByNet, Tokyo, Japan) to manufacture PDL spheroid blocks (77). In 7 studies (43, 48, 53, 58, 72, 73, 76), 3D cell models were constructed from PDL cell sheets rolled into 3D tissue-like structures. Singhatanadgit et al. (73) and Yang et al. (43) developed 3D models from cell micro-mass culture. In four papers (50, 64, 65, 71), 3D cell models were developed by self-aggregation of cells into spheroids. Pandula et al. used multilayered cell constructs manufactured from human periodontal ligament stem cells (hPDLSC) and human umbilical vein endothelial cells (HUVEC) sheets (72). In forty-two papers, 3D PDL cell constructs were based on different types of scaffolds, either commercially available (59, 69, 79, 80) or manually crafted (36–42, 44–47, 49, 51, 52, 55–57, 60–63, 66–68, 75, 78, 81–93). Granules made of HA Endobon® Xenograft (59), human spongiosa scaffolds Tutoplast® (Tutogen Medical GmbH, Neunkirchen am Brand, Germany) (80), collagenated block constituted by natural cancellous and cortical porcine bone OsteoBiol® dual-block (Tecnoss® Dental, Coazze TO, Italy) (69), collagen membrane obtained from bovine pericardium BioRipar® (Assut Europe, Italy) (79), were used as commercial scaffolds for 3D cell cultures in selected papers. Among the various types of handmade scaffolds, collagen-based ones were the most common and employed alone or in combination with other compounds in 14 papers (36, 37, 39, 41, 42, 49, 51, 57, 68, 69, 82, 83, 85, 88). Other natural scaffolds used are alginate-based hydrogel (47, 82, 85, 92), gelatin (42, 46, 78, 82, 92), chitosan (62, 90), fibronectin (FN)-based and hyaluronic acid-based matrix (37), fibrin-based hydrogel matrix (40), and agarose (55). Three studies used scaffolds derived from decellularized tissues: a decellularized pulp matrix (37), a decellularized PDL matrix (86), and a combined decellularized pulp- and PDL matrix (36). Dentin slices (49, 83, 91) and decellularized dermal matrix (87) were also used to develop 3D cell culture models. Synthetic hydrogels employed for the development of 3D PDL cell constructs included polycaprolactone (PCL) (42, 52, 81, 83, 84, 86, 89, 91), polyglycolic acid (PGA) (49), poly(lactic-co-glycolic) acid (PLGA) (38, 44, 56, 60, 61, 63, 66, 83, 93), poly-L-Lactic Acid (PLLA) (45, 67).

In 10 of 58 papers, commercially available hPDL cells were used for 3D cell culture work (45, 49, 50, 57, 67, 70, 74, 78, 82, 85). In the other 48 studies, hPDL cells were isolated from dental patients' PDL tissue (see Table 2). Both primary donor cells obtained from patients or commercial cell lines are suitable for *in vitro* cell culture applications according to principles of good laboratory practice. Cells must be characterized by their morphology and should be free from contamination with other cell types, viruses,

or mycoplasma to ensure feasible and reproducible results (94). Though testing primary human cells against pathogens was not mentioned in any articles that employed them. Another critical parameter in cell culture work, particularly primary cell culture, is the passage number. Only 46 of 58 papers mentioned the passage number used in the experiment (see Table 2). The median passage number for primary cells used in manufacturing 3D cell models was 6, with a range between 2 and 10. Another parameter that was analyzed was the age of the tooth donors. Donor age has been shown to affect the differentiation and proliferation potential of stem cells, similar to the passage number (95). The median age of the donors in these studies was 23 years, with a range of 10 to 47 years. The donor's age was not mentioned in 21 papers. In 6 of 58 papers, hPDL cells were co-cultured with various types of dental and non-dental cells, including dental pulp fibroblasts (53), periosteum cells isolated from alveolar bone (36), oral epithelial cells (68), HUVECs (72), bone marrow-derived mesenchymal stem cells (JBMMSCs) (75), and human mesenchymal stem cells (hMSCs) (41).

3.4. Cell viability and proliferation.

Cell viability and proliferation were tested and reported in 34 papers: (38, 41, 42, 45–47, 50, 53, 54, 56, 57, 59–62, 65–67, 70–72, 76–79, 81, 82, 84–87, 89, 90, 92). Scaffolds used in several studies (39, 42, 46, 49, 51, 57, 59, 60, 62, 67, 70, 81, 82, 85, 86, 89, 92) had no or minor effects on cell viability and proliferation. Ge et al. (87) reported that a scaffold based on a decellularized dermal matrix combined with collagen provides better cell viability when hydroxyapatite particles are incorporated, as assessed after 3 days of culture. Manescu et al. (69) reported that cells seeded on a natural scaffold composed of bovine cancellous and cortical bone had lower viability and proliferation rates than the control group at all examined time points up to 7 days. Morgante et al. reported that a scaffold based on a bovine pericardium membrane stimulated the proliferation of PDLSC after one week in culture (79). Similarly, Farag et al. reported that a complex PCL/decellularized hPDL substrate supported cell proliferation compared with the PCL scaffold alone when tested up to 21 days (86). Tian et al. reported that cell viability in a 3D model built in a mixed gelatin/PCL scaffold was significantly higher than in a collagen scaffold when examined up to 14 days (42).

Cell proliferation in scaffold-free 3D cell constructs was assessed in (50, 54, 71, 72). Hoz et al. reported an increased PDL cell proliferation in 3D cell culture supplemented with cementum protein 1 (CEMP1) after 4 days in culture (54). Singhatanadgit et al. (71) reported an increased metabolic activity in 3D cell constructs cultured in osteogenic medium for 7 days, while viability was not changed significantly compared to 2D cell culture. Berahim et al. (50) demonstrated a decrease in cell numbers in spheroids after 14 days in culture. Pandula et al. (72) examined cell viability in 3D models constructed from cell sheets developed from either monocultured PDLSC or co-cultured with HUVEC and reported a slightly higher number of dead cells in the 3D models made from monocultured PDLSC sheets compared to the 3D model developed from sheets of PDLSC co-cultured with HUVEC.

3.5. Histological examination of 3D constructs' morphology.

Histological examination of the morphology of 3D cell models was performed in 33 studies. To examine the morphology of 3D constructs, H&E staining (36, 37, 40, 43, 48–50, 53–58, 63–65, 68, 70–72, 75–77, 82, 83, 88, 90, 91), Masson's trichrome staining in different modifications (41, 70, 74, 82, 83, 88), Richardson and Picrosirius red staining for collagen (51, 67, 70, 82), and PAS or Alcian blue staining for polysaccharides were employed (54, 78, 82). Of all the included papers, only two (50, 68) provided a biopsy of the native dental tissue, which included the PDL, gingiva, and alveolar bone, as a control.

3.5.1. Morphology of 3D scaffold-free constructs.

Two morphologically distinct areas were reported in (48, 50, 53): the central area was densely packed with round cells, and the periphery was composed of cells elongated along the longitudinal axis of the construct. Moreover, Berahim et al. reported cells in the core of spheroids have a round shape (50). Chu et al. (58) employed H&E staining for a comparative examination of organoids derived from primary dental follicle cells (DFCs) and the human PDL-hTERT immortal cell line. The authors showed that DFCs develop 3D structures with well-aligned cell rows. At the same time, organoids developed from immortalized PDL cells showed a more disorganized matrix with rows consisting of multi-cell arrays. Pandula et al. (72) employed H&E staining in their study on vascularization in 3D PDLSC constructs to postulate the formation of rudimentary vessel-like structures when PDL cells were co-cultured with HUVECs. Bone-like structures formed in PDLSC spheroids were detected by H&E staining and confirmed by Alizarin Red (AR) staining (71). Furthermore, a well-organized, differentiated tissue-like structure with stratified cells surrounded by ECM was observed using H&E (54).

