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Review Article

Tooth Regeneration: What We Have Now and What's Next?

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Abstract

Tooth regeneration gained great attention in the past decade. Researchers have explored various aspects, from partial tooth regeneration to whole tooth regeneration. Great progress has been achieved with stem cells and materials developments. However, there are still no reports about successful whole tooth growth in vitro. In this review, the recent progress on the major factors contributing to tooth regeneration were summarized including the cell sources, the scaffolds & materials, the growth factors, and the incubation systems. In addition, the potential issues and resolutions were discussed based on the available info in light of providing a full picture of the field and help for determining future directions.

Keywords: ***Tooth regeneration, iPS cells, Mesenchymal Stem Cell, 3D printing, Dental Origin Stem cells, 3D culture, PRP, PRF.***

Introduction

To fully grow a tooth, several elements are necessary. Cells are one of the key elements. Various types of cells have been explored as seeds for regrowing teeth or repairing parts of the tooth in recent years. Induced pluripotent stem (iPS) cells which are reprogrammed somatic cells, hold potential in regenerating dental structures, though challenges remain in deriving dental pulp stem cells necessary for dentin-pulp complex regeneration [1]. iPSCs can also form ameloblasts for enamel regeneration and have shown success in regenerating periodontal ligament (PDL) tissue in animal models. Mesenchymal stem cells (MSCs) show promise in tooth regeneration as well, including pulp and dentin formation [2]. Adipose-derived MSCs have shown effectiveness in regenerating periodontal tissue in animal models and may be suitable for allogeneic transplants due to their immune-modulating properties [3]. Dental-derived stem cells from human teeth demonstrate potential in regenerating dental pulp and root development [4]. In addition, endogenous stem cells homing showed promising but inconsistent results in clinical trials for regenerative endodontics, particularly in immature teeth [5]. Then, which type of cells would be the best for tooth regeneration? Or a combination of different types of cells would be helpful in the tooth regeneration or regrowth?

Scaffolds play critical roles in tissue engineering, particularly for bone and dental tissue regeneration. Common scaffold types include injectable hydrogels, electrospun scaffolds, and bioprinted scaffolds, each offering unique advantages in facilitating cell integration and bioactive factors delivery [6]. Hydrogel systems show promise for precise integration in dental bone tissue reconstruction [7]. Biomimetic scaffolds mimic natural structures and are effective for periodontal regeneration, demonstrated by studies using decellularized human tooth scaffolds seeded with dental pulp and PDL stem cells, resulting in regeneration of pulp and periodontal tissues in animal models [8,9]. However, it is not sustainable. Synthetic polymers like polylactic acid (PLA), polyglycolic acid (PGA), and bioceramics such as calcium phosphates are also utilized, showing potential in bone defect repair [10]. Recently, nanomaterials have been shown enhancing cell growth and tissue regeneration with biomimetic characteristics, while biopolymers like alginate and chitosan offer eco-friendly alternatives for developing biocompatible scaffolds [11]. In addition, the development of 3D bioprinting enables precise fabrication of complex scaffolds, such as those combining treated dentin matrix with polymers for personalized bio-root regeneration in dental applications [12]. Then, which type of scaffolds would be the one for tooth regeneration?

Growth factors and other acellular factors are also playing key roles in tooth regeneration, recently, extracellular vesicles (EVs) and exosomes become prominent in cell-free therapies, especially in dentistry [13]. EVs offer several advantages over stem cells, such as lower risks of tumorigenicity and immunogenicity, easier handling, and fewer ethical concerns, making them suitable for dental and maxillofacial tissue repair. Studies have demonstrated the angiogenic and regenerative potential of EVs derived from dental pulp stem cells, particularly from teeth affected by periodontal disease, showing enhanced effects on endothelial cell functions and wound healing [14]. Exosomes from mesenchymal stem cells (MSCs) and dental stem cells are recognized for their superior biocompatibility and therapeutic effectiveness in pulp-dentin regeneration, osteogenesis, and intercellular communication, highlighting their potential as effective cell-free therapeutic tools in dental regenerative medicine [15-17]. Furthermore, growth factors in

platelet-rich fibrin (PRF) and platelet-rich plasma (PRP) are critical for dentin-pulp complex regeneration, promoting osteogenesis, tissue ingrowth, and vascularization [18,19]. Both PRP and PRF have demonstrated similar efficacy in regenerative endodontic treatments, with PRP being a viable alternative despite some issues, such as crown discoloration with PRF [20]. Which type of growth factors or acellular factors would be sufficient and more effective for tooth regeneration?

Beyond the cells, the scaffolds, and the growth factors, the incubation system where the tooth can be growing is also very important. So far, 2D cultures [21], 3D cultures [22], and even in vivo systems in animals [23] have been studied. In this review, the crucial aspects of tooth regeneration are summarized, and we aim to provide information to determine what would be the best options or methodical combination for tooth regeneration.

1. Cells for tooth regeneration

Various cell types were investigated for regenerating dental tissues. Including, induced pluripotent stem (iPS) cells, mesenchymal stem cells (MSCs), dental-derived stem cells and endogenous stem cells. In the following, their functions in tooth regeneration were discussed further and their advantages and limitations in tooth regeneration were also summarized (**Table 1**).

1.1. IPS cells and tooth regeneration

IPS cells are derived from reprogramming somatic cells, especially human dermal fibroblasts and peripheral blood mononuclear cells. There have been some successes in obtaining iPS cells from dental tissue which has turned out to be even more efficient than reprogramming from human dermal fibroblasts. iPS cells have been successfully obtained from dental pulp cells, dental pulp stem cells, stem cells from exfoliated deciduous teeth, stem cells from apical papilla, gingival fibroblasts, oral mucosa fibroblasts, and PDL cells (PDL) [1]. IPS cells can differentiate into many cells without limit [1]. Though unsuccessful, there has been crucial work done to regenerate dental pulp tissue. iPS-derived neural crest stem cells (NCs) have been demonstrated to differentiate into odontoblasts and endothelial cells which are all critical stepping stones to regenerating the dentin-pulp complex [1]. Dental pulp stem cells have not been successfully derived from iPS cells which is seen as a necessary step to regenerate the dentin-pulp complex. There is also progress being made on regenerating enamel with successful efforts in deriving ameloblasts, which are important in the formation of enamel, from iPS cells [1]. PDL (PDL) tissue has been demonstrated to be successfully regenerated from iPS cells in a rat periodontal injury model with BMP6 and hydrogel scaffolds [1]. Additionally, iPS cells can become a great source of stem cells for tooth regeneration. Research has demonstrated that iPS cells can differentiate into neural crest-derived mesenchymal stem cells (NC-MSCs) [24].

IPS cells can differentiate into the different types of cells which are necessary for tooth regeneration. This is the advantage of using iPS cells as seed to regenerate teeth. In addition, iPS cells can be generated from a patient's own cells which means there would be no immune rejection after the implantation of regenerated teeth. However, iPS cells also carry a risk of tumorigenesis which hinder its application in clinics. Because iPS cells can differentiate unlimitedly into many types of cells, there have been reports that iPS cells can result in teratomas [24].

1.2. MSCs and tooth regeneration

MSCs are another promising alternative for tooth regeneration. The location where MSCs could be most effective in tooth regeneration would be the oral cavity [2]. Dental Pulp Stem Cells (DPSCs), the first odontogenic MSCs isolated from dental pulp, can generate dental pulp, the vascular system, and dentin during in situ dental pulp regeneration [25-27]. MSC-derived exosomes can also help mediate tissue regeneration [28]. A study had shown that exosomes taken from PDL stem cells (PDLSCs) in healthy tissues (h-PDLSCS) were used on PDLSCs in inflamed periodontal tissues (i-PDLSCS). H-PDLSCS-derived exosomes enhanced the formation of mineralized nodules and increased osteogenic gene and protein expression in i-PDLSCs, restoring their differentiation capacity [28]. In rat models of periodontitis, treatment with h-PDLSCs-derived exosomes resulted in significant bone regeneration in the alveolar bone defects [28]. Therefore, MSCs-derived exosomes promise a cell-free therapeutic approach to periodontitis. Autologous MSCs transplantation is limited by factors such as age, systemic disease, and tissue quality [3]. However, MSCs have low levels of MHC class II molecules and no co-stimulatory molecules such as CD80 and CD86. This characteristic makes them suitable for allogeneic applications without triggering an immune rejection [29]. That's why a study assessed the efficacy of allogeneic transplantation of MSCs derived from adipose tissue, the adipose-derived multi-lineage progenitor cells (ADMPC) [3]. These cells could differentiate into bone, fat, and PDL tissues [3]. They also showed high levels of immune-suppressing factors when treated with certain cytokines [3]. In a pig model, transplanting ADMPC from donors significantly regenerated bone in periodontal defects. The results were comparable to using the pig's own cells [3]. This suggests that ADMPC can modulate the immune response and effectively regenerate periodontal tissue through donor transplants.

