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PhD Thesis

# Strategies for whole tooth regeneration and possible rejuvenation of adult dental pulp stem cells.

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To my two daughters, Cecilia and Anna, who give me the strength to push through each day.

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# 1. INTRODUCTION

## 1.1 Primary and Permanent dentition

Humans have two sets of dentitions during their lifetime: the primary and the permanent. The primary or deciduous dentition is referred to colloquially as milk(lacteal) teeth (Fig.1). Primary teeth first erupt between 6 – 12 months of age. By 3 years old, most children will have a complete dentition which consists of 20 teeth (10 maxillary and 10 mandibular): 2 first incisors, 2 second incisors, 2 canines, 2 first molars and 2 second molars in each jaw. Incisors are essential to biting; canines hold and tear while molars grind food. With time, generally between the ages of 6 and 13 years, deciduous teeth are shed or exfoliated, paving the path for their replacement by permanent successors(*Dental Morphology - 1st Edition*, n.d.).

Permanent incisors and canines replace primary incisors and canines. Primary molars are replaced by permanent premolars, also known as bicuspid teeth. Permanent molars have no deciduous predecessors and erupt behind the primary teeth. Each jaw of the permanent or adult dentition consists of 4 incisors, 2 canines, 4 premolars and 6 molars (including wisdom teeth). The permanent dentition, therefore, consists of 32 teeth. The permanent incisors cut and sheared food into smaller pieces, making it more suitable for mastication. From an aesthetic standpoint, incisors are critical teeth. The permanent canines are used for gripping and tearing food. The premolars, also known as bicuspids, also function in tearing food, while permanent molars are used predominantly for crushing and grinding food during mastication(*Dental Morphology - 1st Edition*, n.d.).

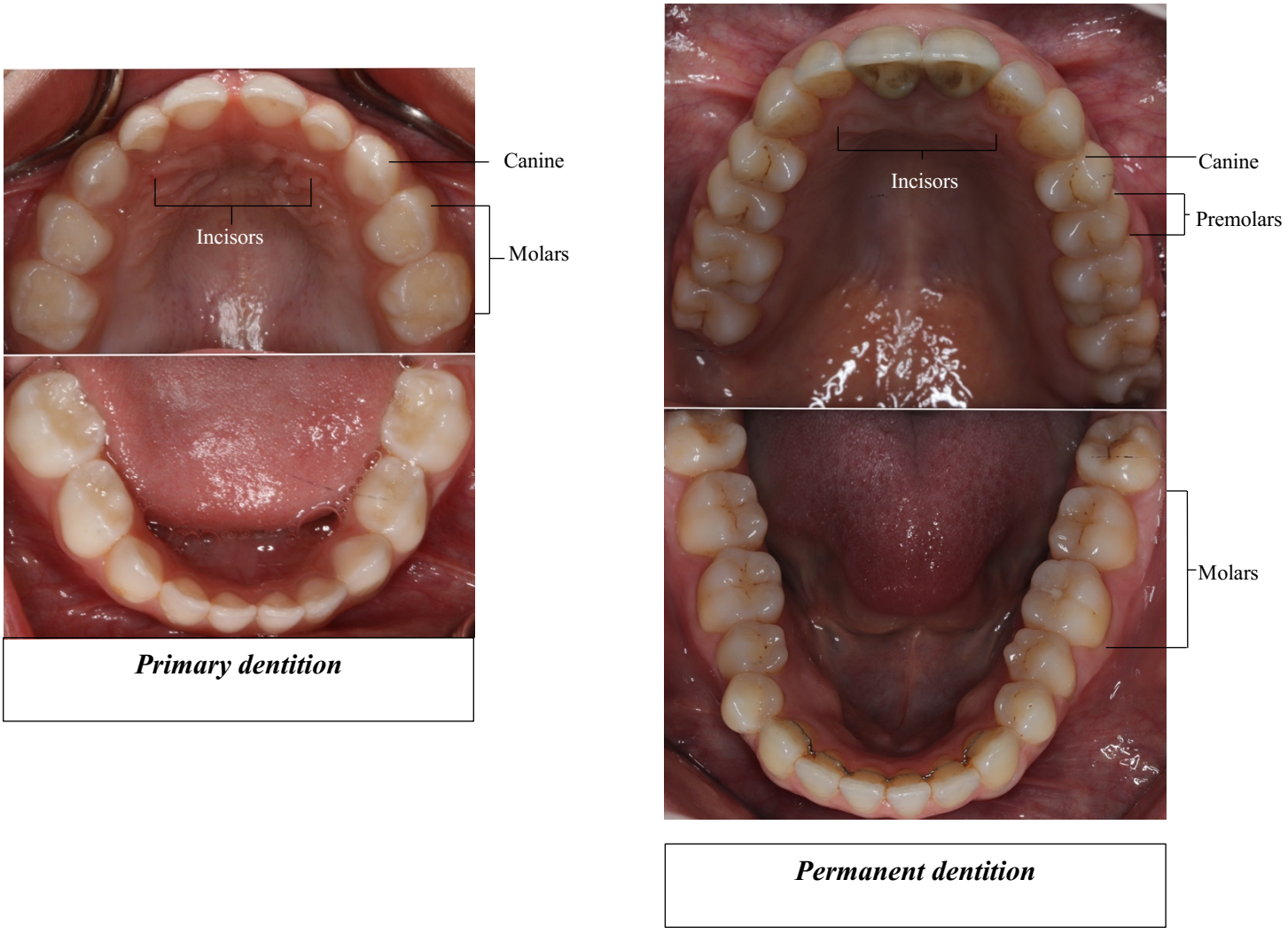


**Figure 1.** Intraoral photographs of the primary and permanent dentition. Human dentition is heterodont and diphyodont. The permanent dentition is heterodont due to having 4 different classes of teeth: incisors, canines, premolars and molars. It is also labelled diphyodont as it has two generations of functional teeth during human life: 20 deciduous and 32 permanent (adult) teeth. The intraoral photograph of primary dentition shows 20 deciduous teeth. The intraoral photograph of the permanent dentition shows complete dentition in the lower jaw; however, in the upper jaw, the patient is missing the upper right wisdom tooth, and the central incisors were missing and replaced by 2 implants.

## 1.2 Tooth Anatomy

A tooth is subdivided into the crown and the root (Fig.2). The clinical crown consists of the portion of the tooth that is exposed in the oral cavity, i.e., the part of the tooth with a surface of the enamel. The tooth's root is the portion encased in the alveolar bone of the maxilla or mandible. The neck of the tooth divides the crown from the root. The enamel is the ivory/ white surface of the tooth that is made of small hexagonal rods parallel to each other and known as enamel prisms. The cementum is a thin layer

on the surface of the anatomic root, which is similar in structure and composition to bone. Underlying enamel and cementum is a major portion of the tooth, known as dentine, composed of dentine tubules. The pulp cavity is located in the innermost portion of the tooth, which contains the dental pulp. The dental pulp is composed mainly of fibroblastic tissue and contains the blood and nerve supply for the tooth. The pulp cavity comprises the pulp chamber and the pulp canals. The pulp chamber is located in the coronal portion of the tooth, whereas the pulp canal is located in the root portion of the tooth(*Netter's Clinical Anatomy - 5th Edition*, n.d.). The tooth is encased in the periodontium, which comprises the gingiva, periodontal ligament (PDL), cementum and alveolar bone(Katancik et al., 2016). This complex structure ensures the root is attached to bone and functions as a protective barrier against pathogenic oral flora(Katancik et al., 2016).