3.5.2. Morphology of 3D scaffold-based constructs.

Well-ordered collagen fiber distribution, detected by Masson's trichrome and H&E staining, was reported in twelve studies (36, 41, 51, 56, 67, 76, 78, 82, 83, 88, 90, 91). Staples et al. and Lee Chang et al. reported PDL-like tissue formation, comprising of well-ordered collagen fiber-like structures reminiscent of the native PDL (88, 91). Proksch et al reported an abandoned collagen fiber distribution, detected by aniline blue staining, with more significant collagen staining in 3D hPDL cell constructs co-cultured with hMSC compared to a monoculture 3D hPDL cell construct (41). Several studies reported new tissue formation with morphologically distinct cell types (36, 51, 78, 83, 88, 90). Uniform cell distribution within a 3D cell structure with no evident differentiation into layers was reported in six papers (40, 49, 55, 63, 67).

3.6. Morphological analysis of 3D cell constructs using spectroscopic techniques.

The morphology of 3D models was characterized using various spectroscopic techniques: scanning electron microscopy (SEM), reflection electron microscopy (REM), transmission electron microscopy (TEM), and vibrating-sample magnetometry (VSM). SEM is the most common approach for structural examination. Data acquired by SEM are presented in 34 papers of the 58 publications (38, 39, 42, 44–47, 50, 52, 55–57, 59–62, 66, 67, 69, 73, 75, 76, 79, 81, 82, 84–87, 89–93). Five papers (39, 55, 69, 78, 80) utilized REM, TEM, or a combination of both, while only one study examined structures using VSM (78).

Seven papers employed a spectroscopic examination in combination with histological staining (H&E, Masson's trichrome, or Picrosirius red), as this combined approach can provide more detailed information about the 3D cell construct morphology (48, 50, 53, 56, 57, 67, 82).

The distribution of fibroblast-like cells with spindle-shaped morphology has been reported in 3D cell scaffold-based models (38, 42, 44, 56, 59, 67, 79, 81, 84, 90, 92, 93). Uniform spindle-shaped fibroblast-like cells distribution inside scaffold-free spheroids was reported by Berahim et al. (50). The presence of differentiated either only osteoblast-like or morphologically heterogeneous osteoblast-like round-shaped and fibroblast-like spindle-shaped cells was reported in 7 papers (47, 57, 60–62, 85, 87).

3.7. Study of mineralization and extracellular matrix deposition.

Newly formed ECM in 3D PDL models was reported by SEM examination after 2–7 (38, 44, 46, 56, 57, 60, 66, 67, 69, 76, 79, 81, 93) or 10–21 days of culture (52, 59, 75, 86, 91), or histological staining with collagen-specific dyes and H&E (40, 41, 43, 49, 50, 53, 57, 58, 67, 70, 74, 75, 82, 91). No ECM deposition in 3D PDL cell models was reported by Berendsen et al. [29] after 5 days of culture in osteogenic medium.

The most commonly used method for studying mineralization is AR staining because it is fast, cost-effective, and doesn't require expensive devices for measurements (96). It was used in 16 papers (48, 51, 53, 54, 59, 61, 63, 65, 69, 71, 72, 81, 83, 85, 88, 89). Other approaches also have advantages. For example, micro-computed tomography (μ CT), used in three studies (48, 53, 69), is a method that reveals well-organized tissue structure and enables non-destructive observation of structure of 3D cell culture models, utilizing X-rays to see inside an object, slice by slice (97). Fourier transform infrared spectroscopy (FTIR) was used in three publications (53, 63, 78), to provide detailed information on the chemical composition of the 3D cell model. The same is true for energy-dispersive spectroscopy, which was used in one investigation (55). Examination of mineral depositions was performed in 16 papers on 3D constructs cultured for 5–28 days in osteogenic medium (48, 53, 59, 61, 69, 71, 72, 81, 83, 85, 88, 89, 91) or without osteogenic inducers (54, 65, 88). More prominent mineralization was detected in cell models grown in scaffolds with embedded HA (59, 70, 78, 81, 87, 89). Only one study (51) detected no mineralization in a 3D cell model embedded in a collagen scaffold as early as 5 days of culture in osteogenic medium. In two studies (48, 53), a similar pattern of mineralization was reported for scaffold-free PDL spheroids: highly mineralized areas were localized in the center of the constructs, whereas the periphery of the constructs completely lacked mineralization as revealed by AR staining. Moreover, the Alizarin-negative region corresponded to the area with a fibroblast cell phenotype seen in H&E staining (48). Calabrese et al. used FTIR to confirm that the mineralized portion of the spheroid contained HA, similar to the mineral of bone, dentin, and cementum (53). Additionally, five studies (60, 61, 63, 83, 88) detected AR-positive areas in a 3D construct based on a PLGA scaffold. SEM examination confirmed that the 3D constructs had deposited a layer of mineral plaques (60, 61, 63), while H&E staining showed osteoblast-like cells localized closely to mineralization nodules (63, 83, 88). In PCL scaffold-based 3D

constructs, mineralization nodules were reported [55, 63, 65], and in collagen-based 3D cell models, mineralization was detected (41, 83, 85, 88), respectively. High mineralization was reported with AR staining in 3D cell constructs developed on OsteoBiol® collagenated scaffold composed of natural cancellous and cortical bone (69) and was further confirmed by μ CT. Mineralization was demonstrated in a 3D cell construct made of sheets of PDL cells co-cultured with HUVECs (72) or hMSCs (41). SEM was used to examine the calcium and phosphorus content in organoid micro-mass cultures, which was further confirmed by AR staining (73). Positively stained nodules of mineralization in a 3D cell construct of PDLSCs cultured in basal culture medium conditioned by apical papilla stem cells were demonstrated using AR staining (43).

3.8. Examination of specific marker expression.

It is well known that PDL tissue comprises fibroblasts, cementoblasts, osteoblasts, osteoclasts, immune cells, and a small number of progenitor fibroblasts that can differentiate into the main cell types comprising PDL when cultured under appropriate conditions. Seo et al. first described this, and it was subsequently confirmed by other authors (98, 99). 3D cell models described in the selected papers were derived from PDL progenitor fibroblasts. They were cultured either in osteogenic medium containing dexamethasone, ascorbate, and β -glycerophosphate, in a basal medium without any osteogenic additives, or in a basal medium supplemented with ascorbic acid, or ascorbic acid with β -glycerophosphate but without dexamethasone (see Table 2). We categorized markers that correspond to particular cell types in the described 3D cell models, and collected the data received by means of PCR, WB, IHC or IF.

3.8.1. Markers of osteogenic lineage differentiation.

Expression of an osteoprogenitor marker, Runt-related transcription factor 2 (RUNX2), was reported in 3D PDL cell models in 17 of 58 papers (41, 46, 49–51, 56–59, 71–75, 77, 84, 85, 87). Furthermore, using IHC staining, RUNX2-positive areas were detected in the peripheral regions of PDL cell models with tight colocalization with other osteoblast-specific markers such as osteocalcin (OCN) (49), osteonectin (OSN), and osteopontin (OPN) (41).