MSCs are gaining attention for their pluripotency and immunomodulatory properties [30]. Despite encouraging results in preclinical animal models, most clinical trials using MSCs therapy for various human diseases have fallen short of expectations [30]. This discrepancy is mainly due to inconsistent criteria for MSCs identification and the cells' inherent heterogeneity [30]. One major challenge in MSCs clinical applications is maintaining consistent quality and characteristics. Factors such as donor variability, tissue source, and culture conditions contribute to MSCs heterogeneity, impacting their therapeutic efficacy. Additionally, MSCs immunocompatibility can be compromised by inflammatory molecules produced from the microenvironment inflammatory responses, which may alter MHC-II expression in MSCs increasing the rejection risk [30]. To overcome these issues, a deeper understanding of the molecular and cellular mechanisms underlying MSCs function and immune interactions is necessary.

Also, the stability of MSCs' stemness and their differentiation capability are vital for therapeutic applications. However, extended culture periods and high passage numbers can lead to cell senescence and reduced regenerative potential. Therefore, it is crucial to develop standardized manufacturing processes and techniques for large-scale MSCs expansion without compromising cell quality. The therapeutic effectiveness of MSCs also relies on their migration and homing abilities, which varies depending on the administration route (local/systemic), injection site, infusion timing, and the cell carrier materials used [30]. It might be improved through genetic modifications and optimized delivery methods [30].

In addition, the paracrine effects of MSCs, involving the secretion of cytoprotective factors and extracellular vesicles (EVs), are significant for their therapeutic action. However, the diverse active components released by MSCs are influenced by the host microenvironment, such as, the inflammation status, hypoxia status, and ECM ect., leading to a wide range of factors that shape the distinct functions of MSCs [29]. So, understanding the interactions

and functions of these secreted components can maximize their potential. Addressing challenges related to MSCs heterogeneity, immunocompatibility, stability, and expansion is essential for translating promising preclinical results into successful clinical applications.

1.3. Dental originated stem cells and tooth regeneration

The human origin of dental-derived stem cells can be used to promote dental pulp regeneration and root development for pulp necrosis [4]. Human pulp stem cells (PSCs) show great promise in endodontic regeneration due to their strong angiogenic, neurogenic, and odontogenic capabilities [31]. They exhibit significant therapeutic potential due to their multipotency and sensitivity to local paracrine activity. A clinical trial study involving 40 patients with pulp necrosis demonstrated that deciduous autologous tooth stem cells could regenerate dental pulp with odontoblasts, blood vessels, and nerves in animal models [11].

1.4. Endogenous stem cells and tooth regeneration

The cell homing feature leverages endogenous stem cells' capacity to migrate and regenerate tissue, guided by physicochemical or biological stimuli or passive blood flow from apical tissue [5]. In 9 clinical trials, the cell-homing in regenerative endodontics shows promising results, particularly in immature teeth, suggesting potential for clinical application [5]. However, results are inconsistent in mature teeth. More studies are needed to further valid the efficacy.

Table 1: Different cell types' contributions to tooth regeneration.

Cell types	Origin	Give rise to cell types	Duration	Advantages	Limits
IPS cells	- PBMC - Skin fibroblast cells	- Dental pulp cells - Dental pulp stem cells - Neural crest stem cells - Odontoblasts - Endothelial cells	4 weeks	- Can differentiate into many different types of cells - Can be taken from your own cells meaning after insertion, there will be no immune response	- Risk of tumorigenesis
MSC	- Bone marrow - Oral cavity - Adipose tissue - Cord blood - Placenta - Period	- Dental pulp - Vascular system - Dentin - Bone - Fat - PDL tissue	8 weeks	- Ability to modulate the immune system	- The homing or migratory ability of MSCs varies depending on the administration route - Immune compatibility can be influenced by environmental inflammatory molecules - Active components released by MSCs are influenced by the host microenvironment (inflammation status, hypoxia, and ECM).
Dental-derived stem cells	- Dental pulp cells	- Odontoblasts - Blood vessels - Nerves	8 weeks	- Exhibits strong capabilities in angiogenesis, neurogenesis, and odontogenesis - Possesses significant therapeutic potential due to its multipotency and responsiveness to local paracrine signals	- The cell source might be limited - Non-autologous applications may trigger immune rejection
Endogenous stem cells	- blood influx, - remaining pulp parts, - stem cells, or - adjacent dentin	- Immature tooth	6 - 42 months	- Autologous, no immune rejection	- Not for mature tooth

2. Scaffolds, 3D printing and tooth regeneration

Common scaffold types include injectable hydrogels, electrospun scaffolds, and bioprinted scaffolds [6]. When the body cannot fully regenerate damaged tissue, bioactive functional scaffolds can aid in bone repair [7]. Scaffolds, constructed from artificial extracellular matrix (ECM), provide a supportive structure for new cell growth and tissue formation. Effective scaffolds must be non-toxic, biodegradable, biocompatible, low in immunogenicity, and safe. Dental tissue engineering has gained attention for utilizing natural or synthetic polymer scaffolds with desirable mechanical properties, such as small pore size and high surface-to-volume ratio, to promote cell regeneration [32]. The application, advantages and limitations of the scaffolds that have been investigated are summarized (**Table 2**) and discussed in detail as follows.

2.1 Biomimetic Scaffolds and tooth regeneration

Biomimetic scaffolds are candidates for scaffolds that could foster periodontal regeneration for both hard and soft tissues [9]. A study demonstrated the use of a decellularized human tooth as a possible scaffold for regenerating PDL and pulp tissue, creating this scaffold by removing cells from a human tooth [33]. They then seeded it with periodontal ligaments stem cells (PDLSCs) and dental pulp stem cells (DPSCs) obtained from human teeth. These seeded scaffolds were transplanted into immunosuppressed mice. After 9 weeks, the PDLSCs exhibited regeneration of the cementum/PDLs complex, while the DPSCs showed potential for pulp regeneration through gene expression associated with revascularization and hard tissue formation. The findings suggest that a decellularized human tooth scaffold, in combination with PDLSCs and DPSCs, can effectively regenerate PDLs and pulp tissues.

Another study explores the use of decellularized tooth buds (dTBs) from unerupted porcine teeth as potential scaffolds for bioengineering whole teeth [34]. Porcine tooth buds were decellularized using sodium dodecyl sulfate/Triton-X cycles and categorized into four types for implantation: 1) acellular dTBs, 2) recellularized dTBs with porcine dental epithelial cells, human dental pulp cells, and human umbilical vein endothelial cells, 3) dTBs seeded with bone morphogenetic protein (BMP)-2, and 4) non-decellularized natural tooth buds. These implants were placed into the mandibles of Yucatan mini-pigs and grown for 3 or 6 months. The histological analysis and micro-computed tomography (CT) revealed that recellularized dTBs and natural tooth buds developed organized dentin, enamel-like tissues, and cementum while acellular dTBs and dTBs with BMP-2 did not. Decellularized human tooth scaffolds also helped in the formation of tooth root structures. After 6 months, the implants formed bioengineered teeth comparable in size to natural teeth. This study is among the first to demonstrate the potential of dTBs for functional whole tooth regeneration.

2.2. Synthesized materials and tooth regeneration

Synthetic polymers include polylactic acid (PLA), polyglycolic acid (PGA), polycaprolactone (PCL), poly (ethylene glycol) (PEG), Poly Lactic-co-glycolic Acid (PLGA) and Zwitterionic polymers [10].