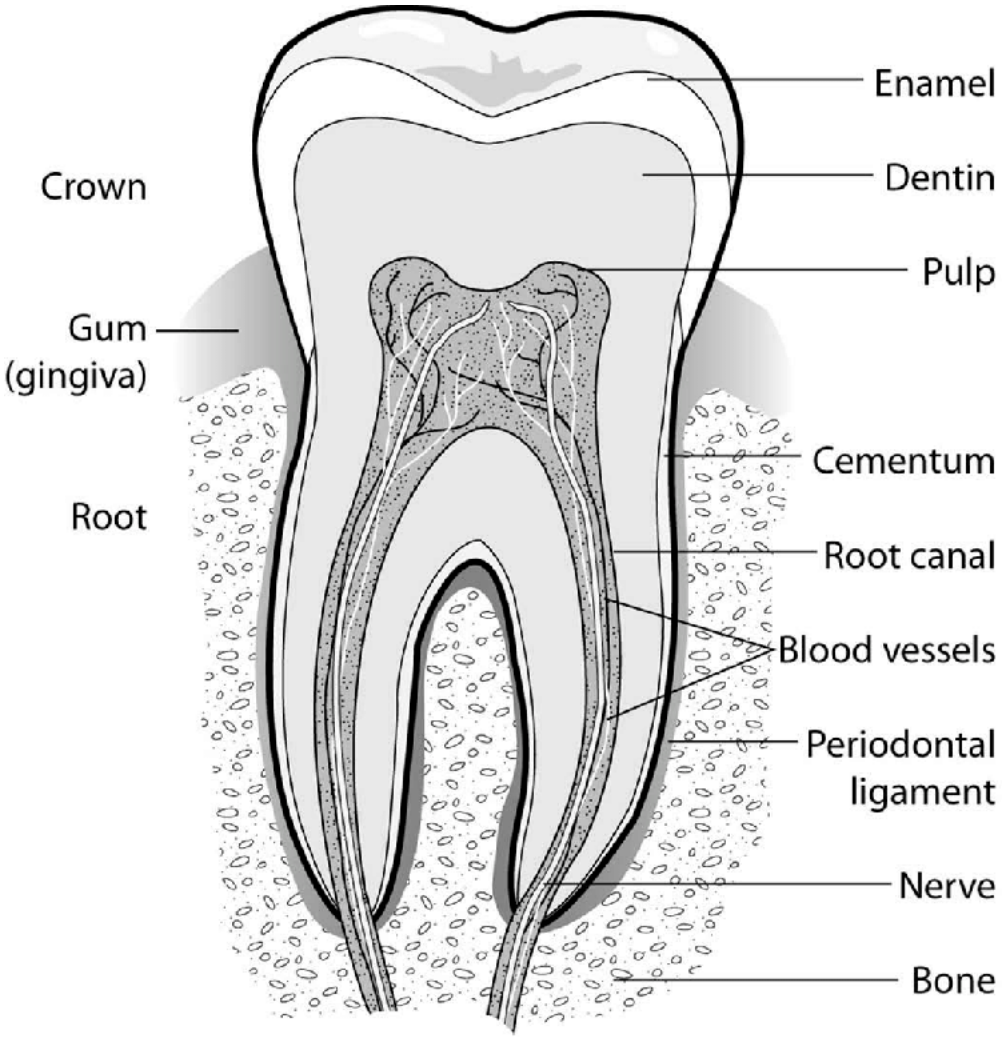


**Figure 2.** Diagram of a cross-section through an adult human molar tooth. Enamel, dentine, dental pulp containing the pulp and nerve supply, cementum, periodontal ligament, gingiva and alveolar bone are the main components of a dental tooth. This diagram is taken from (Pergolizzi et al., 2020)

## 1.3 Tooth morphogenesis

Understanding regenerative dentistry relies on the full comprehension of tooth morphogenesis. By replicating morphogenesis and embryonic developmental processes, regenerative dentistry can construct dental tissue that is structurally, mechanically, and functionally similar to the native tissues (Contessi Negrini et al., 2021). Tooth morphogenesis, or early tooth development, is regulated by interactions between the epithelium and the underlying neural crest-derived mesenchyme(Thesleff & Tummers, 2008). Tooth development occurs through the classical stages – epithelial thickening, dental lamina, bud, cap and bell (Fig.3).

### *1.3.1 Epithelial thickening*

In the 5th week of embryonic development, an epithelial thickening can be evidenced in each facial process: medial nasal, maxillary and mandibular before fusion occurs (Ooé, 1957). Six islands of thickened dental epithelium have been noted on the oral surface of paired medial nasal, maxillary and mandibular processes. As the processes fuse, these islands coalesce to form the upper and lower jaw arches(Nery et al., 1970).

### *1.3.2 Dental lamina*

Following fusion, the thickened epithelium (primary epithelial band) grows into the adjacent mesenchyme. On the inside of the dental arch, the primary epithelial band gives rise first to the dental lamina (DL). Shortly after, the outside of the dental arch gives rise to the vestibular lamina. Both the DL and the vestibular lamina grow into the underlying ectomesenchyme. The DL represents the region that would eventually give rise to the future tooth rows. The vestibular lamina gives rise to the vestibule or sulcus between the cheek and the jaw in the oral cavity. Within the DL, placodes form, which appears as localized thickenings (Thesleff & Tummers, 2008). These placodes function as the first signalling centres of the tooth, which will initiate the formation of individualized tooth germs(Kwon & Jiang, 2018).

### *1.3.3 Bud stage*

The subsequent stages of tooth morphogenesis are labelled as a result of the shape of the tooth germ when viewed in frontal sections(Kwon & Jiang, 2018). In the 8th week of embryonic development, there is a budding of the dental epithelium from the DL further deeper into the ectomesenchyme, which itself undergoes condensation around the bud, forming a mound along the mesial-distal aspect of a jaw arch(Hovorakova et al., 2018).

### *1.3.4 Cap stage*

As the name suggests, the cap stage has an epithelial component of the tooth germ, resembling a cap on top of a spherical ectomesenchyme aggregation(Kwon & Jiang, 2018). This epithelial “cap” is known as the enamel organ, while the aggregation gives rise to the dental papilla. The dental papilla will give rise to odontoblasts which differentiate in dental pulp and dentine. The enamel organ differentiates into ameloblasts which secrete the enamel proteins, enamelin and amelogenin, which will

later mineralize to form enamel. Encapsulating both the enamel organ and the dental papilla is the dental follicle. The peripheral dental follicle made of ectomesenchyme cells will later give rise to cementum and periodontal tissues(Thesleff & Tummers, 2008).

Just before the transition from the bud stage to the cap stage, at the distal tip of the tooth bud is an important signalling structure called the primary enamel knot (PEK) (Kwon & Jiang, 2018). In the cap stage, the enamel knot appears as a cluster of non-dividing cells in the enamel organ. It is believed that the enamel knot sends signals to its surrounding dental epithelial cells, thereby regulating the folding of the enamel organ to form the "cap"(Kwon & Jiang, 2018). Each tooth germ has one PEK at the cap stage.

### *1.3.5 Bell stage*

The enamel organ develops into a bell shape as its undersurface deepens and encases the dental papilla. The dental follicle continues encapsulating the enamel organ and the dental papilla. The enamel organ differentiates into 4 distinct cell groups: the inner enamel epithelium (IEE), stratum intermedium, stellate reticulum, and outer enamel epithelium (OEE). The OEE and the IEE are a continuous structure, and where they intersect to form the rim of the bell, which is coined the cervical loop(Kwon & Jiang, 2018). Cervical loop cells continue to proliferate through the crown stage and play crucial roles in root formation. The enamel organ, via folding of the IEE layer, becomes the future tooth crown. During the transition from the cap stage to the bell stage, the primary enamel knot undergoes apoptosis, and secondary enamel knots appear, which regulate the position and size of the tooth cusps in molar teeth(Kwon & Jiang, 2018).