Late osteogenic marker OCN, which appears concomitantly with mineralization, was reported in 20 of 58 papers (36, 37, 42, 43, 46, 47, 49, 61–63, 70, 71, 73–75, 77, 78, 80, 85, 87). In five studies, OCN expression was localized in cells with a round osteoblastic shape in the periphery of 3D PDL cell constructs (49, 61, 62, 70, 75), while in three papers (36, 37, 78) an abundant distribution of OCN was reported all over the 3D model section. Another late osteogenic marker, bone sialoprotein (BSP), is a protein marker of terminally differentiated osteoblasts. Its expression is upregulated at the onset of mineralization (100). BSP upregulation was detected in twelve studies (43, 48, 54, 61, 62, 71, 72, 75, 76, 80, 83, 88). However, Ern et al. demonstrated lower BSP transcript expressions in 3D PDL cell models cultured with Prostaglandin E2 (PGE2) (80). Cho et al. and Lee et al. both reported strong BSP expression in 3D PDLF-based cell models cultured with bone morphogenetic protein (BMP) (83, 88). Furthermore, the BSP-positive area was colocalized with AR positivity (88). Hoz et al. reported the

upregulation of BSP in PDL spheroids when CEMP1 was present (54). Both BMP2 and CEMP1 are known to promote mineralization (91). Yang et al. and Singhatanadgit et al. demonstrated moderate BSP expression in spheroids derived from PDL cells, but only when cultured in osteogenic medium (43, 71). Inanc et al. (61, 62) reported strong colocalization of BSP with OCN in osteoblast-like cells within 3D PDL cell constructs. Pandula et al. noted significant BSP upregulation in 3D cell sheets derived from PDLSC and HUVEC co-culture compared to PDLSC monoculture cell sheets (72). Basu et al. (48) observed BSP localization of in the central region of the 3D scaffold-free construct, where cells exhibited osteoblast morphology.

Expression of another marker of differentiated osteoblasts and osteocytes, OPN, was measured in 12 studies: (36, 37, 41, 47, 51, 59, 61–63, 76, 81, 87). In all the above-mentioned studies, OPN upregulation was observed in 3D cell models, except for Berendsen et al., who reported no OPN upregulation under mechanical loading conditions compared to control, which might be due to a short differentiation period (51). Additionally, more prominent OPN upregulation was observed in the presence of HA in culture medium at the same time points (37, 47, 59, 62, 81, 87). Moreover, Inanc et al. reported prominent localization of OPN-positive areas on and around the cells and cell clusters with osteoblast morphology. Finally, several studies have reported a higher OPN expression in 3D PDL cell models cultured in osteogenic medium compared to those in basal medium (47, 59, 61–63).

Expression of Osterix, a transcription factor specific to osteoprogenitor cells and differentiated osteoblasts, was also detected by two other studies (70, 74).

Alkaline phosphatase (ALP), another early osteogenic marker, was measured in 21 studies: (37, 43, 46–48, 54, 59, 63, 65, 67, 71, 72, 74–76, 80, 81, 85, 87, 89, 92). ALP up-regulation was reported in most of these studies (37, 43, 46–48, 54, 59, 63, 67, 71, 72, 75, 81, 85, 87, 89, 92). Furthermore, in (48), ALP staining was localized to the center of the 3D construct, in the region with prominent mineralization. More prominent ALP staining was observed in 3D cell models based on decellularized ECM substrate (37). Wu et al. reported ALP upregulation on 3rd day of culture, with significant downregulation up to day 7 (46). Two other studies reported a subsequent downregulation of ALP in the 3D PDL cell model at the end of culture (74, 80).

Expression of another osteogenic marker, secreted protein acidic and rich in cysteine (SPARC, also known as OSN), was also analyzed (41, 47, 61, 62). In (61), OSN expression in 3D PDL cell models was detected at low levels, in contrast to (47, 62), where significant OSN overexpression was reported. Furthermore, in (47), OSN expression was co-localized with collagen type 1, confirming OSN binding affinity for collagen.

3.8.2. Collagens type 1 and 3.

Collagens are a principal component of PDL fibers and ECM, which bind glycoproteins and fibroblasts. The most abundant collagens in PDL are types 1 and 3. They comprise 75% and 25% of all collagens in

PDL, respectively (101). Both types of collagens are synthesized by either fibroblasts or by mature osteoblasts.

Collagen 1 expression was detected in 25 studies: (40–43, 47, 50–52, 57, 58, 61, 67, 69, 70, 74–77, 80, 81, 83, 85, 86, 88, 91, 102). Enhanced collagen 1 expression was reported in 3D PDL cell models in 13 papers: (41, 42, 50, 51, 67, 74, 75, 77, 80, 81, 83, 85, 88, 91, 102). Zhang et al. and Proksch et al. demonstrated enhanced collagen 1 expression in 3D cell models constructed of hPDL cells co-cultured with JBMMSCs (75) or with hMSC (41) compared to PDLF monoculture 3D models. Wu et al. reported significantly enhanced collagen 1 expression in PDLSC spheroid blocks compared to PDLSC spheroids or PDLSC monoculture (102). In the 3D model described in paper (88), based on IHC data, the distribution pattern of collagen fibers resembled that of native PDL. In one paper (83), collagen 1 upregulation was observed only in the presence of BMP2 and BMP7, which are known osteogenic inducers. A dense network of collagen fibers in PDLF spheroids described in (50) resembles that of the native PDL but lacks the typical fiber orientation found in mature tissue. Berendsen et al. reported enhanced collagen 1 expression in 3D cell models subjected to mechanical loading (51). And finally, Abdal-hay et al. reported upregulation of collagen 1 in 3D constructs based on PCL scaffold with incorporated HA compared to control 3D models without HA (81). However, no difference was detected in collagen 1 expression between 3D cell models grown with and without HA using an alginate/polyvinyl alcohol scaffold (47).

Chu et al. compared the expression of collagens 1 and 3 in organoids derived from DFC and PDL cells, showing significantly higher collagen 1 transcript expression and collagen 1 protein distribution in constructs developed from PDL cells. In contrast, collagens 3 and 5 were expressed more considerably in the DFC-based model (58). In the PCL scaffold-based 3D cell constructs described by Daghrery et al. (84), collagen 3 overexpression was detected in 3D cell constructs with aligned fiber orientation, unlike those with randomly oriented fibers. Collagen 3 expression was enhanced in spheroids developed from PDL cells compared to 2D cell culture (70).

3.8.3. Enamel, dentin, and cementum tissue markers.

Seven papers (42, 53, 54, 70, 83, 88, 91) investigated the expression of CEMP1 in 3D PDL cell models. The localization of CEMP1 expression in areas with high mineralization was also analyzed in 3 papers (53, 81, 89). Moreover, in one paper (83), CEMP1 was significantly upregulated in a phase of the 3D cell constructs containing encapsulated BMP7 and BMP2, while no expression was seen in other phases. Hoz et al. found that cementum attachment protein (CAP) mRNA expression occurred only when 3D PDLSC cell constructs were cultured with human recombinant CEMP-1. CAP expression was detected at a late stage of differentiation in 3 studies (42, 54, 80). However, Berahim et al. (49, 50) reported no CAP expression in PDLF spheroids after day 20 of culture.

Expression of dental sialophosphoprotein (DSPP) was measured in 4 studies (36, 37, 53, 87). DSPP is highly expressed in dentin, making it a valuable marker for this mineralized tissue (103). Overexpression of DSPP was reported in all of the abovementioned studies (36, 37, 53, 87). Ivanov et al. reported intense

DSPP staining only in 3D constructs made of hPDLSC co-cultured with periosteum cells (36). Calabrese et al. (53) and Lee Chang et al. (88) showed that DSPP expression is localized to highly mineralized Alizarin-positive areas. In contrast, Ivanov et al. detected DSPP expression only in bioengineered 3D PDL cell constructs grown on decellularized matrix (36, 37).