2.2.1. Polylactic acid (PLA) and PLA derivatives for tooth regeneration.

The use of adult dental pulp stem cells on PLA scaffolds for regenerating teeth has been studied and possible [35]. A study involving extracting teeth from both sides of the lower jaws of two adult dogs. Dental pulp stem cells were isolated and placed on 3D-printed hydroxyapatite/PLA (HA/PLA) scaffolds, then transplanted into the left lower jaw of each dog. The right lower jaw received cell-free scaffolds as a control group. Mineralization was monitored using polychrome sequential labeling and dental cone beam computed tomography (CBCT). Nine months post-surgery, the results indicated that mineralization was significantly higher in the experimental group with cell-seeded scaffolds compared to the control group. However, the HA/PLA scaffolds did not fully absorb in either group [35]. The study concluded that dental pulp stem cells play a crucial role in tooth regeneration, and that despite PLA scaffolds' partial successes, faster-absorbing scaffold materials are needed for effective tooth regeneration. PLA scaffolds have also been used to regenerate dental cementum [36].

Also, traditional treatments for periodontal and other bone defects typically involve using barrier membranes for guided tissue regeneration (GTR) and guided bone regeneration (GBR) [37]. However, these membranes often lack the ability to actively influence the bone healing process. To resolve this issue, a biomimetic bone tissue engineering strategy using a novel Janus porous polylactic acid membrane (PLAM) was introduced. The PLAM was created through unidirectional evaporation-induced pore formation, followed by the self-assembly of a bioactive metal–phenolic network (MPN) nano interface. The PLAM-MPN has dual functions: a barrier function on its dense side and a bone-forming function on its porous side. In vitro tests showed that the MPN nano interface significantly reduced proinflammatory responses in mice bone marrow-derived macrophages (BMDMs), promoted angiogenesis in human umbilical vein endothelial cells (HUVECs), and improved the attachment, migration, and osteogenic differentiation of human periodontal ligament stem cells (hPDLSCs). When implanted into rat periodontal bone defects, PLAM-MPN significantly enhanced bone regeneration. This bioactive MPN nanointerface within a Janus porous membrane offers versatile capabilities to regulate cellular activities that promote bone regeneration, showing great potential for clinical GTR and GBR applications [37].

2.2.2. Application of PLGA for Periodontal Regeneration

PLGA polymers are valued for their biodegradability, biocompatibility, and ease of manipulation, making them ideal candidates for GTR membranes [38]. Preclinical research in the 1990s highlighted the success of PLGA membranes in accelerating periodontal tissue regeneration in primates with defects similar to human conditions. Studies on rhesus monkeys with intrabony defects revealed that PLGA membranes significantly promoted the formation of new cementum, bone, and connective tissue adhesion compared to flap surgery alone [38]. Clinical trials in humans, starting in the late 1990s, demonstrated that PLGA membranes effectively improved clinical attachment levels, reduced pocket probing depths, and increased bone fill in intrabony and Class II furcation defects [38]. Also, the clinical performance of PLGA membranes has been compared with other non-resorbable and resorbable membranes. A ten-year study comparing PLGA and ePTFE (a typical non-resorbable membrane used in clinic) membranes showed significant gains in tissue attachment and bone formation with both materials [38]. Although PLGA membranes often resulted in higher reductions in pocket probing depths and greater clinical attachment level gains, these differences were not statistically significant due to small sample sizes and subject variability. Comparisons with other resorbable membranes, such as collagen, indicated similar outcomes in periodontal regeneration, particularly in promoting the regeneration of cementum and PDLs [38].

PLGA can also serve as an active reagents carrier for tooth regeneration. It has been utilized as a carrier for delivering growth factors, antibiotics and genetic material to enhance periodontal regeneration. Growth factors such as PDGF and rhGDF-5 have been delivered using PLGA-based systems, resulting in improved alveolar bone and PDL formation. Core-shell PLGA microspheres have been designed to achieve the sequential release of different drugs [38], mimicking physiological patterns and enhancing regenerative outcomes. Advanced techniques, including surface coating and electrospinning, have been developed to achieve sustained and controlled drug release, further promoting the regeneration of cementum and PDLs [38]. Study has shown topographic cues from artificial scaffolds regulate cell function, focusing on the roles of Yes-associated protein (YAP) and β-catenin signaling in the differentiation of dental pulp stem cells (DPSCs) [39]. Researchers used a PLGA membrane to investigate spontaneous odontogenic differentiation of DPSCs. Results indicated that the topographic cues of the PLGA scaffold enhance odontogenic differentiation of DPSCs and pulp tissue through the YAP/β-catenin signaling pathway [39]. Another study showed that PLGA scaffolds loaded with plasmid DNA encoding fibroblast growth factor-2 (pFGF-2) on human PDL cells (hPDLCs) in vitro, and evaluated the scaffold's ability to promote PDL regeneration in a beagle dog teeth avulsion model [40]. Results showed that the PLGA/pFGF-2 scaffold facilitated the formation of more regular PDL-like tissues and reduced root surface resorption compared to the PLGA alone scaffold. These results present a promising method of scaffolds and active reagents combinations for improving the treatment prognosis of replanted teeth, offering a novel approach for enhancing tissue regeneration.

Table 2: Different scaffolds materials application in tooth regeneration.

Scaffold Materials	Were used to regenerate tooth components					
	Cementum periodontal complexes	pulp	root	enamel	Periodontal ligament (PDL)	Dentin
PLA (Polylactic Acid)	Yes	Yes	Not reported	Not reported	Yes	Not reported
Poly Lactic-co-glycolic Acid (PLGA/PLG)	Yes	Yes	Not reported	Not reported	Yes	Not reported
Decellularized human tooth	Yes	Yes	Yes	Yes	Yes	Yes
Treated Dentin Matrix (TDM)	Yes	Yes	Yes	Yes	Not reported	Yes

2.3. Biopolymers and tooth regeneration

In recent years, biopolymers have gained significant attention for their potential to develop high-performance biocomposites with low environmental impact [41]. These materials are valued for their abundance, renewability, eco-friendliness, and lightweight properties. By blending biopolymers with suitable additives, desired properties can be achieved through effective polymer-filler interaction. The chemical composition, degradation kinetics, and mechanical properties of biopolymer composites can be tailored to meet specific application needs. Interfacial interactions between the biopolymer and the nanofiller significantly influence the mechanical properties of these composites. Natural polymers include alginate, cellulose, chitosan, silk, collagen, gelatin, fibrin, laminin, decellularized extracellular matrix, and hyaluronic acid [10].

For tissue engineering, biomaterials must be safe, non-immunogenic, and effective [12]. Treated dentin matrix (TDM) is promising for tooth regeneration, though the effects of sterilizing allogenous TDM are unclear. A study evaluated autoclaved TDM (a-TDM) and its interaction with dental pulp stem cells (DPSCs) through various microscopy techniques and molecular analyses in vitro, along with tests in mouse and goat models in vivo. The findings revealed that a-TDM effectively promoted the formation of new dentin-pulp-like tissues, enamel dental pulp, and cementum periodontal complexes. Key markers for dentin, enamel, and periodontal tissues were identified in the regenerated structures through immunohistochemistry. Consequently, a-TDM with DPSCs serves as a potent scaffold for cell proliferation and differentiation, highlighting its potential as a biomaterial for tooth regeneration [12].

2.4. 3D printing application with different materials for tooth regeneration

The advent of bio-three-dimensional (bio-3D) printers has significantly advanced regenerative medicine, enabling the creation of three-dimensional constructs like spheroids that closely mimic physiological conditions through the secretion of extracellular matrix proteins by cells [42]. One study aimed to develop a 3D construct as a model for the dentin-pulp complex using O9-1 cells derived from the cranial neural crest of mice. The 3D construct was formed by assembling spheroids onto a needle array with a bio-3D printer. Results showed enhanced expression of extracellular matrix proteins tenascin C and DMP1 in the spheroids compared to two-dimensional cultures. Notably, tenascin C was prevalent in the outer layer of spheroids cultured in embryonic stem cell medium, while DMP1 expression was induced in a calcification-induction medium and localized to the outermost layer. These findings suggest that the 3D constructs with polarized expression of tenascin C and DMP1 resemble the dental papilla, indicating their potential as artificial models for studying odontogenesis.