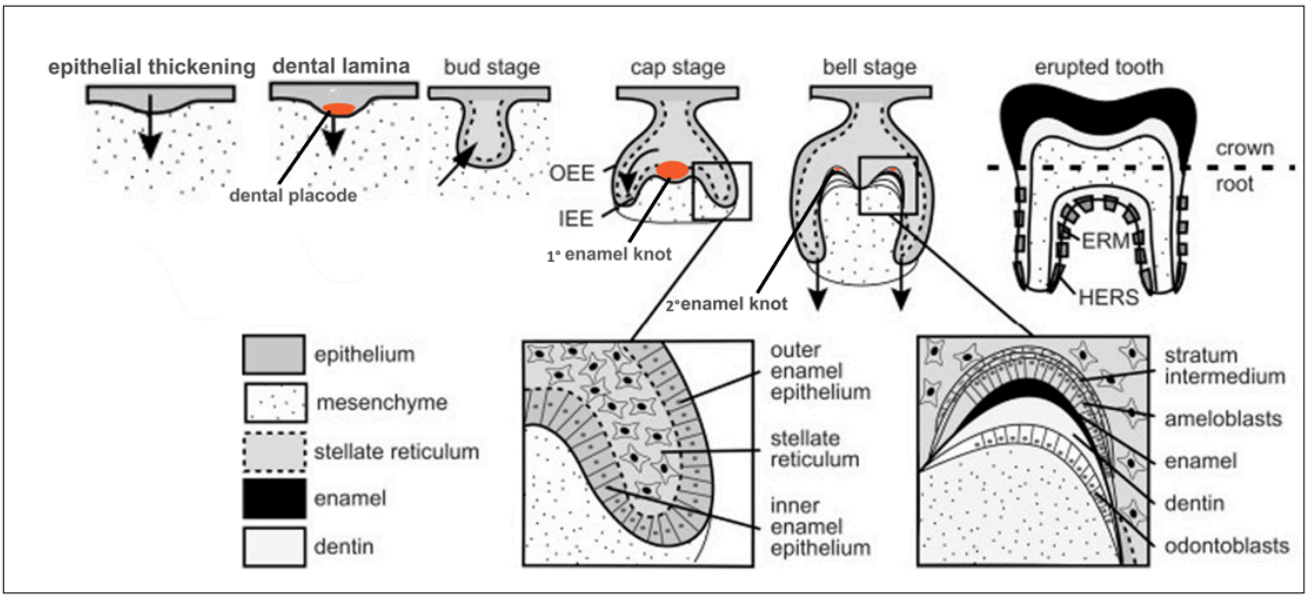


**Figure 3.** The stages of tooth morphogenesis. From initiation to the eruption of the tooth: epithelial thickening, dental lamina, bud stage, cap stage and bell stages. Hertwig's epithelial root sheath (HERS), epithelial cell rests of Malassez (ERM). Modified from(Thesleff & Juuri, 2015)

## 1.4 Tooth loss

### *1.4.1 Acquired tooth loss*

Edentulism is the state of being edentulous or without natural teeth(Al-Rafee, 2020, p.). Complete edentulism is an oral cavity without teeth. Partial edentulousness is the state in which one or more but not all, natural teeth are missing(Vidhya & Krishnan, 2015). A systematic analysis of the Global Burden of Disease Study 2017 stated that edentulism and severe tooth loss affect about 267 million people around the globe(James et al., 2018). According to United Kingdom historian Professor Joanna Bourke, as early as the Victoria era and the early 20th century, having all your teeth removed was considered the perfect 21st birthday gift or present for a new bride as a result of the excruciating pain associated with going to the dentist and the costliness of the dental procedures (Lee, 2015). Nowadays, dentistry has radically changed, but caries, periodontal disease, trauma, endodontic treatment complications, impactions, orthodontic treatment, pre-prosthetic treatment, and neoplastic and cystic lesions are still among the top reasons teeth are extracted(Vidhya & Krishnan, 2015). Many surveys on different populations cite caries, periodontal diseases and wisdom tooth extractions as the top reasons for extractions(Aljafar et al., 2021)(Bikash et al., 2019)(Passarelli et al., 2020). Tooth loss of this nature is acquired during a person's lifetime, hence known as acquired tooth loss.

### *1.4.2 Congenital tooth loss*

Congenitally missing teeth is also known as hypodontia or tooth agenesis and, in layman's term, mean to be born without certain teeth. Despite the exact genetic mechanism leading the hypodontia being poorly understood, it is accepted that its multifactorial etiology has a polygenetic mode of inheritance with epistatic genes and environmental factors(Thesleff, 2000). Environmental factors like the exposure of a developing tooth germ to infection, trauma and drugs may contribute to its disturbance in combination with genetic factors. Congenitally missing teeth may occur in isolation (non-syndromic) or as part of a recognized genetic syndrome for which there are approximately 120 syndromes such as the more commonly known cleft lip, cleft palate or both, ectodermal dysplasia, and down syndrome(Rakhshan, 2015).

## 1.5 Consequences of tooth loss

Losing one or more natural teeth have been acknowledged as a serious life event(Bergendal, 1989). The consequences of this have a significant impact on one's daily life (Fig. 4). The physical aspects of losing one or more teeth have been extensively studied, which include drifting, rotating of the adjacent tooth, and supraeruption of the opposing tooth into the edentulous space(Petridis et al., 2010). Loss of posterior support from the loss of multiple posterior teeth can lead to facial changes. Loss of multiple or all teeth with the accompanying alveolar bone loss can reduce the facial height and cause premature ageing(Tallgren et al., 1991). The physical consequences may happen with time, but the social-psychological impact of tooth loss may be immediate with profound outcomes. The feeling of bereavement, loss of self-confidence, and concerns about appearance can impact one's day-to-day existence, interpersonal relationships, the ability to get a job, etc (Dosumu et al., 2014). The loss of one or more teeth can impair a person's ability to masticate, lead to restrictions in food choice, avoidance of eating in public and reduced enjoyment of food. In a recent quantitative assessment of the emotional effects of tooth loss in a United Kingdom population, 45% of the participants had difficulty accepting the loss of their teeth(Davis et al., 2000). Of the range of emotions patients experienced with losing the last remaining tooth, the top four emotions were sadness, losing part of myself and feeling depressed and old(Davis et al., 2000). Of course, a small percentage of patients experienced relief as a tooth was usually extracted due to associated pain or pathology.


**Figure 4.** Diagram of the consequences of tooth loss. A combination of socio-psychological, physical and emotional effects leads to severe restriction in one's daily life.

## 1.6 Prosthetic replacement for missing teeth

Prosthetic replacement for missing teeth has remained unchanged for decades, albeit improvements have been made in materials and techniques(Yelick & Sharpe, 2019). Dentures, bridges and implants remain the mainstay of tooth replacement treatment options. A denture can be fixed or removable, partial or complete, and is usually made of synthetic materials such as acrylic or metal. Commonly known disadvantages of dentures include poor aesthetics and ill fit. Removable dentures require removal for cleaning overnight. Ill-fitting dentures can slip out, making essential daily functions such as eating and speaking difficult. A denture can also lead to an increased risk of periodontal disease, which often causes damage to the adjacent teeth(Bergman, 1987). A denture is not a one-off solution and needs replacement over time(Schwass et al., 2013). A dental bridge is a prosthesis that uses the

adjacent teeth to attach and support a replacement tooth or teeth. They provide pleasing aesthetics and function; however, they generally fail after 5-15 years and need replacement(Schwass et al., 2013). Over time, bone resorption under the artificial replacement tooth may lead to gaps under the bridge. As cleaning this prosthesis is not always easy for many patients, the biological cost, if not cleaned well, includes predictable decay around the adjacent tooth(Valderhaug, 1991). Unlike dentures and bridges, dental implants, usually made of titanium, osseointegrate with bone and, therefore, can have superior aesthetics and function; however, they are also not without problems. Peri-implantitis is a site-specific inflammatory disease which leads to bone loss surrounding the implant and can lead to implant failure.

# 2. AIM OF THE THESIS

**General aim**

This thesis aims to deepen our knowledge of the strategies used for whole tooth regeneration, which include dental tissue engineering, stimulation of third dentition formation, cell-tissue recombination, and gene-manipulated tooth regeneration. In doing so, a more specific focus is placed on adult dental pulp stem cells whose regenerative potential declines with age. Our study further investigated if it were possible to rejuvenate aged human dental pulp stem cells (hDPSCs) using the microenvironment of young hDPSCs.

**Specific aims**

To examine the strategies used for whole tooth regeneration, which include dental tissue engineering, stimulation of third dentition formation, cell-tissue recombination, and gene-manipulated tooth regeneration.