Upregulation of BMP2, a well-known mediator of odontogenic differentiation, was reported in 2 papers (70, 74). Expression of another marker of dentin tissue, dentin matrix acidic phosphoprotein (DMP1), which regulates matrix mineralization, was not altered in (51). The upregulation of amelogenin and ameloblastin, the key enamel matrix proteins (104), that are crucial for enamel mineralization, was mentioned in (54).

3.8.4. Fibroblast markers.

Periostin (POSTN), which is typically expressed in fibroblasts, is a secreted adhesion-related protein located in the periosteum and periodontal ligaments (105). POSTN expression was examined in 14 studies (39, 42, 48–50, 56–58, 68, 74, 75, 82–84). A comparative analysis of POSTN gene expression in two scaffold-free 3D organoids, derived from either DFC or PDL cells, revealed that POSTN expression is significantly higher in DFC-based organoids compared to PDL cell-based organoids (58). Berahim et al. (49) detected a higher level of POSTN expression in the peripheral region of the PDLF spheroids adjacent to the dentin substrate. Moreover, Cho et al. reported intense POSTN staining in the part of the organoid that is positive for cluster of differentiation (CD) CD146, which corresponds to PDL (83).

Periodontal ligament-associated protein (PLAP-1), also known as asporin, is an extracellular proteoglycan uniquely located in PDL tissue and expressed by fibroblasts (106). Detection of PLAP-1 has been reported in 6 studies, indicating a significant upregulation of PLAP-1 expression (48, 53, 58, 67, 70, 81). Basu et al. reported colocalization of POSTN and PLAP-1 fluorescence in the periphery of the 3D PDLSC construct. This suggests that cells in the peripheral area of the organoid exhibit a periodontal ligament fibroblast phenotype, suggesting that the organoid's peripheral regions acquire PDL tissue features during tissue maturation (48).

Increased expression of fibroblast-specific protein (FSP) was detected in 3D cell culture models in two papers (60, 81), and a significant FN expression was reported in five studies (60, 74, 75, 79, 86).

3.8.5. Other markers.

Osteoprotegerin (OPG) and its counterpart, the receptor activator of nuclear factor- κ B Ligand (RANKL), play an essential role in bone metabolism and remodeling by regulating osteoclastogenesis (27). Overexpression of OPG and RANKL in 3D PDL cell culture was reported in 7 studies (38, 39, 44, 45, 56, 66, 93). A similar timeline of regulation of OPG and RANKL in 3D cell models was noted in different studies when organoids were grown under compression (38, 44, 56, 66, 93): the highest expression of RANKL was detected after 6 hours of compression, followed by RANKL downregulation and the highest expression of OPG was detected after 72 hours, which suggests a shift from osteoclastogenesis towards inhibition favoring osteogenesis. Moreover, expression of STRO-1 and CD44, markers of

mesenchymal stem cells, was reported in 2 studies (37, 78). The overexpression of cytokines interleukin (IL) IL-11, IL-6, and IL-8 was reported in 5 papers (38, 40, 42, 55, 66).

3.9 3D PDL cell models for functional research.

The effects of mechanical forces mimicking tooth movements in 3D PDL cell constructs were tested in 9 papers (38, 39, 44, 45, 51, 56, 66, 82, 93). Compression was examined in 8 papers (38, 39, 44, 45, 51, 56, 66, 93), while only one paper investigated tensile forces (82). Li et al. (38, 66) and Mahdi Souzani et al. (39) reported that cells exhibiting spindle-shaped morphology grew in a well-organized multi-layered fashion, with more densely packed fibers and deposited ECM, when 3D cell constructs were cultured for three days under mechanical loading conditions. A negative impact on cell proliferation was observed in 3D cell culture after prolonged or high-magnitude compression (38, 39). In contrast, low-load compression stimulated proliferation (39). Under mechanical loading, overexpression of RANKL and OPG (38, 39, 44, 56, 66, 93) as well as POSTN (39) was detected in 3D PDL cell models. While Li et al. reported POSTN downregulation in PDL organoids after 6 h of compression (56). Overexpression of the principal mediator of inflammation, PGE₂, and cyclooxygenase (COX), an enzyme that regulates PGE₂ production, was detected in 3D cell models subjected to mechanical loading (38, 45, 51, 56, 66, 93). Overexpression of IL-11, -8, -24, fibroblast growth factors (FGF) 2 and 7, and proteins that regulate cell cycle and calcium homeostasis were found in 3 papers (38, 56, 66). Berendsen et al. showed that in 3D cell models cultured for 5 days and subjected to mechanical loading for 4 days, collagen 1, OPN, RUNX2, and DMP1 expression was unaffected (51).

The effect of stretching was studied in one paper (82). Chiu et al. reported modifications in collagen fiber orientation, enhanced proliferation, and significant overexpression of POSTN in 3D PDL cell models subjected to tensile forces (82).

Another aspect of dental physiology directly related to OTM is hypoxia. When orthodontic force is applied to a tooth, the cells on the compressed side of PDL tissue undergo hypoxia due to occluded blood vessels (107). The effect of hypoxia was tested in three papers (64, 65, 93). Elevated hypoxia-inducible factor (HIF)-1 α protein levels in organoids cultured under hypoxic conditions were reported in 3 papers (64, 65, 93).

Vascularization and angiogenesis in 3D PDL cell culture models were explored in 5 studies (64, 65, 70, 72, 79). Pandula et al. reported rudimentary vessel-like structures in 3D models developed from PDLSC and HUVEC co-culture and PDLSC monoculture, in a comparative study (72). Ono et al. reported increased expression of Vascular endothelial growth factor A (VEGFA) and Syndecan-1 (70). Morgante et al. (79) detected a significant increase in the expression of integrins, VEGF, and VEGF receptors and a decrease in E-cadherin expression in 3D PDL cell models. Increased expression of essential signaling factors in angiogenesis, angiopoietin-like 4 and angiogenin, was reported (64, 65).

The influence of bacterial infection and inflammatory mediators was investigated in 3 papers (40, 55, 80). Makkar et al. studied the effects of common constituents of the human oral microbiome,

specifically *Streptococcus mitis*, *Streptococcus oralis*, and *Fusobacterium nucleatum* (40). 3D PDL cell models showed minimal response to *Streptococcus mitis* and *oralis*, whereas *Fusobacterium nucleatum* significantly increased IL-6 and IL-8 production. Ern et al. investigated how PGE2, a key mediator of inflammation, affects the osteoblastic differentiation of PDLSCs in a 3D model. Ern et al. reported that PGE2 significantly reduced ALP, collagen type 1, and OCN expression, but did not impact BSP expression (80). Additionally, neither cell morphology nor proliferation was affected by PGE2. Expression of stemness genes and interleukins, proliferation, and cell viability in PDLSC spheroids after infection with the periodontal pathogen *Porphyromonas gingivalis* (*P.gingivalis*) were also tested by Zhao et al. (55). The authors detected significant downregulation of NANOG and Sex Determining Region Y (SRY)-Box Transcription Factor (SOX) 2 and increased IL-6 and IL-8 production. Cell viability in PDLSC spheroids was substantially impaired by *P.gingivalis* (55).