Another feature of 3D bioprinting is allowing precise deposition of cells in supportive bioinks to create complex scaffolds for targeted tissue repair [43]. One study introduces a personalized scaffold with multiple bioactivities, such as promoting stem cell proliferation and differentiation, biomimetic mineralization, and angiogenesis [44]. A novel bioink system was developed using a biocompatible and biodegradable polymer enhanced with treated dentin matrix (TDM) from dentin tissue. The inclusion of TDM improves the scaffold's microstructure, hydrophilicity, and mechanical strength. When combined with dental follicle cells, the personalized TDM scaffold mimics the native tooth root's anatomy and physiology three months after transplantation in beagles. The results indicate that 3D-printed TDM scaffolds have significant bioactivities and hold great clinical potential for treating tooth loss.

3D-printing also has relevance to alveolar ridge preservation techniques [45]. Using a bone substitute is crucial to prevent alveolar ridge resorption. However, developing materials that offer sufficient mechanical strength, bioactivity, osteoinductivity, and compatibility with the tooth extraction socket remains challenging. One study introduces a new photo crosslinked composite ink made from nacre, polyurethane (PU), and polyhedral oligomeric silsesquioxane (POSS) to create 3D porous scaffolds for alveolar ridge preservation. The nacre/PU/POSS (NPP) composite was evaluated for its rheological behavior, mechanical properties, and surface hydrophilicity. In vitro experiments confirmed the biomineralization of the NPP scaffolds, which also supported the homogeneous distribution and proliferation of MC3T3-E1 cells. When grafted into extraction sockets, the NPP scaffolds reduced alveolar bone resorption and promoted new bone formation [45]. These findings suggest that NPP composites are promising materials for alveolar ridge preservation and the 3D printing of bone grafts in future applications.

Digital light projection (DLP) printing of hydroxyapatite (HAp) bioceramic offers a promising method for creating high-resolution, personalized bio-tooth root scaffolds [46]. Compared to natural decellularized dentine (NDD) scaffolds, the DLP-printed bio-tooth roots had precise shapes, excellent structure, and smooth surfaces, meeting various personalized requirements. Sintering the bioceramic at 1250 °C improved HAp's physicochemical properties and nearly doubled its elastic modulus compared to NDD scaffolds [46]. Additionally, hydrothermal treatment applied a nano-hydroxyapatite whisker (nano-HAw) coating, which enhanced mechanical properties and surface hydrophilicity, promoting dental follicle stem cells' (DFSCs) proliferation and osteoblastic differentiation [46]. Subcutaneous transplantation in mice and in-situ transplantation in rat alveolar sockets showed that nano-HAw-containing scaffolds facilitated DFSCs differentiation into PDL-like structures [46]. So, optimizing sintering temperature and applying a nano-HAw coating through hydrothermal treatment make DLP-printed HAp bioceramic scaffolds a promising option for personalized bio-root regeneration. It might be applicable to other materials as well to improve the properties of the scaffolds.

Another challenge in bioengineered tooth roots is long-term functional stability due to an ineffective cervical sea when using biomaterial scaffolds, though they mimic the mechanical strength of natural tooth roots [12]. One study explores a bioinspired method by incorporating two critical odontogenic growth factors, TGF-β1 and BMP4, into a treated dentin matrix (TDM) scaffold [37]. This setup

promotes functional bio-root regeneration by creating a spatial interface gradient. The study examined how TGF-β1/BMP4 affected dental follicle stem cells (DFSCs) through gene and protein expression analysis, as well as subcutaneous and jaw bone transplantation. An artificial crown with TDM/TGF-β1/BMP4/DFSCs was tested on beagles to evaluate occlusal function. TGF-β1 and BMP4 together enhanced DFSC proliferation, migration, and osteoinductive differentiation, boosting the expression of markers like Periostin, BSP, OPN, ALP, Vinculin, and Paxillin. The TDM/TGF-β1/BMP4/DFSCs composites provided a consistent inductive interface for neovascularization and enthesis regeneration. After more than five months, the bio-roots in beagles exhibited excellent biomechanical properties and healthy gingiva akin to natural teeth [37]. This approach presents a novel strategy for biomimetic tooth regeneration.

3. Acellular Factors supporting tooth regeneration

Acellular factors play a pivotal role in tooth regeneration through various mechanisms. Growth factors are crucial for tissue repair and regeneration, promoting cell proliferation, differentiation, and tissue organization, although their clinical application is limited by issues like short lifespan and rapid diffusion. Extracellular vesicles (EVs), nanoscale membrane structures filled with lipids, proteins, and nucleic acids, facilitate intercellular communication and have emerged as promising agents in tissue regeneration due to their lower tumorigenic and immunogenic risks compared to stem cells. Exosomes, another form of acellular factors, offer significant advantages due to their cost-effectiveness, biocompatibility, and low immunogenicity, making them valuable for dental pulp regeneration and periodontal repair. Platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) are also utilized for enhancing tissue regeneration, with PRP showing effectiveness in regenerative endodontics and PRF demonstrating benefits in bone growth and vascularization. Together, these acellular factors provide a range of therapeutic options for advancing tooth regeneration, each with unique advantages and limitations (Table 3).

3.1. Growth Factor and tooth regeneration

Growth factors (GFs) are essential for driving tissue repair and regeneration, making them key elements in the field of regenerative medicine [47]. Growth factors (GFs) are essential bioactive molecules that orchestrate a wide range of cellular processes, including cell proliferation, migration, differentiation, and tissue organization during development and repair. Despite their potential demonstrated in preclinical studies, their translation into clinical practice has faced significant obstacles, such as their short lifespan, rapid diffusion away from target sites, and lack of cost-effectiveness. How GFs can be sustainable or slow release are the challenges.

Growth factors can stimulate reparative dentinogenesis and pulp-like tissue formation [48]. Some possible growth factors are enamel matrix derivative, platelet-derived growth factor, platelet concentrates, and fibroblast growth factor-2 [40]. Concentrated growth factor (CGF), derived from platelets, is an excellent scaffold for addressing dentin pulp disorders due to its natural origin, ease of use, and biodegradable nature [49]. Its application is expanding in both medical and dental fields for bone repair and soft tissue healing. Research in vitro has shown that CGF significantly boosts the proliferation and migration of dental stem cells [49]. Another study proposed platelet-derived growth factor-BB (PDGF-BB) is a promising treatment to prevent tooth ankylosis which is a pathological condition involving improper PDL restoration after tooth replantation [16]. Using a rat tooth replantation model, researchers found that PDGF-BB pretreatment significantly reduced ankylosis and restored functionally organized PDL collagen fibers. The mechanical strength of the healing PDL reached 76% of that in normal, non-replanted teeth after 21 days. In vitro, PDGF-BB enhanced the proliferation and migration of human periodontal fibroblasts, downregulated RUNX2 and ALP mRNA expressions, and inhibited Wnt3a-induced upregulation of β-catenin, AXIN2, RUNX2, COL1A1, and ALP mRNA expressions [16]. These findings suggest that topical PDGF-BB treatment promotes cell proliferation and migration, inhibits canonical Wnt signaling, and aids in the functional restoration of the PDL after tooth replantation, indicating its potential clinical application to reduce ankylosis and promote proper PDL regeneration.

Nerve growth factor (NGF) is crucial for the development and differentiation of both neuronal and non-neuronal cells [50]. A study's results underscore NGF's vital role in both dental injury response and regeneration, highlighting its potential for use in pulp regeneration therapies.

The study explored the presence of NGF and its receptors, p75NTR and TrkA, in human teeth under normal and pathological conditions. In healthy teeth, NGF, p75NTR, and TrkA are minimally expressed in dental pulp fibroblasts and odontoblasts but are prominently present in the nerve fibers of the dental pulp. In contrast, injured and decayed teeth show a marked increase in NGF and TrkA expression in odontoblasts near the damage, linking NGF signaling to dental repair. During the differentiation of primary human dental mesenchymal cells into odontoblasts, NGF and TrkA expression also rises significantly. Furthermore, applying NGF to cultured human tooth slices led to significant axonal growth and Schwann cell migration.