**Study**

To investigate if aged hDPSCs can acquire rejuvenation features through contact with factors secreted by young hDPSCs.

# 3. WHOLE TOOTH REGENERATION STRATEGIES - HISTORICAL BACKGROUND, CURRENT APPLICATIONS, AND FUTURE CONSIDERATIONS

## 3.1 Introduction

Treatment for tooth replacement in clinical dentistry is still based on prostheses made from synthetic materials that, unfortunately, have limited lifespans, each having inherent weaknesses and not based on tissue biology. Instead, recent research is trending towards a more biologically driven direction known as regenerative dentistry. Regenerative dentistry uses cell and molecular biology knowledge to create biologically driven dental therapies that repair and regenerate dental tissues(Sharpe, 2020). There are two main regenerative pathways for teeth. The first is the biological repair of individual dental tissues, for example, the regeneration of dentine or pulp. The second regenerative pathway, for which this chapter of the thesis focuses, is the regeneration of a whole tooth that is healthy, vital, innervated, vascularized and has pleasing aesthetics and function.

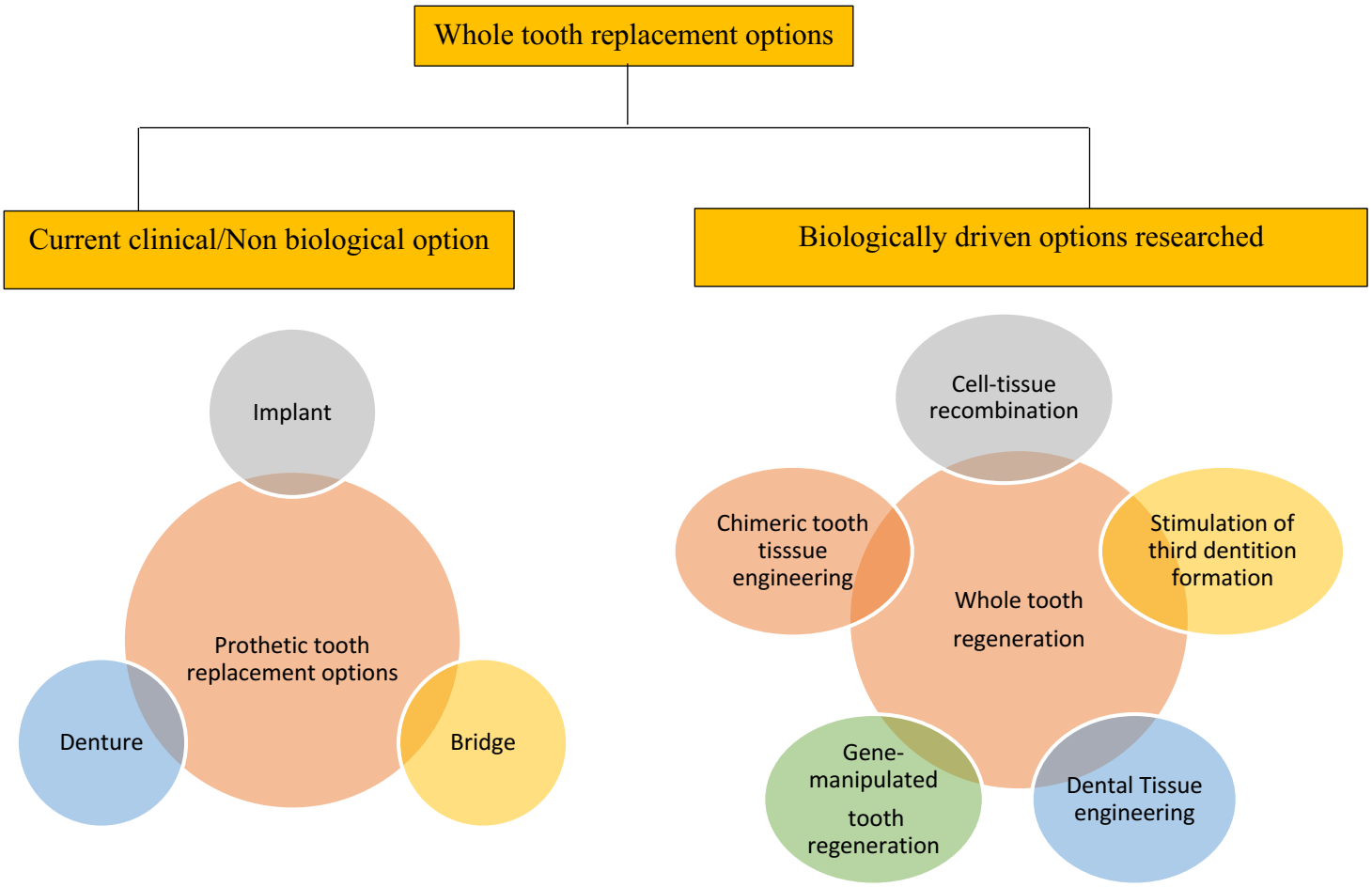


**Figure 3.** Non-biological vs biological options for replacing a whole tooth. The three main biological options include replacing one or several missing teeth with an implant, bridge or denture. Regenerative dentistry offers the biological driven likelihood of using strategies such as cell- tissue recombination, stimulation of the third dentition formation, dental TE, gene manipulated tooth regeneration and chimeric tooth TE to regenerate a whole tooth.

The stem cells primarily used for these regenerative strategies are classified according to their source and cell potency. The three main sources of stem cells are embryonic stem cells (ESCs), adult stem cells and induced pluripotent stem cells (iPSCs). ESCs are pluripotent stem cells commonly derived from the inner cell mass of embryos(Hassani et al., 2019). Their pluripotent cell potency means they can give rise to all tissue types. Adult stem cells are extracted from mature tissue. They are multipotent, meaning they can only give rise to a limited range of cells within a tissue type. Unlike naturally occurring ESCs and adult stem cells, iPSCs were recently generated artificially in 2006(Shi et al., 2017).

Human dental pulp stem cells (hDPSCs) were first isolated and cultivated in 2002 (Gronthos et al., 2000). In particular, postnatal human dental pulp stem cells have high proliferative activity, self-renewal potency, and multi-lineage differentiation ability. hDPSCs can differentiate into hepatocytes, osteoblasts, odontoblasts, myocytes, adipocytes, endocrine cells, chondrocytes, and neural and endothelial cells(Al Madhoun et al., 2021). Following this outstanding discovery, other sources of oral adult stem cells or dental-derived mesenchymal stem cells (DMSCs) have since been discovered and are labelled according to their tissue of origin: tooth germ progenitor cells (TGPCs)(Ikeda et al., 2008), dental follicle stem cells (DFSCs)(Handa et al., 2002), salivary gland stem cells (SGSCs)(Feng et al., 2009), stem cells from human exfoliated deciduous teeth (SHED)(Miura et al., 2003), stem cells from the apical papilla (SCAP)(Sonoyama et al., 2008), periodontal ligament stem cells (PDLSCs)(Seo et al., 2004), oral epithelial stem cells (OESCs)(Papagerakis et al., 2014), gingival-derived mesenchymal stem cells (GMSCs)(Q. Zhang et al., 2009) and periosteal stem cells (PSCs)(Debnath et al., 2018) (Fig.5). SHEDs and DPSCs are ideal sources of these stem cells as they are readily available: deciduous teeth exfoliate and can therefore be collected for harvesting stem cells. Meanwhile, many adults have wisdom or supernumerary teeth extracted or require orthodontic extractions, i.e., all teeth extracted without pathology. Instead of routinely discarding these teeth, stem cells can be harvested and stored for future use in tooth bioengineering.