4. Discussion

Mesenchymal stem cells, particularly PDLSC or PDL-derived progenitor cells, have been extensively studied, with the prospect of their utility in treating periodontal diseases and dental tissue regeneration (22, 23). These cells exhibit superior multilineage differentiation capacity (22, 24). Many studies have demonstrated that stem cells from PDL tissue, under certain inductive conditions, can differentiate into the main cell types that comprise PDL tissue: osteoblasts, cementoblasts, and fibroblasts (25–27). Despite the advantages of cell therapy with 2D cell cultures, reduced differentiation potential and lack of long-term retention of implanted cells *in vivo* make tissue regeneration with 2D cell cultures challenging (30). 3D cell culture attracts considerable attention as a prospective approach in tissue engineering and regeneration, as it preserves cell stemness and promotes differentiation upon either *in vivo* transplantation in animal models or *in vitro* culture, compared to monolayer culture (108). Interestingly, gene expression of cells behaves differently in 3D and 2D culture (109). The above-mentioned points and the advantages of 3D cell constructs for mimicking complex interactions between cells and ECM make them valuable tools for examining various physiological aspects of dental and periodontal tissue functions and for dental tissue regeneration. Though, eighteen studies have employed 3D cell culture models to investigate functional aspects of PDL (OTM, hypoxia, bacterial colonization, and vascularization of PDL tissue, (see paragraph 3.9 for details) most studies, 40 of 58, employed 3D cell constructs to explore their potential application in tissue engineering and to test different scaffold materials that can potentially be used in dental tissue regeneration.

The fabrication of multicellular tissue-like structures employs two technologically distinct approaches: the scaffold-free technique, which uses cell self-assembly, and the scaffold-based technique, which uses a biocompatible natural or synthetic substrate to support the growth of a 3D cell construct. The most conventional approach to 3D cell culture involves using a scaffold that acts as a substrate for cell growth. Various scaffolding materials, including HA granules, natural hydrogels, synthetic polymers, decellularized matrices, or their combinations, were used to construct 3D PDL cell models. 3D cell constructs based on decellularized matrices as a scaffold exhibited the highest level of biocompatibility (36, 37, 86, 87). These scaffolds lack cellular components but preserve ECM components that support

cell migration, adhesion, and proliferation. Numerous studies confirmed the efficiency of decellularized matrices in facilitating dental tissue regeneration (110, 111). Though it was postulated that some scaffolds have limitations that can negatively affect cell stability and behavior during incubation (112), data extracted from selected studies using decellularized matrices as a scaffold for 3D cell culture show that these scaffolds do not inhibit cell viability, support proliferation (86, 87), promote cell differentiation (36, 37, 49, 83, 86, 87), and enhance extracellular matrix formation (49, 86, 91).

Cell commitment to osteogenic lineage differentiation was evidenced by the expression of typical osteogenic markers that correspond to early and late differentiation phases: an initial proliferative phase lasting several days after confluence, followed by a phase of collagen deposition and extracellular matrix maturation, and terminal differentiation marked by the mineralization of the mature matrix (113). Accordingly, overexpression of early osteogenic markers RUNX2 and Osterix was reported during the first week of osteogenic induction (41, 50, 57, 59, 70–72, 75, 84, 87), while late osteogenic markers OCN, BSP, and OPN, which are up-regulated at the onset of mineralization, were reported later, after the second or third week of culture (36, 37, 41, 42, 47, 49, 61–63, 71, 74, 80, 81, 85, 87). Increased OPN and OCN expression was observed in the presence of HA around cells with osteoblast morphology (37, 61, 62, 70, 78, 81). OPN expression is characteristic of end-stage extracellular matrix deposition and the onset of mineralization. It is one of the mineral-associated adhesion molecules. Interestingly, the upregulation of late osteogenic markers was reported together with significant mineralization (41, 59, 61, 63, 81, 85, 87) and ECM deposition (41, 59, 81). Moreover, DSPP overexpression, a marker of dentin formation, known to occur at the early stage of PDL development, was reported in the second week of 3D PDL cell culture (36, 37, 53). Whereas overexpression of CEMP1, a protein specific to cementoblasts, was reported after 28 and 42 days of culture, respectively, corresponding to the late phase of mineralization (83, 91). Interestingly, demineralized and chemically treated dentin matrices can be used as scaffolds due to their good mechanical properties, their ability to serve as a pool of dentinogenic and osteogenic growth factors, and their ability to mimic ECM. Dentin has been used as a scaffold for 3D cell models in two studies (49, 83). Whereas Cho et al. found that culturing PDLSC embedded in PLGA and grown on dentin slices promotes their differentiation into osteoblasts and cementoblasts (83), Berahim et al. reported only a limited effect of dentin substrate on PDL cells differentiation (49). Overall, all studies that employed various decellularized matrices as scaffolds for 3D culture reported that these scaffolds stimulated the osteogenic differentiation of PDL progenitor cells (36, 37, 49, 83, 86, 87, 114).

It is particularly intriguing that HA, combined with various hydrogels or other substrates, has demonstrated beneficial effects on cell proliferation and differentiation (47, 62, 78, 81, 87, 88, 92). The chemical similarities between HA and natural bone might explain its osteoinductive properties. A significantly increased OCN marker, which appears alongside mineralization, was observed only in the presence of HA during the first week of 3D PDL cell culture, compared to control samples without HA (62, 70, 78, 87, 89). Notably, HA also promoted ECM deposition and mineralization, as evidenced by the overexpression of collagen type 1 during the first week of culture; in contrast, collagen type 1 expression in samples without HA was only observed after the second week of culture. Moreover, the differentiation of cementoblasts from PDL cells grown on HA scaffolds was confirmed by the overexpression of

CEMP1 (70, 88). When HA was combined with PCL hydrogel, it significantly stimulated metabolic activity in 3D cell models and increased ALP activity along with higher expression levels of OPN and collagen type 1, compared with 3D cell models embedded in PCL hydrogel alone (81). Additionally, HA combined with alginate or gelatin gels accelerated the expression of specific markers associated with pre- and mature osteoblast stages, including osteocalcin, ALP, and osteopontin (47, 92). And finally, the stimulatory effects of HA on mineralization in 3D models were confirmed in several studies (59, 62, 70, 78, 87, 89). The acceleration of osteogenic marker expression and mineralization by HA could be of great interest for bone research.

Strategies to improve the development of 3D PDL cell models include incorporating various bioactive molecules. Lee et al. reported the emergence of distinctive tissue phenotypes resembling native periodontal tissue on a complex, three-phase, region-specific scaffold (88). This scaffold contained encapsulated amelogenin, connective tissue growth factor (CTGF), and BMP2, and was seeded with either dental pulp stem cells (DPSCs), PDLSCs, or alveolar bone stem cells (ABSCs). After four weeks of *in vitro* culture, the following distinctive tissue phenotypes were observed: 1) in the scaffold seeded with PDLSC, a PDL-like structure rich in collagen fibers formed, with a mineralization pattern specific to native PDL in the CTGF phase, 2) a highly mineralized alveolar bone-like structure developed in the BMP2 phase, when seeded with ABSC, 3) in the amelogenin phase, a dentin-like tissue structure was formed when cultured with DPSC. In a study by Cho et al., the same type of multiphase scaffold with encapsulated CTGF, BMP2, and BMP7 and seeded with PDLSC was utilized (83). After six weeks of culture, *de novo* tissue formation expressing specific cementum and osteogenic markers was reported (83). A scaffold with gene-activated matrix encoding platelet-derived growth factor (PDGF) was also described by Peng et al. (90). The authors reported that PDL cells retained their fibroblast morphology and formed structures resembling native PDL (90).