3.2. Extracellular Vesicles (EVs) and tooth regeneration

EVs are membrane structures containing lipids, proteins, and nucleic acids (miRNA, mRNA, and DNA), crucial for intercellular communication, with size ranging from 30 nanometers (nm) to 10 micrometers (μm) [13]. Recently, EVs have gained attention for their potential in tissue regeneration through cell-free therapies in stomatology. EVs play roles in repairing and regenerating dental and maxillofacial tissues, including dental and periodontal tissues, maxilla and mandible bones, temporomandibular joint cartilage, peripheral nerves, and soft tissues. One study explored the angiogenic potential of EVs from dental pulp stem cells (DPSCs) of teeth affected by periodontal disease (P-DPSCs) in comparison to those from healthy teeth (H-DPSCs) [14]. Both types of conditioned media (CM) and EVs promoted angiogenesis in endothelial cells (ECs) in both lab and animal models, with the effect being reduced when EV secretion was inhibited. Notably, EVs from P-DPSCs (P-EVs) were more effective at enhancing EC proliferation, migration, tube formation, and angiogenesis-related gene and protein expression than EVs from H-DPSCs (H-EVs). P-EVs also significantly improved wound healing and new blood vessel formation in a skin defect model [14]. These results suggest that P-DPSCs are a promising source for regenerative medicine and cellular therapies. Meanwhile, this result also suggests to us that the origin of the EVs are very important, and in some cases the health source may not be the right one to go with.

The formation of the dentin-pulp complex involves intricate interactions between Hertwig's epithelial root sheath cells (HERS) and dental papilla cells (DPCs) [51]. One study explored the role of exosome-like vesicles (ELVs) secreted by HERS in this process, hypothesizing that ELVs might mediate HERS functions and induce specific differentiation of dental mesenchymal cells. ELVs derived from a HERS cell line (ELVs-H1) were characterized and tested for their ability to influence DPC behavior. In vitro experiments

demonstrated that ELVs-H1 promoted DPC proliferation, migration, and odontogenic differentiation, while activating Wnt/β-catenin signaling and supporting tube formation and neural differentiation. In vivo, ELVs-H1 combined with DPCs in a collagen gel facilitated the regeneration of dental pulp-dentin-like tissue, including both reparative dentin-like hard tissue and soft tissue with blood vessels and neurons [51]. These results indicate that ELVs-H1 can create a conducive microenvironment for dental mesenchymal stem cell differentiation and may be valuable for dental pulp-dentin tissue regeneration.

3.3. Exosomes and tooth regeneration

Exosomes, key paracrine effectors and nanoscale EVs essential for intercellular communication, have gained attention for their role in pulp-dentin complex regeneration [52]. They are seen as promising therapeutic tools for dental pulp regeneration due to their cost-effectiveness, wide availability, good biocompatibility, and high safety [52]. Recent advances in exosome research highlight their benefits over traditional cell therapies, including "cell-free" properties, low immunogenicity, high biological safety, and effective action [53]. These advantages help overcome issues like immune rejection, donor cell scarcity, heterogeneity, and ethical concerns. While traditional root canal therapy is the primary treatment for irreversible pulpal disease, it does not fully restore pulp function. Stem cell transplantation shows promise but faces challenges like cell heterogeneity and poor regeneration. "Cell-free" exosome therapies, particularly those derived from odontogenic stem cells, offer significant potential for dental pulp regeneration, showing superior therapeutic effects compared to non-odontogenic stem cell-derived exosomes. Despite the promise, few studies have comprehensively reviewed the critical factors in this process.

3.3.1 MSCs origin exosomes in tooth regeneration

Exosomes could be from different stem cells origin, which also play different roles in the tooth regeneration. Studies have demonstrated that MSCs can promote bone regeneration through exosome-delivered messages. While the exact osteogenic mechanisms are not fully understood [54], it is evident that MSC-derived exosomes (MSC-Exos) play a crucial role in bone regeneration. They can directly influence osteogenesis-related cells, carry substances that affect cellular activators or the local environment, or bind to materials to activate the osteogenic framework. Another study investigated the effects of MSC exosomes on dental pulp cells (DPCs), demonstrating that MSC-Exos enhance DPC migration, proliferation, and odontogenic differentiation. These enhancements occur via exosomal CD73-mediated activation of adenosine receptors, which in turn activates AKT and ERK signaling pathways [17]. In a rat pulp defect model, MSC-Exos increased the expression of dentin matrix proteins and promoted the formation of dentin-like tissue. Additionally, MSC-Exos facilitated the recellularization of pulp-dentin tissues in human premolars implanted subcutaneously in mice [17]. These findings suggest that MSC-Exos have a significant impact on DPC functions and hold promise as a cell-free therapeutic alternative for pulp-dentin regeneration.

In another study examined the therapeutic effects of human MSC exosome-loaded collagen sponges on periodontal intrabony defects in an immunocompetent rat model [17]. Compared to control rats, those treated with exosomes showed more efficient repair of defects, including the regeneration of periodontal tissues such as new bone and PDL. This improvement was associated with increased cellular infiltration and proliferation, suggesting that MSC exosomes promote regeneration by enhancing cellular mobilization and proliferation. In PDL cell cultures, MSC exosomes were found to boost PDL cell migration and proliferation via CD73-mediated adenosine receptor activation of pro-survival AKT and ERK signaling pathways. Blocking AKT or ERK phosphorylation inhibited these effects. This study is the first to demonstrate that MSC exosomes enhance periodontal regeneration by increasing PDL cell migration and proliferation, indicating that MSC exosomes are a viable, ready-to-use, cell-free therapeutic option for periodontal defects.

3.3.2. Exosomes from dental origin stem cells and tooth regeneration

Exosomes were derived from human dental pulp stem cells (hDPSCs-Exos) that had undergone different periods of osteogenic differentiation, purified, and loaded onto the scaffolds. Scaffolds loaded with exosomes from osteogenically differentiated hDPSCs showed superior bone regeneration within 10 weeks compared to those with exosomes from undifferentiated hDPSCs. RNA sequencing revealed that osteogenic exosomes carried specific cargo, including upregulated miRNAs (Hsa-miR-29c-5p, Hsa-miR-378a-5p, Hsa-miR-10b-5p, Hsa-miR-9-3p) linked to osteogenesis and downregulated anti-osteogenic miRNAs (Hsa-miR-31-3p, Hsa-miR-221-3p, Hsa-miR-183-5p, Hsa-miR-503-5p) [16]. This study concludes that exosomes from osteogenically differentiated stem cells present a novel, promising strategy for cell-free bone regeneration.

In addition to promoting bone regeneration, hPDLSCs-Exos also play therapeutic roles for treating H2O2-induced osteoblastic damage. This was due to the hPDLSC-Exos promote hFOB1.19 cell proliferation, migration, and osteogenic differentiation, inhibit H2O2-induced apoptosis, and activate the PI3K/AKT and MEK/ERK pathways. Also, study in periodontitis showed that hPDLSCs-Exos can modulate macrophage phenotypes and are effective in treating periodontitis. This study demonstrated that incorporating hDPSCs-Exos into chitosan hydrogel (hDPSCs-Exos/CS) accelerates the healing of alveolar bone and periodontal epithelium in mice with periodontitis [55]. hDPSCs-Exos/CS treatment improves periodontal lesions by reducing inflammation and modulating the immune response. Specifically, hDPSCs-Exos/CS promotes the conversion of macrophages from a pro-inflammatory to an anti-inflammatory phenotype, potentially due to the presence of miR-1246 in hDPSCs-Exos. These findings highlight the therapeutic mechanism of hDPSCs-Exos/CS and provide a foundation for developing effective treatments for periodontitis.

Also, exosomes derived from rabbit dental pulp stem cells (rDPSCs-Exos) exhibit the effects of on cell homing and angiogenic differentiation for pulp regeneration [56]. rDPSCs-Exos were derived from rabbit dental pulp stem cells cultured under growth (Exo-G) or angiogenic differentiation (Exo-A) conditions and characterized using nanoparticle tracking analysis and an antibody array. rDPSCs-Exos at a concentration of 5 × 10^8/mL significantly promoted cell proliferation and migration. Gene expression analysis revealed that rDPSCs-Exos increased the expression of angiogenic markers, including VEGFA, FLT1, and PECAM1. Additionally, key exosomal microRNAs in Exo-A were identified as crucial for cell homing and angiogenesis. The study concludes that exosome-based strategies for cell homing and angiogenic differentiation hold considerable therapeutic potential for pulp regeneration.