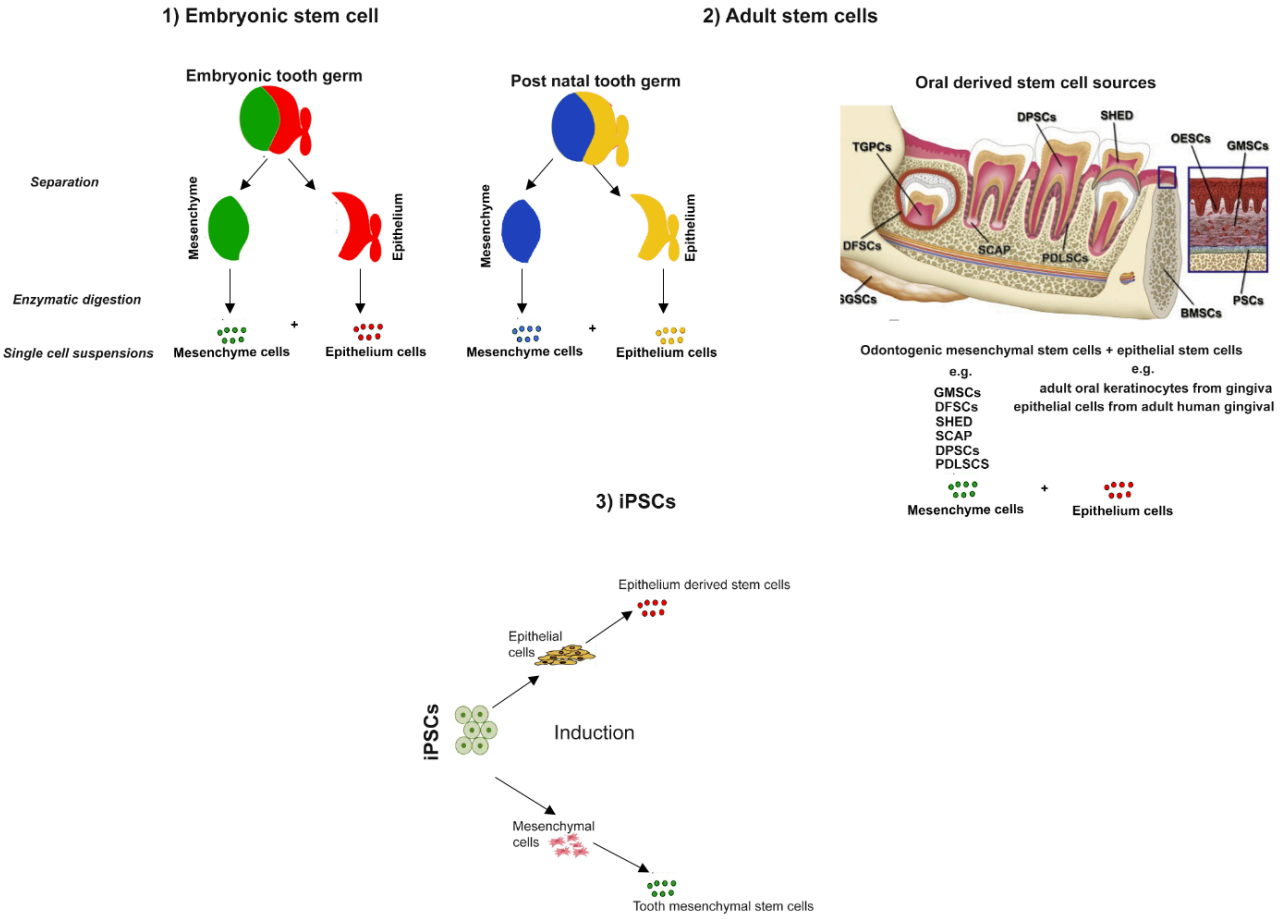


**Figure 5.** The diagram illustrates 3 main cell sources used in regenerative dentistry 1) embryonic stem cells from embryonic tooth germ, 2) adult stem cells isolated from post-natal tooth germ or from oral-derived stem cell sources. DPSCs: dental pulp stem cells; SHED: stem cells from human exfoliated deciduous teeth; PDLSCs: periodontal ligament stem cells; DFSCs: dental follicle stem cells; TGPCSs: tooth germ progenitor cells; SCAP: stem cells from the apical papilla; OESCs: oral epithelial stem cells; GMSCs: bone marrow stem cells; BMSCs: gingival-derived mesenchymal stem cells; PSCs: periosteal stem cells  Taken from (Egusa et al., 2012) 3) iPSCs

In this part of the thesis, we explore the various strategies researchers have investigated to move the benchmark in dentistry for whole tooth regeneration, which includes dental tissue engineering (TE), stimulation of third dentition formation, cell-tissue recombination, gene-manipulated tooth regeneration, and briefly touches on chimeric tooth TE (Fig.4).

## 3.1 Material and Methods

*Protocol and Search Strategy*

The literature reports for whole tooth regeneration procedures were systematically searched in PubMed (National Library of Medicine, NCBI), Embase, Web of Science and Scopus until August 2022. The keywords were initially used: tooth AND (regeneration OR tissue engineering) OR stem cells. A manual review of the articles' references was also performed.

The identified studies were filtered by strategies used to regenerate a whole tooth; "Tissue recombination experiments," "Cell-tissue recombination", "Cell-Cell recombination," "Collagen drop method/organ germ method," "Scaffold free approach", "Cell-scaffold complex" and "Targeted molecular therapy".

*Eligibility criteria*

The following inclusion criteria were applied: publications reporting whole tooth regeneration in tooth bioengineering strategies and targeted molecular therapy. The exclusion criteria applied included: narrative or systematic reviews, meta-analyses, comments, editorials, letters to the editor, study protocols; articles aimed to regenerate solely individual dental tissues such as enamel, dentine or pulp; articles which did not undertake in vivo experiments.

*Information sources*

Using the keywords MEDLINE (PubMed), Embase, Web of Science, and Scopus were surveyed up to August 2022. Finally, the studies' references were surveyed to identify potential additional records.

*Selection of sources of evidence and data charting process*

The studies were screened independently, and any disagreement was resolved through discussion with

the supervisor. Rejected studies were recorded, whereas studies that fulfilled the inclusion criteria were processed for data extraction.

*Data items*

Data on the following issues were extracted and recorded depending on the strategy used for whole tooth regeneration: citation, publication status, and year of publication; the study consisted of constructing a whole tooth. Studies in which whole tooth regeneration was the objective were included regardless of whether it did not regenerate all dental tissues. It was rejected if a study aimed to bioengineer dental constituents only, such as the dentine-pulp complex. If a study did not include in vivo experiments was rejected. The main variables of this review, such as cell origin, cell donor, type of cell, culture method, scaffold, implantation histology and morphology, eruption, vascularization, and nociceptive potential, were extracted and tabulated.

## 3.2 Whole tooth regeneration strategies

### *3.2.1 Revitalizing the lost potential of the dental lamina - "Why can't we have more than two sets of teeth like some other vertebrates."*

In humans, the first dentition is the primary or deciduous dentition, more colloquially known as baby teeth. The primary dentition comprises 20 teeth: incisors, canine, and molars(Lu et al., 2017). The subsequent permanent dentition comprises 32 teeth: incisors, canines, premolars and molars(Hovorakova et al., 2018). Some people have one or more teeth in excess of the standard 20 primary or 32 permanent teeth, defined as supernumerary teeth. Supernumerary teeth may occur unilaterally or bilaterally, be erupted or impacted, and occur in one or both jaws (Lu et al., 2017). The prevalence of supernumerary teeth has been reported to range between 0.04% to 2.29% (Pippi, 2014). Supernumerary teeth can occur sporadically, i.e., in non-syndromic cases or are more frequently associated with some syndromic diseases. Syndromes associated with supernumerary teeth include Amelogenesis Imperfecta, Cleidocranial dysplasia, Gardner's syndrome and many more(Lu et al., 2017). The proposed aetiologies for supernumerary teeth formation included atavism, dichotomy, hyperactivity of the DL, heredity, progress zone theory and unified aetiology (Anthonappa et al., 2013).

The theory of hyperactivity of the DL is most widely accepted; however, the cause of the hyperactivity is unknown.