The selected papers employed various natural and synthetic hydrogels to develop 3D PDL cell culture models. Hydrogels are attractive matrices for tissue engineering due to their good biocompatibility, ability to mimic natural ECM, and capacity to facilitate the formation of self-organized cells clusters and promote differentiation (115). More significant expression of PDL-specific markers in a 3D model based on PCL/gelatin scaffold was detected compared to collagen scaffold in (42). Furthermore, several studies reported good viability and proliferation of 3D PDL cell models developed in collagen hydrogels (41, 50, 57, 82, 85). Moreover, Ivanov et al. postulated that a collagen gel combined with a decellularized matrix formed a microenvironment that promoted both osteogenic and odontogenic differentiation in 3D PDL cell models (36, 37). In comparative studies by Berahim et al., collagen matrix stimulated more cell proliferation than PGA, without affecting cell viability (49, 57). Lu et al. effectively used 3D PDLF organotypic culture grown in collagen matrix and co-cultured with epithelial cells to study the development of periodontal-epithelial junctions (68). It is worth noting that the implementation of co-culture is intriguing and promising, as different types of progenitor cells in co-culture can give rise to more complex and specific tissue-like structures. Co-culturing of different cell types on a multilayered scaffold could promote the development of 3D cell culture models with a complex, well-organized, multilayered structure that is morphologically and functionally more akin to native tissue.

The advantages of synthetic hydrogels used in the selected papers include their nontoxicity and biological inertness, as claimed by the authors (49, 57, 60, 67, 81, 89, 92). However, they lack the biological cues found in the native ECM, which may affect 3D cell culture development. For example, PDL cells viability was impaired in the PCL scaffold alone but was significantly improved by adding HA nanoparticles (81). Promising results were reported for synthetic hydrogels blended with natural scaffolds such as alginate and gelatin (42, 47). Polyvinyl alcohol hydrogel blended with alginate demonstrates high biocompatibility and promotes PDLSC differentiation (47). The same results were observed for the PCL scaffold: greater expression of PDL-specific markers in a 3D model based on the PCL/gelatin scaffold was detected compared to the collagen scaffold (42).

Finally, it is noteworthy that hydrogels have proven to be stable under compression, that's why they are used for studying OTM (38, 39, 51, 66, 116). For example, collagen and PLGA hydrogels were successfully used to study the effects of hydrostatic pressure on 3D culture of PDL cells (38, 39, 51, 66).

Scaffold-free cell constructs developed using the well plate method were also investigated (50, 64, 65, 70). A dense collagen network formation resembling native PDL was reported, along with the expression of POSTN, collagen 1, and RUNX2, which are known to be specific to native PDL (50). This approach has limitations related to the size of organoids and the accessibility of nutrients. If organoids exceed 200 μm in diameter, the apoptotic or necrotic core can emerge after long-term culture because of a lack of oxygen and nutrients in the central parts of organoids (117). This might be the reason for the reduction in organoids' size at the end of the culture (41, 50, 58, 70). This drawback can be overcome using the rotational method of organoid culture (62, 74). This method facilitates the diffusion of nutrients and oxygen to the organoid. Yang et al. and Inanc et al. reported the expression of osteogenic markers and ECM deposition in PDL cell spheroids manufactured in the rotational cell culture bioreactor and cultured for two and three weeks, respectively (62, 74).

Basu et al. and Calabrese et al. described organoids developed from a confluent cell sheet by natural detachment from the plate and contraction toward two tiny pins positioned in the center of the dish. Organized PDL-cementum-like complex engineered tissues, derived either from PDL cell monoculture (48) or PDLSC and DPSC co-culture (53) were reported after 2 weeks in an osteogenic medium. In a comparative study by Chu et al., organoids were constructed from DFSC and immortalized PDL cells monolayers, respectively, by lifting and rolling them into rod-like structures. The expression of a wide panel of PDL-related genes was reported. The results showed that both cell types successfully formed scaffold-free 3D organoids, though DFSC organoids exhibited superior morphology and cell-row order (58).

The mimicking of biochemical and biomechanical microenvironments makes 3D dental cell culture a more plausible model for studying dental physiology than monolayer culture. 3D cell constructs are a convenient model for examining mechanical loading in dental tissues. Orthodontic tooth movements are inherent phenomena in periodontal tissue physiology that stimulate cell proliferation and differentiation. PDL tissue is crucial for converting mechanical force into biological signals and for maintaining the

periodontium by balancing bone formation and resorption. As 3D cultures enable application of compression in a multi-directional manner, as occurs in natural PDL tissue, they are a more efficient model than 2D culture. In 2D cell culture, mechanical loading can be applied only in one direction. A hydrostatic pressure applicator developed and employed by Mahdi Sousani et al. enables the application of pressure to a column containing organoids in culture medium using compressed air, and its volume determines the magnitude of the mechanical load (39). Li et al. used a simpler “weight” method: cover glasses were placed on top of the PDLF-PLGA 3D construct, and load strength was determined by the weight of the lead granules (38, 66). Regardless of the method of compression used, both groups reported changes in cell proliferation, ECM deposition, and collagen fiber morphology in 3D constructs after compression (38, 39, 66).

Another physiological aspect we identified in the selected studies was the approaches used to develop 3D cell constructs suitable for studying vascularization in PDL. Constructing scaffolds capable of forming vascular networks is a challenging task in tissue engineering. Adequate and sufficient vascularization is crucial for the long-term survival of implanted 3D tissue, as it provides nutrients and oxygen while preventing necrosis. Various approaches were employed to address this subject (117). Though Janjic et al. (64, 65) failed to detect the expression of angiogenin and angiopoietin, which play a crucial role in vascularization, after one day of spheroid culture. That might be explained by a relatively short period of culture. In contrast, other studies demonstrated signs of vascularization after 1–3 weeks in culture (70, 72, 79). 3D cell sheets developed from PDLSC co-cultured with HUVEC were used in (72), a 3D cell model from PDLSC monoculture grown on BioRipar® scaffold in (79), or the floating cell culture technique was employed in (64, 65, 70). The authors reported the formation of vascular-like structures and a significant increase expression of angiogenesis-promoting proteins, such as VEGF, VEGF receptor, and Syndecan-1, which may provide insights into the development of angiogenesis in PDL (70, 72, 79). However, more studies on this subject are needed to conduct a thorough analysis.

5. Limitations

Generally, data on various approaches to performing 3D cell culture reveal a high level of heterogeneity, indicating that there is no precise superior technique for constructing the optimal 3D PDL model. This review aimed to summarize all currently described 3D cell models derived from PDL-derived progenitor cells and systematically analyze them with respect to their construction, cell composition, and applications. All the described 3D cell models have their advantages and limitations. In particular, some studies tested a limited panel of markers. This limitation makes it impossible to draw a plausible conclusion regarding the emergence of differentiated cell types. Not providing the age of donors for PDL cells and the passage number in some selected papers creates uncertainty about the quality of the cells used and the validity of the data. In particular, the passage number is a critical parameter in cell culture work. As it increases, cells accumulate mutations that can lead to a loss of their typical morphological and/or functional features (118). This could influence the proliferation rate and differentiation potential, rendering the data unreliable. The median number of passages for primary human PDL cells used in the extracted papers was 6, with a range of 2–10. That means only part of the studies were conducted

within the appropriate passage range, limiting the validity of the data. The lack of standardization in protocols, particularly regarding culture times, composition of the culture medium, utilization of various types of scaffolds, and different approaches to developing 3D cell models, makes it impossible to perform accurate comparisons between different 3D cell models. More studies using standardized characterization methods and broader marker evaluation are required to develop optimized 3D PDL cell culture protocols, thereby improving the accuracy of analysis and comparison across investigations.