3.3.3. Exosomes from other dental cells in tooth regeneration.

Regenerative endodontic procedures (REPs) offer a new treatment option for dental pulp and periapical diseases in permanent teeth with open apices, although they primarily generate cementum- or bone-like tissues rather than the actual dentine-pulp complex [57]. Enhancing dentine-pulp complex regeneration is thus a key research

focus. Stem cells from the apical papilla (SCAP) can differentiate into primary odontoblasts and dental pulp cells, essential for root dentine and dental pulp production. One study introduced SCAP-derived exosomes (SCAP-Exos) into root fragments with bone marrow mesenchymal stem cells (BMMSCs) and transplanted them into immunodeficient mice, resulting in dental pulp-like tissue formation and new dentine deposition. In vitro, SCAP-Exos significantly increased the gene and protein expression of dentine sialophosphoprotein and mineralized nodule formation in BMMSCs. SCAP-Exos were endocytosed by BMMSCs, enhancing their dentinogenesis. Therefore, exosomes from dental stem cells show potential as a therapeutic approach for dentine-pulp complex regeneration in REPs.

3.3.4. Limitations of Exosomes in tooth regeneration application

Regenerative therapy is needed for various dental issues such as craniofacial, osteochondral, periodontal, nerve, pulp injuries, endodontic problems, and osteoarthritis [58]. While stem cell use in dental tissue engineering has gained attention, clinical application remains challenging. As an alternative, cell-free tissue engineering using stem cell-derived exosomes is being explored. However, exosome use is limited by cells' secretion capabilities. To address this, researchers are studying efficient strategies for large-scale clinical applications, such as using ceramics-based scaffolds to enhance exosome production and secretion. More research is needed to refine these strategies for future regenerative treatments.

While exosomes have potential in tissue engineering, their rapid clearance in vivo limits their effectiveness. To overcome this, hDPSCs-Exos were embedded into a hydroxypropyl chitin (HPCH)/chitin whisker (CW) thermosensitive hydrogel, creating an exosome-loaded hydrogel (HPCH/CW/Exo). This hydrogel can be easily injected into irregular endodontic spaces and gelates in situ. In vitro experiments showed that the exosome-loaded hydrogel significantly enhanced odontogenesis and angiogenesis, and in vivo animal studies demonstrated the

formation of new dental pulp-like tissue in an implanted tooth root model. This system offers a promising alternative to keep exosome effects [59].In addition, there are significant challenges to their clinical translation. These include the need for standardized methods for exosomes separation and purification, large-scale pharmaceutical-grade production, and long-term biosafety evaluations. The heterogeneity of MSC-Exos, due to varying protein and RNA contents, affects their therapeutic outcomes, making it necessary to develop more precise methods to classify and produce uniform exosome subgroups. Additionally, the way MSCs are pretreated to stimulate exosome release can alter their properties, further complicating standardization. Optimizing factors such as dosage, timing, and transplantation site is crucial for maximizing their effectiveness in regenerative medicine.

3.4. Platelet-rich plasma(PRP)/Platelet-rich fibrin(PRF) and tooth regeneration

3.4.1 PRF and tooth regeneration

PRF is composed of a fibrin complex containing leukocytes, cytokines, and glycoproteins like thrombospondin [19]. It has demonstrated high success rates in procedures such as sinus lifts, healing extraction sockets, and treating periapical abscesses. Additionally, PRF is more cost-effective and easier to prepare than platelet-rich plasma, making it suitable for routine clinical use. PRF, either alone or combined with bone grafts, enhances bone growth and vascularization by facilitating the migration, attachment, and proliferation of osteoblasts, ultimately leading to bone formation [60]. Revascularization, which involves inducing a blood clot in the root canal space, has been a clinical success. PRF acts as an autologous scaffold in this process, helping repair iatrogenic perforations and revascularize immature teeth with necrotic pulps. It supports tissue ingrowth and offers benefits such as thickening dentinal walls, lengthening roots, reducing periapical lesions, and achieving apical closure.

3.4.2. PRP and tooth regeneration

PRP, derived from autologous blood and rich in key cytokines and growth factors, has garnered significant attention for its regenerative potential [18] as well. PRP delivers these essential components to targeted areas, promoting tissue regeneration. This method is now being applied to regenerate various tissues, including liver, bone, cartilage, tendon, and dental pulp. A study investigated the effectiveness of PRP as a scaffold in regenerative endodontic treatment compared to the conventional blood clot (BC) scaffold [61]. Twenty necrotic, single-rooted immature teeth were treated with either PRP or BC after disinfection with triple antibiotic paste, and covered with white mineral trioxide aggregate. Over an 18-month period, clinical and radiographic evaluations showed that all teeth remained asymptomatic, though one BC-treated tooth developed periapical pathosis. The study concluded that PRP is an effective scaffold for regenerative endodontic treatment, though outcomes were similar to those with the conventional BC scaffold. Another study uses PRP versus BC as scaffolds in regenerative endodontics (RET) for immature permanent anterior teeth with necrotic pulps in 26 patients, aged 12.66 ± 4.47 years [62]. Results are the same, both scaffolds showed significant improvements in these parameters with no significant difference between PRP and BC groups. Another study explored tissue regeneration in the pulp space of immature dog teeth with apical periodontitis using different treatments: BC, DPCs, PRP, and a combination of DPCs and PRP [63]. Fifty-six roots from beagle premolars were divided into four experimental groups and two control groups. After inducing apical periodontitis, root canals were disinfected and filled with the specified materials, sealed with MTA and composite, and analyzed after 90 days. Radiographic and histologic evaluations showed no significant difference in periradicular bone healing among the experimental groups, but those with DPCs exhibited greater root thickening and more mineralized tissue formation, particularly in the apical third. The PRP groups formed more tissues in the canals, and the DPCs + PRP group showed bone-like tissue growth from the periapical area into the canal. The study concluded that combining DPCs with PRP enhances vital tissue regeneration in the root canals of immature teeth with apical periodontitis.

Another case report examines a regenerative endodontic procedure using PRP in a 12-year-old boy's maxillary second premolar, 14 months post-treatment [64]. The patient presented with pain and sensitivity to cold, leading to a diagnosis of reversible pulpitis and normal periapical tissues. Despite the regenerative treatment, root canal therapy was performed due to the patient's complaints and guardian's insistence. Histological examination of tissue removed from the root canal revealed vital pulp-like connective tissue with minimal inflammation and no bone. These findings suggest that PRP can successfully generate pulp-like tissue in human teeth during regenerative endodontic procedures.

3.4.4. Comparing and Contrasting PRF and PRP

One study aimed to compare the regenerative potential of PRP and PRF in treating necrotic immature permanent maxillary central incisors [20]. In a double-blinded, parallel randomized controlled trial with 30 patients, 26 met the study requirements. Group I received PRP treatment and Group II received PRF treatment, with follow-ups every three months for one year. Clinical outcomes of pain,

mobility, swelling, and sinus/fistula, and radiographic outcomes for root length and width, bone density, and apical diameter were assessed. Results showed tooth survival at 12 months. PRP resulted in a marginal increase in root length and width, periapical bone density, and a decrease in apical diameter, with no significant difference compared to PRF. However, PRF led to more crown discoloration. None of the treated teeth responded to the sensibility test at the study's end. The study concluded that PRP is a viable alternative to PRF for revascularizing necrotic immature teeth, showing excellent 12-month prognosis.

Table 3: Acellular factors' role in tooth regeneration and their applications.

Acellular Factors	Origin	Supported tissues/cells	Application	Duration	Advantages	Limitations
Growth Factor	- Cells cultures - Plasma-rich fibrin	- Pulp-dentin complex - Dental nerves - PDL	- Rat tooth replantation model	4 weeks	- orchestrate a wide range of cellular processes, including cell proliferation, migration, differentiation, and tissue organization during development and repair	- Short half-life - Rapid dispersion from the target area - Poor cost-efficiency
Extracellular Vesicles (EVs)	- Immunocytes - Blood cells - MSCs - Urine - Breast milk - Saliva -Synovial fluid - plasma	- Dental pulp	- Mice skin defect model	2-4 weeks	- provide enhanced therapeutic efficacy and regenerative capacity - avoid risks like immune rejection, tumor formation, and blood vessel blockages - easier to store and handle	- Short half-life - Rapid dispersion from the target area - exosome use is limited by cells' secretion capabilities - heterogeneity affects their therapeutic outcomes
Exosomes	- Dental mesenchymal stem cells - Dental pulp stem cells - PDL stem cells	- Dental Pulp - Alveolar Bone - PDL - Dentin-pulp tissue	- In vivo rat studies			
PRP/PRF	- blood	- Dental pulp	- Clinical trial with 30 patients	12 months	- Serve as scaffold and provide concentrated growth factors simultaneously - Autologous avoid immune rejection	- No reports

4. Incubation System for tooth growing

4.1. In vitro incubation system and tooth regeneration

4.1.1. 2D culture system application in tooth regeneration

Explant culture is essential for developmental biology as it allows manipulation of developing organs at specific time points, but accessing and monitoring developing tissues ex vivo can be challenging [21]. Slice culture provides a solution by enabling observation of morphogenetic movements and targeting specific cell populations for manipulation or lineage tracing.