The occurrence of supernumerary teeth has been attributed as evidence that the third dentition exists in humans (also known as the partial third dentition). The molecular mechanisms associated with supernumerary teeth in mice have been extensively studied. The investigation of syndromic diseases associated with the presentation of supernumerary teeth has revealed key pathogenic genes responsible in humans(Groden et al., 1991). In mouse mutant models, numerous studies have been able to implicate several signalling molecules such as bone morphogenetic protein (BMP), fibroblast growth factor (FGF), tumor necrosis factor (TNF) and molecules of the sonic hedgehog (Shh) and wingless-related (Wnt) pathways involved in the formation of supernumerary teeth(Lu et al., 2017). A review by Lu et al. provided in-depth insight into these molecular mechanisms(Lu et al., 2017).

In mice, specifically in the toothless regions between the incisors and molars, many transient vestigial tooth buds develop but later undergo apoptosis. Many studies in mice mutant models modulated the signalling pathways expressed in these vestigial tooth buds, which in essence, rescued vestigial tooth rudiments. They, therefore, led to supernumerary tooth formation where there would have usually been a toothless region(Tummers & Thesleff, 2009). Other studies have instead investigated *de novo* tooth formation in mice, which instead of rescuing vestigial tooth buds, used primary tooth germs of the DL (Nakamura et al., 2008). Though extensively studied, there may be better models to investigate tooth replacement than mice. Mice are monophyodonts, with only one set of teeth throughout their life; however, their incisors exhibit continuous growth (Fig.6). Polyphyodonty vertebrates continuously regenerate teeth throughout their life span (Fig.6a). The COVID-19 pandemic has reminded us of the interconnectedness of humans and other vertebrates. Researchers have widened their focus to encompass other vertebrates that can continuously regenerate teeth to understand whole tooth regeneration further.

Within the vertebrate phylogeny, there is great diversity in tooth regenerative capacity. The shark dentition continuously regenerates throughout life and is, therefore, classified as a polyphyodont vertebrate (Rasch et al., 2020). For each tooth position, many replacement teeth are made beforehand, so sharks are known as having a conveyor belt dentition(Rasch et al., 2020). Bony fishes also exhibit polyphyodonty dentition(Fraser et al., 2013). While some vertebrates with polyphyodonty dentition can have many teeth made in advance to replace one tooth, in others, only one tooth is made in advance for each tooth position(Fraser et al., 2013). There is significant variation in the dentition of reptiles; for

example, some snakes have polyphyodont dentition, whereas others are monophyodonts, meaning they only have one set of teeth throughout their life (Buchtová et al., 2012).

Similarly, mammals also exhibit great diversity in their tooth regenerative capacity. Monophyodont mice have been historically used to research tooth morphogenesis and regeneration; however, they exhibit continuously growing incisors while their molars are never replaced (Fig.6c)(Rasch et al., 2020). In most mammals, such as humans and pigs, dentition is diphyodont, meaning they have two generations of teeth, i.e., humans can only regrow teeth once (Fig.6b). The fossil record of early mammals showed a gradual reduction in tooth replacement(Kielan-Jaworowska et al., 2004). This evolutionary theory has stirred interest among scientists as to whether this lost tooth replacement capacity in mammals could be restored, i.e., instead of humans being diphyodonts, they could exhibit polyphyodonty dentition whereby teeth are continuously replaced.

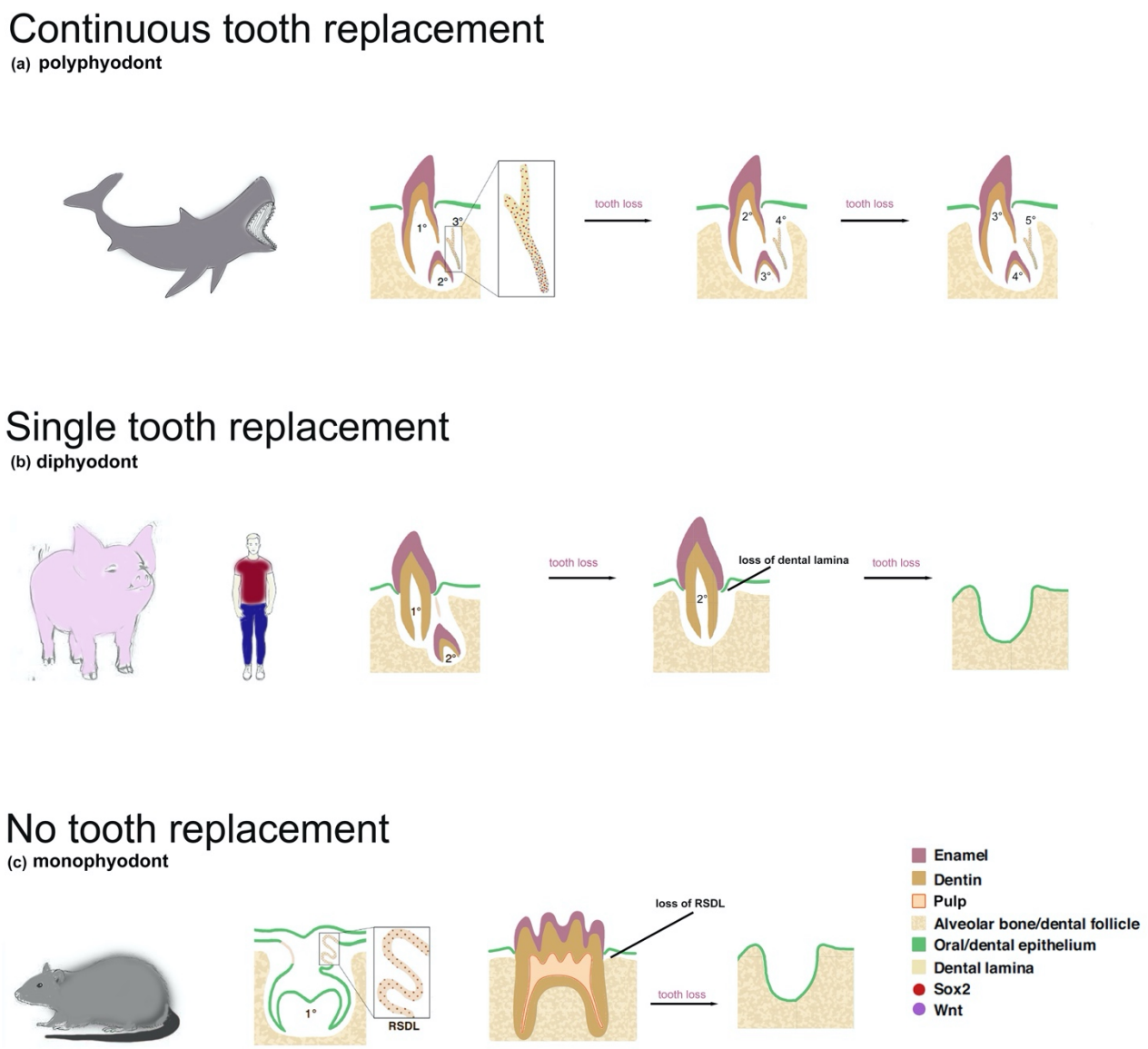


**Figure 6.** Diagram illustrates the relationship between the number of tooth generations and the dental lamina (DL) in polyphyodonty, diphyodont and monophyodont species. (a) A polyphyodont has several tooth generations and has the capacity for continuous tooth replacement, and the DL persists. The cells in the successional dental lamina (SDL) express SRY (sex determining region Y)-box 2 (Sox2) and Wnt signalling. (b)Diphyodonts have two generations of teeth; however, the DL undergoes apoptosis after the initiation of the permanent teeth. (c)Monophyodonts have only one tooth generation; the rudimentary successional dental lamina (RSDL) undergoes repression by the first tooth generation. Modified from (L. Li et al., 2019)

The DL is a specialized epithelial structure arising from the initial thickened oral epithelium along the jaw, which protrudes into the mesenchyme and develops after the initiation of the first tooth generation. In vertebrates, tooth replacement is initiated from the end of the DL, called the successional dental

lamina (SDL)(Dosedělová et al., 2015). The SDL has been identified as the structure responsible for tooth replacement in diphyodont and polyphyodonty vertebrates. A morphologically similar structure, coined the rudimental successional dental lamina (RSDL), has been identified in monophyodonts such as mice. In diphyodonts, the DL starts to disintegrate by undergoing apoptosis and fragments after the initiation of the permanent tooth bud(Štembírek et al., 2010).