Abbreviations

μCT	micro-computed tomography
3D	3-dimensional
ABSC	alveolar bone stem cells
ALP	alkaline phosphatase
AR	Alizarin Red
BMP	bone morphogenetic protein
BSP	bone sialoprotein
CAP	cementum attachment protein
CD	cluster of differentiation
CEMP1	cementum Protein 1
COX	cyclooxygenase
CTGF	connective tissue growth factor
DFC	dental follicle cells
DFSC	dental follicle stem cells
DMP1	dentin matrix acidic phosphoprotein 1
DPSC	dental pulp stem cells
DSPP	dental sialophosphoprotein
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
FN	fibronectin
FSP	fibroblast-specific protein
FTIR	Fourier-transform infrared spectroscopy
H&E	hematoxylin & Eosin
HA	hydroxyapatite
HA-PADM	hydroxyapatite-coated porcine acellular dermal matrix
HIF	hypoxia inducible factor
hGF	human gingival fibroblasts
hMSC	human mesenchymal stem cells

hPDL	human periodontal ligament
hPDLF	human periodontal ligament fibroblasts
hPDLSC	human periodontal ligament stem cells
HUVEC	human umbilical vein endothelial cells
IF	immunofluorescence
IHC	immunohistochemistry
IL	interleukin
NANOG	transcriptional factor
OCN	osteocalcin
OPG	osteoprotegerin
OPN	osteopontin
OSN	osteonectin (SPARC)
OTM	orthodontic tooth movement
PADM	porcine acellular dermal matrix
PAS	periodic acid schiff
PCL	polycaprolactone
PCR	polymerase chain reaction
PDL	periodontal ligament
PDLSC	periodontal ligament stem cells
PDLF	periodontal ligament fibroblasts
<i>P. gingivalis</i>	<i>Porphyromonas gingivalis</i>
PGE2	prostaglandin E2
PLAP-1	periodontal ligament-associated protein 1
PLGA	poly(lactic-co-glycolic) acid
PLLA	poly-L-lactic acid
POSTN	periostin
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RANKL	receptor activator of NF- κ B ligand
REM	reflection electron microscopy

RUNX2	runt-related transcription factor 2
SEM	scanning electron microscopy
SOX2	sex determining region Y (SRY)-Box transcription factor 2
SPARC	secreted protein acidic and rich in cysteine
SRY	sex determining region Y
STRO-1	monoclonal antibody, a marker for mesenchymal stem cells
TEM	transmission electron microscopy
VEGFA	vascular endothelial growth factor A
VSM	vibrating-sample magnetometry
WB	western blot
WoS	Web of Science

Declarations

Ethics declarations

Ethics approval and consent to participate: Not applicable.

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Tables

Table 1. Keywords used for database search

Pubmed	((("3D cell culture"[Title/Abstract]) OR (organoid*[Title/Abstract]) OR (spheroid*[Title/Abstract]) OR ("Three D cell culture"[Title/Abstract])) AND ((hPDL*[Title/Abstract]) OR ("periodontal ligament"[Title/Abstract])) Filters: from 2000 - 2026
Scopus	TITLE-ABS-KEY ((hpdI* OR pdI* OR periodontal AND ligament) AND (organoid* OR spheroid* OR 3d AND cell AND culture)) AND PUBYEAR > 2000 AND PUBYEAR < 2026
WoS	periodont* AND spheroid* (Topic) OR PDL* AND spheroid*(Topic) OR periodont*AND organoid* (Topic) OR PDL* AND organoid* (Topic) OR periodont* AND 3D cell culture (Topic) OR PDL* AND 3D cell culture (Topic) OR periodont* AND Three-D cell culture (Topic) OR PDL* AND Three-D cell culture (Topic) NOT in vivo (Topic) NOT neur* (Topic) NOT retina* (Topic) NOT epithel* (Topic) NOT skin (Topic) NOT mammary (Topic). Index date: 2000 - 2026
Google Scholar	3D hPDL cell culture hPDL organoid hPDL spheroid hPDL three D cell culture human periodontal ligament spheroid human periodontal ligament 3D cell culture human periodontal ligament organoid human periodontal ligament three D cell culture

Table 2 is not available with this version.

Table 3. Questions for risk of bias and quality assessment.

	Description	Grade
1	Title	(0) Inaccurate/no concise (1) Concise/adequate
2	Abstract: either a structured summary of background, research objectives, key experiment methods, principal findings, and conclusion of the study or self-contained (should contain enough information to enable a good understanding of the rationale for the approach)	(1) Clearly inadequate (2) Possibly accurate (3) Clearly accurate
3	Introduction: background, experimental approach, and explanation of rationale/hypothesis	(1) Insufficient (2) Possibly sufficient/some information (3) Clearly meets/sufficient
4	Introduction: primary and secondary objectives for the experiments (specific primary/secondary objectives)	(1) Not clearly stated (2) Clearly stated
5	Methods: study design explained number of experimental and control groups, steps to reduce bias (demonstrating the consistency of the experiment (done more than once), sufficient detail for reproducing, etc.)	(1) Clearly insufficient (2) Possibly sufficient (3) Clearly sufficient
6	Methods: precise details of experimental procedure (i.e., how, when, where, and why)	(1) Clearly insufficient (2) Possibly sufficient (3) Clearly sufficient
7	Methods: How sample size was determined (details of control and experimental group) and sample size calculation.	(1) No (2) Unclear/not complete (3) Adequate/clear
8	Methods: Details of statistical methods and analysis (statistical methods used to compare groups)	(1) No (2) Unclear/not complete (3) Adequate/clear
9	Results: explanation for any excluded data, results of each analysis with a measure of precision as standard deviation or standard error or confidence interval	(1) No (2) Unclear/not complete (3) Adequate/clear

10	Discussion: interpretation/scientific implication, limitations, and generalizability/translation	(0) Clearly inadequate (1) Possibly accurate (2) Clearly accurate
11	Statement of potential conflicts and funding disclosure	(0) No (1) Yes
12	Publication in a peer-reviewed journal	(0) No (1) Yes

Table 4. Risk of bias assessment. Papers were assessed according to the grade scale represented in Table 3.

First author	1	2	3	4	5	6	7	8	9	10	11	12	sum
Abdal-hay,	1	2	3	2	1	3	2	3	3	2	1	1	24
Bahrami	0	2	1	1	3	3	3	3	3	1	1	1	22
Basu	1	2	2	1	3	3	3	N/A	3	2	0	1	21
Berahim, (2015)	1	2	2	2	3	3	3	N/A	3	2	1	1	23
Berahim, (2013)	0	3	1	1	2	3	2	2	3	2	0	1	20
Berahim, (2011)	1	3	3	2	1	3	3	2	3	2	0	1	24
Berendsen	1	3	2	1	2	3	2	1	2	2	1	1	21
Blaudez	1	2	3	1	3	3	3	N/A	3	2	1	1	23
Calabrese	1	2	2	2	3	3	3	N/A	3	2	1	1	23
Chiu,	1	3	2	2	3	3	3	3	3	2	1	1	27
Cho	0	3	3	2	3	3	3	3	3	2	1	1	27
Chu	1	3	3	2	3	3	3	3	3	2	0	1	27
Daghrery	1	2	3	2	3	3	3	3	2	2	1	1	26
Diomede,	1	3	2	1	3	3	3	3	3	1	0	1	24
Elango	1	3	3	1	3	3	3	3	3	1	1	1	26
Ern	1	3	3	1	1	3	3	3	2	2	1	1	24
Farag	0	3	3	2	2	3	3	3	3	2	1	1	26
Ge	1	2	2	1	3	3	3	3	3	2	1	1	25
Hoz	1	3	3	1	1	3	3	3	2	2	0	1	23
Inanc, (.2006)	1	3	3	2	1	3	3	3	3	2	0	1	25
Inanç (2009)	0	3	3	2	3	3	3	3	3	2	0	1	26
Inanç (2007)	1	3	2	2	3	3	3	3	3	2	0	1	26
Ivanov,(2023,Biomolecules)	1	3	3	2	3	3	3	3	3	2	1	1	28
Ivanov, (2023, Cells)	1	3	3	2	3	3	3	3	3	2	1	1	28
Jianru	1	3	3	2	3	3	3	3	3	2	1	1	28
Jafar	1	3	2	2	3	3	3	3	3	2	0	1	26
Janjić(2019, JPeriodontol	0	3	3	1	3	3	3	3	3	2	0	1	25
Janjić, (2019, JPeriodontal	0	3	3	1	3	3	3	3	3	2	0	1	25