In 2D models, studying cell-cell and cell-matrix interactions is limited, whereas 3D models can more accurately replicate these interactions in vitro [22]. Despite these drawbacks, 2D cultures remain highly appealing for laboratory use due to their simplicity and cost-effectiveness [65].

4.1.2. 3D culture application in tooth regeneration

The development of 3D cell cultures has significantly advanced in vitro studies, bringing them closer to mimicking animal models [22]. These cultures offer more biologically accurate structures, allowing for the study of complex interactions that were previously unachievable with traditional 2D cultures. One study aimed to develop an in vitro tooth germ culture model using a 3D rotary cell culture system (RCCS) to determine its suitability for tooth germ development [66]. Mandibular first molar tooth germs from 1-day-old mice were cultured in RCCS for 3, 6, and 9 days. Development was monitored using histology, stereoscopic microscopy, and quantitative real-time PCR. Results showed that the tooth germs maintained their spatial shape and vascular structures, with thick layers of dentin and enamel forming over time. No significant differences in DMP1 or FGF10 expression were observed compared to tooth germs grown in vivo [66]. The study concluded that RCCS effectively supports tooth germ development, maintaining spatial morphology and enhancing growth, making it a promising tool for investigating odontogenesis mechanisms.

3D cell culture systems better mimic tissue structures than traditional 2D models, and organs-on-chips (OoCs) are increasingly efficient 3D models [67]. It could also be used as a tool for testing cell differentiation or selecting materials. A study aimed to create a simplified dentin-on-a-chip using microfluidic chip technology and tissue engineering for screening dental materials. A microfluidic device with three channels was designed to create 3D dental tissue constructs using stem cells from the apical papilla (SCAP) and gelatin methacrylate (GelMA). The study explored the effects of different cell densities and GelMA concentrations on the constructs' features within the chip. Cell viability and distribution were assessed through various staining techniques, and osteo/odontogenic potential was evaluated via ALP and Alizarin red staining. Results showed that a cell seeding density of 2 × 10^4 cells/μL in 5% GelMA was optimal for cell proliferation, even distribution, and higher osteo/odontogenic differentiation compared to 10% GelMA. The study concludes that GelMA concentration regulates SCAP

differentiation, recommending the use of 2 × 10^4 cells/μL in 5% GelMA for constructing a simplified dentin-on-a-chip, which can serve as a well-defined biological model for regenerative endodontics and a potential testing platform for cell differentiation[67].

In addition, 3D culture has been used for investigating how stem cells maintain their stemness as well. Previous research has identified a specific subset of dental pulp stem cells (hDPSCs) characterized by STRO-1, c-Kit, and CD34 markers, showing a strong propensity for neural lineage commitment. However, maintaining these biological properties under conventional adherent culture conditions has posed challenges [68]. A study by the same researchers aimed to evaluate whether cultivating these cells as 3D floating spheres could better preserve their embryological and biological characteristics. The study focused on examining the expression of inwardly rectifying potassium channel Kir4.1, as well as Fas and FasL proteins in hDPSCs derived from 3D spheres. The results demonstrated that hDPSCs derived from 3D spheres retained their fibroblast-like morphology, sustained expression of stemness markers, and robust proliferation capability. These cells also exhibited expression of neural crest markers and Kir4.1 in their undifferentiated state, indicating maintenance of their differentiation potential. Notably, Fas and FasL proteins were detected in undifferentiated hDPSCs from sphere cultures, with FasL showing sustained expression post neurogenic commitment, significantly higher compared to osteogenic and myogenic commitments. These findings underscore the potential of 3D sphere culture as a favorable microenvironment for preserving the biological properties of neural crest-derived hDPSCs, suggesting promising applications in regenerative medicine [68].

4.2 In vivo tooth regeneration applications

One study created and investigated an in vivo incubation system in chicken eggs. Mouse tooth germ development is studied using three main approaches: wild-type and mutant mouse lines, transplantation of tooth germs to ectopic sites, and organ culture [23]. While in vivo methods are the most physiological, they lack accessibility for experimental manipulation. Organ cultures offer accessibility but do not support full development and are suitable only for short-term analysis. To address these limitations, a new approach was developed by implanting embryonic day 12 mouse tooth germs into the lateral mesenchyme of day 4–5 chick embryo wing buds. The eggs were reincubated, and implanted tissues were monitored using histochemistry and in situ hybridization. The tooth germs progressed through normal growth and development stages, mimicking native temporal patterns, reaching the cap, bell, and crown stages in approximately 3, 6, and 10 days, respectively. To investigate the mechanisms regulating development, tooth germ fragments were microinjected with neutralizing antibodies to Sonic hedgehog (Shh) and examined over time, revealing delayed development and poor morphogenesis. This model system demonstrated that the limb bud is an effective, accessible, and economical environment for studying tooth germ development and the role of Shh signaling [23].

Another study, they developed a 3D culture technique to grow functional HERS cells into spheroids and compared them to 2D monolayer HERS cells [69]. Then, proliferation, self-renewal, stemness, and the capacity for differentiation and dentin formation, both in vitro and in vivo were analyzed. Transcriptome analysis was used to uncover the molecular mechanisms behind these differences. The results showed that HERS spheroids had superior biological traits and functionality compared to 2D cells. When transplanted to rat In vivo, HERS spheroids generated more mineralized tissue and, when combined with dental papilla cells (DPCs), facilitated the formation of dentin-like tissue. The study also highlighted the critical role of the HIF-1 pathway in the generation and function of HERS spheroids. This method of creating HERS spheroids significantly enhances the expansion of functional HERS cells, making them a valuable resource for tooth regeneration and further research [69].

Tooth organoids can be generated by self-organizing dental epithelial and mesenchymal cells in a specific culture system or through tissue engineering by seeding stem cells on a scaffold that promotes odontogenic differentiation [70]. One study developed two injectable double-network (DN) hydrogel-based 3D cell culture systems for dental pulp regeneration [71]. The DN hydrogels, compared to single-network (SN) hydrogels, showed better injectability, mechanical properties, and longer degradation times. Encapsulating human dental pulp stem cells (hDPSCs) in DN hydrogels promoted their differentiation and mineralization in vitro. In vivo, DN hydrogels matched the properties of pulp-like tissue, suggesting their effectiveness for dental pulp tissue regeneration [71].

So, the above results show that the arrangement of cells within a tissue is crucial for organogenesis, including tooth development. Recent progress in tooth regeneration involves reassembling dissociated embryonic dental cells and implanting them in mice [72]. Study had tested the hanging drop method to reorganize mixed epithelial and mesenchymal cells in a liquid medium to facilitate tooth histogenesis and organogenesis. This method allowed precise control over cell proportions and numbers, resulting in uniformly sized microtissues. The liquid medium promoted better cell migration compared to collagen gels. Three protocols were compared, and the one combining hanging drop and semisolid medium cultures before in vivo implantation yielded the best results. Implanted teeth developed with well-formed crowns, dentin and enamel mineralization, and initial root formation. Vascularization and mesenchymal cell heterogeneity were similar to natural molar development. Additionally, co-implantation with a trigeminal ganglion resulted in innervation of the dental mesenchyme and odontoblast layer [72]. The main advantage of this technique is the small number of cells required to create a tooth, making it useful for studying tooth development, physiology, metabolism, toxicology, and testing alternative cell sources.