In contrast, the DL remains intact in animals with polyphyodont dentition, enabling continuous tooth replacement (Fig.6). Furthermore, it was shown that an odontogenic source of stem cells in lizards was responsible for tooth replacement instead of the DL (Tucker & Fraser, 2014). Taken together, continuous tooth replacement depends not only on the preservation of the DL but also on maintaining an epithelial dental stem niche within the DL(M. M. Smith et al., 2009).

In alligators, polyphyodonty animals with a lifelong renewal of teeth, specific segments of the DL were maintained through Wnt signalling; the DL can be activated by increasing Wnt/ β-catenin signalling, which led to the initiation of tooth formation(Wu et al., 2013). In snakes, activation of the Wnt/β-catenin pathway led to changes in the DL, resulting in the loss of the organized emergence of tooth germs(Gaete & Tucker, 2013). These studies highlight the importance of Wnt signalling in polyphyodont. A breakthrough study using monophyodont mice revealed that stimulation of Wnt signalling in the DL led to the formation of a new tooth in transgenic mice and in vitro culture (Popa et al., 2019). This study further postulated that the adjacent tooth might regulate the tooth renewal capacity, as removing a molar tooth activated Wnt activity (Fig.7). In other words, the study showed the formation of the first molar represses the development of any replacement tooth at the RSDL. The supposition is that in humans who also have a DL, the adjacent molar sends a Wnt-inhibitory signal to the surrounding dental tissues, which prevents Wnt activity in the DL and leads to its apoptosis, restricting the lifelong renewal of teeth (Popa et al., 2019). Therefore, further research is needed to expand on this theory and provides clues on the possibility of continuous tooth regeneration in humans.

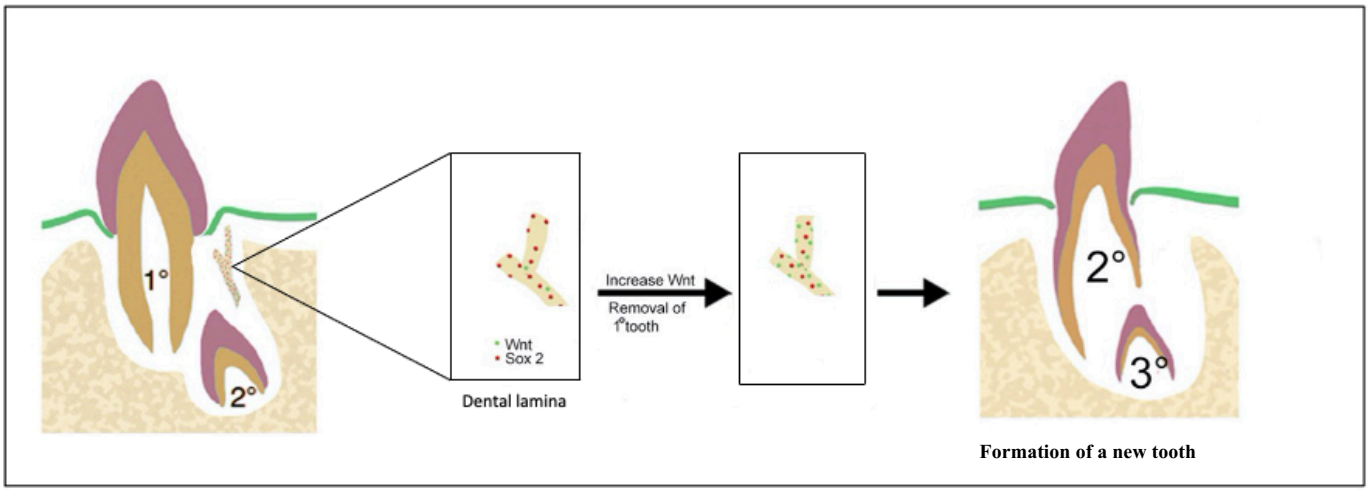


**Figure 7.** The diagram illustrates hypothetical stimulation of the third dentition in humans through modulation of Wnt signalling or by removing an adjacent tooth-activated Wnt activity in the DL, which may lead to the formation of a new tooth.

### *3.2.2 Dental Tissue engineering*

One of the top strategies for whole tooth regeneration involves TE. Classically, TE aims to regenerate, repair, or replace damaged tissues and organs. There is significant research in the field of dental TE which focuses on the repair of individual damaged or diseased dental tissues such as enamel, dentine and pulp; however, this section of the thesis focuses on the TE strategies aimed at regenerating a whole tooth that may have been lost due to dental diseases such as caries or periodontal disease, trauma or congenitally missing.

Classic TE triad generally involves the selection of an adequate cell population, which is then combined with a scaffold to which soluble factors can be added. This combination is called a construct that is first cultured in vitro and then implanted in vivo to regenerate tissue that mimics the mature native tissue structurally, mechanically, and functionally. A new TE paradigm known as developmental TE or developmental engineering has emerged. In addition to regenerating tissue structurally, mechanically, and functionally similar to the native tissues, it replicates morphogenesis and the embryonic developmental processes (Contessi Negrini et al., 2021). Whole tooth developmental TE aims to produce vascularized and innervated, morphological and functional teeth composed of enamel, dentine, pulp, and cementum surrounded by a functional periodontium made up of gingiva, PDL, cementum and alveolar bone.

The development of a mammalian tooth is largely dependent on epithelial-mesenchymal interactions that are sequential and reciprocal(Slavkin et al., 1984). Furthermore, a cell population that has retained inductive capability is cocultured with another cell population competent in receiving the inductive signals(Takeo & Tsuji, 2018). Dental TE techniques involve one of the following strategies: cell-scaffold complex, cell-cell recombinations and cell tissue recombinations. The coculture of epithelial-mesenchymal cells is performed via three main strategies: scaffold-free, cell-seeded scaffolds or the collagen drop method/ hydrogel-based strategy (Contessi Negrini et al., 2021).

### *Tissue recombination*

The reciprocal interactions between epithelium and mesenchyme are the hallmark of tooth development(Thesleff & Jernvall, 1997). With this knowledge, scientists attempted to regenerate teeth by combining dental epithelial (DE) and dental mesenchymal (DM) tissues harvested from embryonic tooth buds at different stages of development and subsequently recombined with either dental or non-dental tissues (Fig.8). Numerous recombination experiments led to further understanding of odontogenesis(Huggins et al., 1934)(Kollar & Baird, 1969)(Kollar & Baird, 1970a)(Mina & Kollar, 1987). The embryonic mouse dental epithelium, before day 12, possessed odontogenic potential and, when combined with non-odontogenic, neural-crest-derived mesenchymal cells of the second arch, elicited the formation of a dental papilla(Mina & Kollar, 1987). After embryonic day (ED11.5), the odontogenic potential was demonstrated to reside with the DM, which can initiate tooth development when combined with dental and non-dental epithelium (W. Zhang, Vázquez, et al., 2017). Kollar et al. successfully regenerated tooth structure when tissue from the incisor enamel organ/lip-furrow epithelium and incisor/molar papilla of a mouse at ED 14-17 were recombined and transplanted into the anterior chamber of the eye(Kollar & Baird, 1970a).