Res														
Le	1	3	3	1	3	3	3	3		3	2	1	1	27
Lee	1	3	2	1	1	3	3	3		3	2	1	1	24
Li 2016	1	3	3	2	2	3	2	3		2	2	0	1	24
Li 2013	1	3	3	2	3	3	3	3		3	2	1	1	28
Li 2011	1	2	2	1	3	3	2	1		2	1	1	1	20
Liang	1	2	3	1	3	3	3	3		3	2	1	1	26
Liao	1	3	3	2	3	3	3	3		3	1	0	1	26
Lu	1	3	3	2	3	3	3	3		3	2	0	1	27
Mahdi	1	3	3	2	3	3	3	3		3	2	1	1	28
Makkar	1	3	3	2	3	3	3	3		3	2	1	1	28
Manescu	1	3	3	1	1	3	3	3		3	2	0	1	24
Morgante	1	3	3	2	1	3	3	3		3	2	1	1	26
Ni	1	3	3	2	3	3	3	3		3	2	1	1	28
Nowwarote	1	3	3	2	2	2	3	3		2	2	1	1	25
Ono	1	3	3	2	3	3	3	3		3	2	0	1	27
Pandula	1	3	3	1	1	3	3	3		3	2	1	1	25
Peng	1	2	3	2	1	3	3	3		3	2	1	1	25
Proksch	1	3	3	2	3	3	3	3		3	2	1	1	28
Sano	1	2	3	2	3	3	3	2		2	2	1	1	25
Singhatanadgit 2009	1	3	3	1	1	3	3	3		3	2	1	1	25
Singhatanadgit, 2013	1	3	3	2	3	3	3	3		3	2	0	1	27
Staples	1	3	3	1	3	3	3	3		3	2	1	1	27
Tian, Y. 2021	1	3	3	1	1	2	3	3		3	2	1	1	24
Tian Z.2025	1	3	3	2	3	3	3	3		3	2	1	1	28
Vurat	1	3	3	1	3	3	3	3		3	2	1	1	27
Wu	1	3	3	2	3	3	3	3		3	2	1	1	28
Yang Y.2023	1	3	3	2	3	3	3	3		3	2	1	1	28
Yang Z.2009	1	3	3	2	1	3	3	3		3	2	1	1	26

Zhang	1	3	3	1	3	3	3	3	3	2	1	1	27
Zhao	1	2	3	1	1	3	3	3	3	2	0	1	23

Figures

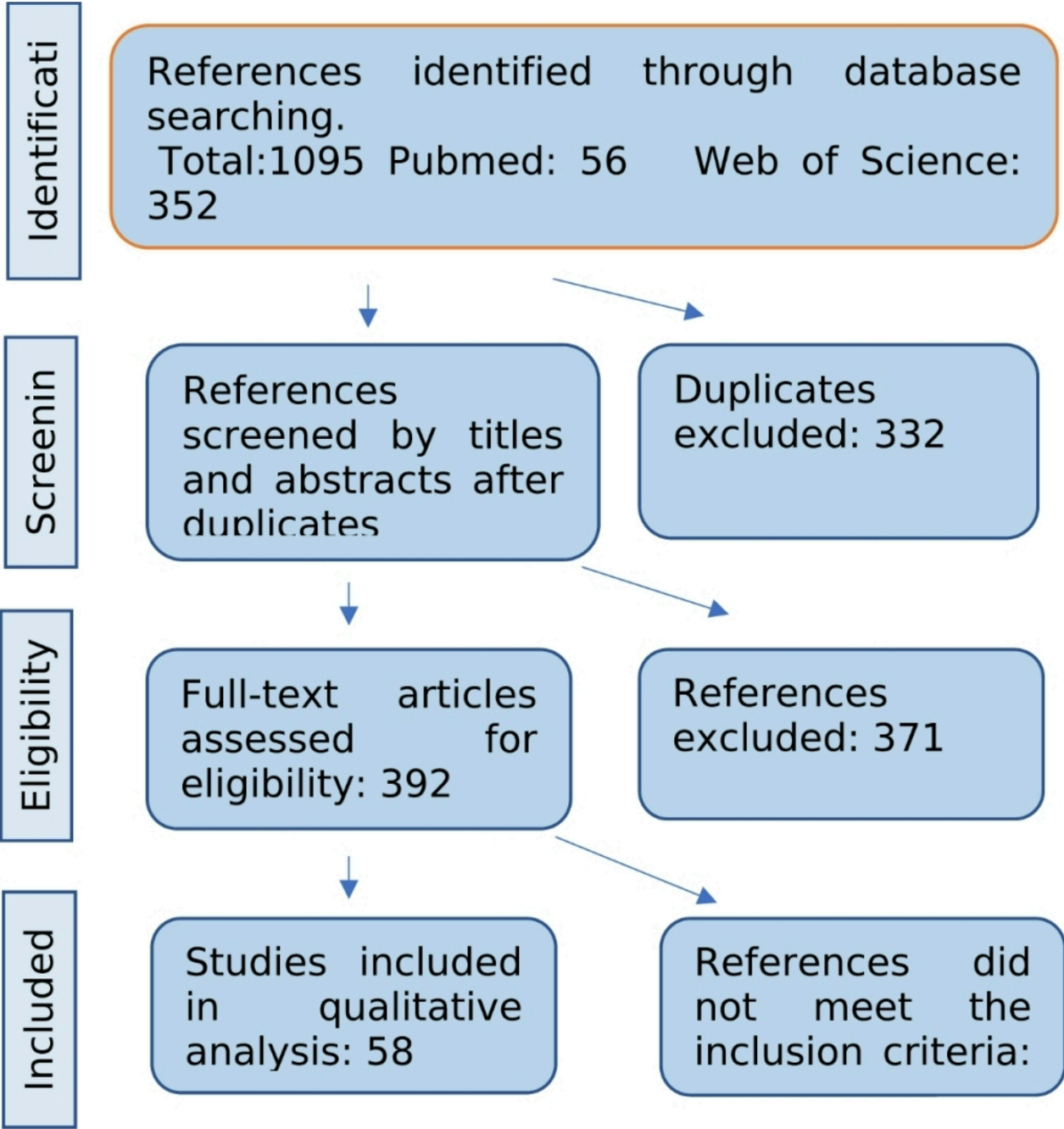


Figure 1

PRISMA chart

Supplementary Files

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- [Additionalfile1.xlsx](#)