5. Conclusion and perspectives

In conclusion, tooth regeneration is a multifaceted field benefiting from advancements in cell types, scaffold materials, acellular factors, and incubation systems. There is great progress in all these aspects, such as, iPS cells, MSC cells, decellularized tooth scaffolds, and new biomaterials for 3D printing are all very promising (**Figure 1**). However, there are also quite a few limitations. So, future studies might be focusing on addressing the limitations of each type of stem cell, scaffold, and acellular factor. Or how the current available system could be used effectively. For example, even though a decellularized human tooth scaffold could regenerate many of the tooth's components such as the cementum, pulp, root, enamel, PDL, and dentin, extracting a living human tooth from a person is inconvenient and impractical. Instead, the other types of scaffolds, which don't regenerate all the tooth components individually, could be combined. For example, treated dentin matrix cannot regenerate treated PDL but can regenerate the cementum, pulp, root, enamel, and dentin. If the treated dentin matrix is combined with PLA which can regenerate PDL, this new type of material could be used as a scaffold for all of these tooth components. Advancing research in these combinations could significantly enhance the field of tooth regeneration.

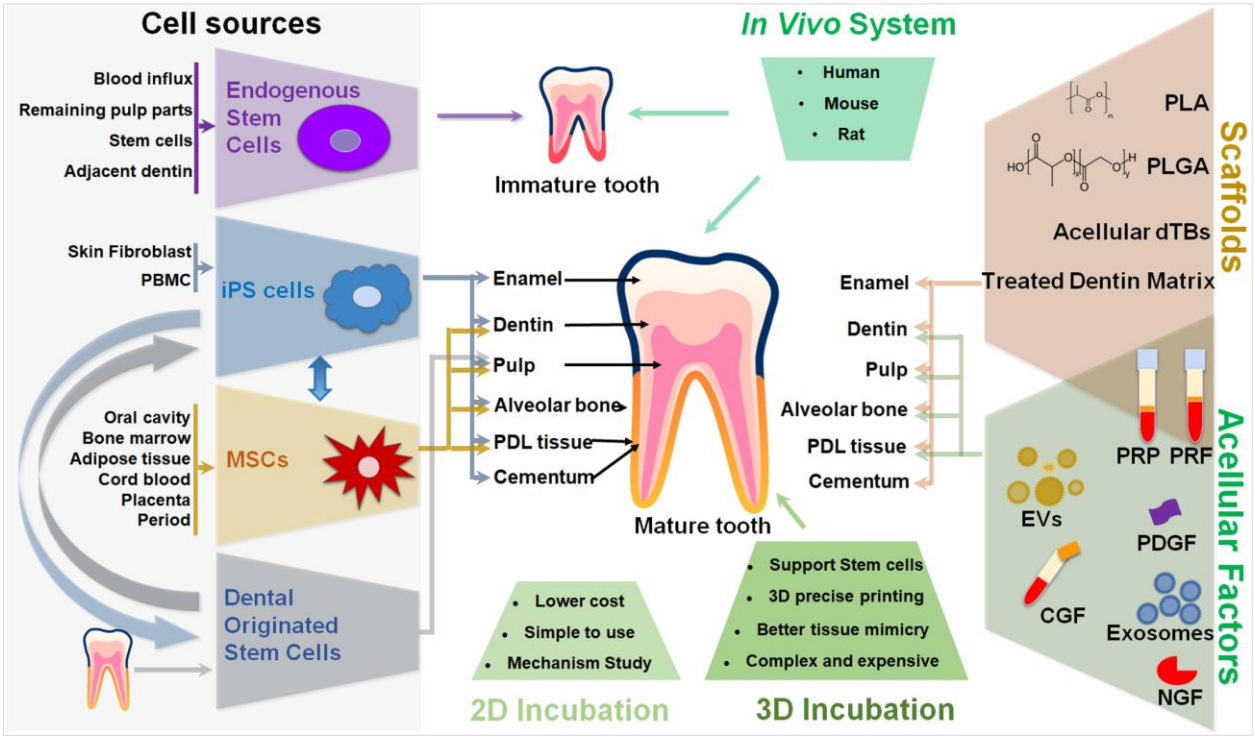

Figure 1. Factors involved with tooth regeneration. All the cells and their origins at the left panel, the incubation and growth system in the center panel, and the scaffolds and accelluar components on the right panel were all investigagted for their roles in tooth regeneration. iPS cells: induced pluripotent stem cells; MSCs: mesenchymal stem cells; PLA: polylactic acid; PLGA: poly lactic-co-glycolic acid; PRP: platelet-rich plasma; PRF: platelet-rich fibrin; EVs: extracellular vesicles; CGF: concentrated growth factors; PDGF: platelet-derived growth factor; NGF: nerve growth factor.

List of abbreviations

ADMPC: Adipose derived multi lineage progenitor cells
aTDM: Autoclaved TDM
BC: Conventional blood clot
Bio 3D: Bio three dimensional
BMP: Bone morphogenetic protein
BMDMs: Bone marrow derived macrophages
BMMSCs: Bone marrow mesenchymal stem cells
CBCT: Computed tomography
CGF: Concentrated growth factor
CM: Conditioned media
CT: Micro computed tomography
CW: Chitin whisker
DLP: Digital light projection
DN: Double network
DPSCs: Dental Pulp Stem Cells
DFSCs: Dental follicle stem cells
DPC: Dental pulp cells
DPCs: Dental papilla cells
dTBs: Decellularized tooth buds
ECM: Extracellular matrix
ECs: Endothelial cells
ELVs: Exosome like vesicles
ELVs H1: ELVs derived from a HERS cell line
EVs: Extracellular vesicles
ExoA: rDPSCs Exos were rabbit dental pulp stem cells cultured angiogenic differentiation
Exo G: rDPSCs Exos were rabbit dental pulp stem cells cultured under growth
GBR: Guided bone regeneration
GelMA: Gelatin methacrylate
GFs: Growth factors
GTR: Guided tissue regeneration
HA/PLA: Hydroxyapatite/PLA
HAp: Hydroxyapatite
hDPSCs: Dental pulp stem cells from healthy teeth
hEVs: EVs from H DPSCs
hDPSCs: Human dental pulp stem cells
hDPSCs Exos: Exosomes were derived from human dental pulp stem cells
hDPSCs Exos/CS: Incorporating hDPSCs Exos into chitosan hydrogel
HERS: Hertwig's epithelial root sheath cells
HPCH: Hydroxypropyl chitin
HPCH/CW/Exo: Exosome loaded hydrogel
hPDLSCs: Human periodontal ligament stem cells
HUVECs: Human umbilical vein endothelial cells
iPDLSCS: PDLSCs in inflamed periodontal tissues
iPS: Induced pluripotent stem
MPN: Metal–phenolic network
MSC Exos: MSC derived exosomes
MSCs: Mesenchymal stem cells
Nacre/PU/POSS: NPP
NDD: Natural decellularized dentine
NC MSCs: Neural crest derived mesenchymal stem cells
NCs: Neural crest stem cells
Nano HAw: Nano hydroxyapatite whisker
NGF: Nerve growth factor
nm: Nanometers
OoCs: Organs on chips
PCL: Polycaprolactone
PDGF BB: Platelet derived growth factor BB
PDLSCs: PDL stem cells
pDPSCs: Dental pulp stem cells of teeth affected by periodontal disease
PDL: Periodontal ligament tissue
PEG: Poly (ethylene glycol)

PGA: Polyglycolic acid
pEVs: EVs from pDPSCs
pFGF 2: Plasmid DNA encoding fibroblast growth factor 2
PLA: Polylactic acid
PLAM: Polylactic acid membrane
PLGA: Poly Lactic co glycolic Acid
POSS: Polyhedral oligomeric silsesquioxane
PRF: Platelet rich fibrin
PRP: Platelet rich plasma
PSCs: Human pulp stem cells
PU: Polyurethane
RCCS: Rotary cell culture system
REPs: Regenerative endodontic procedures
RET: Regenerative endodontics
rDPSCs Exos: Exosomes derived from rabbit dental pulp stem cells
SCAP: Stem cells from the apical papilla
SCAP Exos: SCAP derived exosomes
Shh: Sonic hedgehog
TDM: Treated dentin matrix
YAP: Yes associated protein
μm: Micrometers

Ethics approval

Does not apply as this is a literature review of already published data. We have not performed any experiments and directly collect or use any data from a patient.

Consent to participate

Does not apply as this is a literature review of already published data. We have not performed any experiments and directly collect or use any data from a patient.

Data Availability

We did not have any additional data to share. All the data is from published research and cited appropriately.

Conflicts of Interest

The authors declare no any conflict interest.

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Authors' contributions

E.L. and F.F. conceived the idea, E.L. did the literature search, E.L. and F.F. organized the data, E.L. write the manuscript, and E.L. and F.F. revised and final approved the manuscript.

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