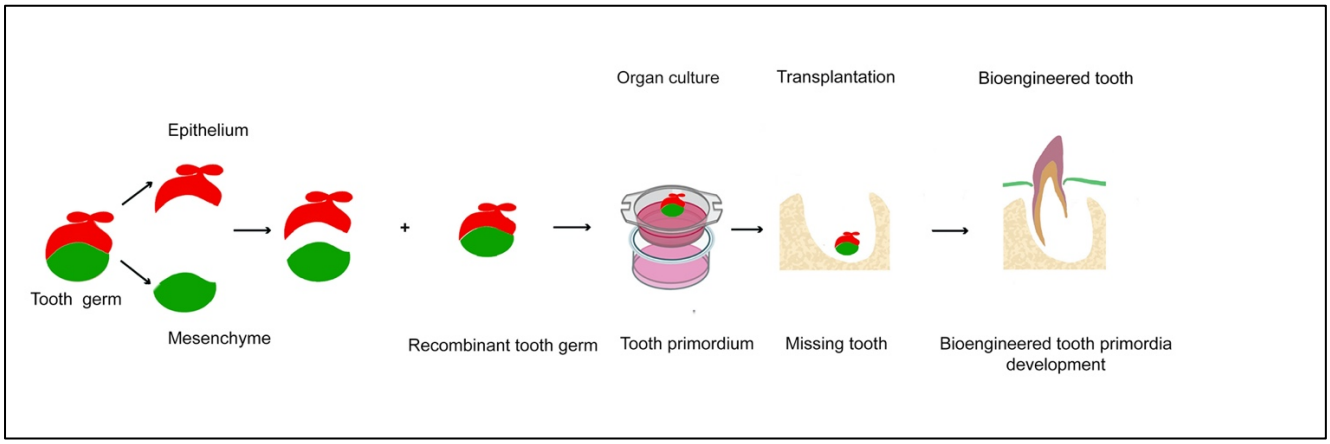


**Figure 8.** The diagram illustrates the tissue recombination strategy for regenerating a whole tooth previously missing.

Many studies have successfully regenerated teeth using tissue recombinations (Table 1). Investigating tooth regeneration using tissue recombination encompassed the variation in each aspect of the methodology; the most suitable site for transplantation (eyes, abdominal wall, torso, kidney capsule, spleen, cheek), mainly using embryonic tissues but also postnatal tissues, performing different types of transplantation (autologous, xenoplastic), and performing different types of recombinations (homologous, heterogenous, interspecific or heterospecific) (Table 1).

| Author | Source of cells | Method | Transplanted | Findings | Significance |
|---|---|---|---|---|---|
| (Huggins et al., 1934) | Unerupted permanent canine tooth germs (3 to 6 weeks) | Recombination of different parts of the tooth germ element | Autogenous transplantation into the connective tissues of the abdominal wall of young dogs | 31 days post-transplantation-tooth structure developed consisting of dentin and enamel only when both components were transplanted. The epithelial layer did not produce enamel in the absence of the mesenchymal layer | The combined importance of dental ectomesenchyme in guiding the formation of tooth |
| (Kollar & Baird, 1970a) | 1. Enamel organ and lip-furrow epithelium 2. Incisor or molar dental papillae both isolated from incisor and molar tooth germs (14- to 17-day mouse embryos) | Recombination of different components of the tooth germ element | Anterior chambers of homologous host eyes | 2-3 weeks post-implantation-reconstructed teeth are typical in all respects; enamel and dentin matrices are deposited | Inductive interaction between the epithelium and the dental papilla. The dental papilla determines the shape of the reconstructed tooth germ. |
| (Kollar & Baird, 1970b) | 1. Epithelium from footplate (ED 14-15) and snout (ED 12- | Dental mesenchyme combined with foot epithelium | Anterior chambers of homologous mice eyes | 2 weeks post-transplantation post-implantation-toothlike structures | Confirmed inductive role of dental mesenchyme |

| | | | | | |
|---|---|---|---|---|---|
| | 13) 2. Incisor/molar mesenchyme of mouse (ED 14-16) | | | present- formation of dentine and enamel | |
| (Howes, 1977) | Tooth buds from Rana pipens frog | Transplanted teeth of all stages | Autologous transplantation within the premaxilla | The tooth grew to normal size and shape in the absence or presence of a jaw | Confirmed possibility of tooth regeneration in frogs |
| (Howes, 1979) | Tooth buds from the green iguana | Individual buds of a known stage of development and small blocks of the entire upper or lower jaw were transplanted | Autologous transplantation into a subcutaneous site in the gular region of an iguana's neck or anterior chamber of the eye. | 45 days post-implantation, the tooth achieved full growth | Confirmed possibility of tooth regeneration in iguanas |
| (Al-Talabani & Smith, 1978) | Maxillary second molar tooth germ from 5-day-old hamsters | Intact tooth germ of a Syrian hamster transplanted | Cheek pouch | 5 or 6 weeks post-transplantation -8 out of 11 transplants harvested had completed development and a mature maxillary second molar with bone, PDL and cementum | The spleen seems to be a suitable site for the transplantation of tooth germs |
| (Al-Talabani & Smith, 1979) | Maxillary second molar tooth germ from 5-day-old hamsters | Tooth germs are transplanted at different stages of their development | Cheek pouch | Mature molar teeth had formed after 6 weeks in the cheek pouch. | Successfully produce teeth even when genetically distinct (allogeneic) mice of the same strain are used. |
| (Morio, 1985) | Mandibular first molar tooth germs of mice | Recombinants of the cervical loop and the dental papilla mesenchyme were prepared. | Under the kidney capsule of syngeneic male mice of 2 months | 20 days after transplantation, the recombinant developed into an almost intact molar tooth. | The role of the cervical loop |
| (Ishizeki et al., 1987) | First-molar tooth germs (gestational day 13) from mouse embryo | Tooth germs, together with surrounding tissues, mandibular bone and occasionally Meckel's cartilage grafted intact | The spleen of six- to eight-month-old isogeneic adult mice | 60 after post-transplantation, fully developed and mineralized mature teeth were obtained | The spleen seems to be a suitable site for the transplantation of tooth germs. |
| (Mina & Kollar, 1987) | The first and second branchial arches were dissected from mouse embryos of 9-13 days gestational age | Heterotypic recombinations of the mandibular arch and second branchial arch tissues.Homotypic control recombinations | Anterior chambers of eyes of male mice | Tooth formation only when 9 to 1 l-day-old mandibular epithelium was used in recombinants: combinations of the epithelium (non-odontogenic epithelium) with mesenchyme did not result in tooth formation from tissues up to 11 days of age. | Oral epithelium as providing instructive information for the initiation of mouse tooth formation |
| (Ishizeki et al., 1989) | Dental pulpal tissues of lower first molar tooth germs obtained | Pulp cells enclosed by intact odontoblasts transplanted | Spleen of isogeneic adult mice | 40 days post-implantation, grafts formed tubular dentine and osteodentine | The role of dental pulpal tissue in tooth regeneration |

| | | | | | |
|---|---|---|---|---|---|
| | from neonatal mice | | | | |
| (Ishizeki et al., 1990) | Mandibular first molars tooth germs obtained from neonatal mice within 12 hours after birth | Undifferentiated ectomesenchymal cells of dental papilla transplanted | Transplanted to the isogenic spleen | 3-7 days post-implantation, transplants formed osteodentin-like material, thus demonstrating the capacity of papilla cells to produce calcified tissue | The role of ectomesenchymal cells |
| (Cho, 2003) | Tooth germs from wild-type mice | Intact tooth germs transplanted | Tooth germ transplantation into the subcapsular layer of the kidney of adult lacZ transgenic mice for 1 week | Reconstructed toothlike structures, including predentin and dental pulp | |
| 1/31/23 1:54:00 PM(Isogawa et al., 2004) | 1. Human dental papilla from third molar tooth germs age (6-12yrs) 2. Human or mouse molar epithelium (E16) | Homologous transplantation of intact tooth germs | Subcutaneous implantation into the dorsal aspect of immunocompromised mice | 3-4 weeks post-implantation transplants formed thin dentin and immature enamel layers. Extremely small structures 8 weeks post-implantation | The first study of the combination of human tooth germ components with those of other animals as transplants |
| (Yamamoto et al., 2005) | E13.5 mouse mandible first molars | Intact tooth germs transplanted | Kidney capsule | Transplanted tooth germ closely resembled normal mandible first molar: crown and advanced root development. | The microenvironment plays an important role in determining the structure of the enamel |
| (Higuchi et al., 2008) | E13.5 mandibular first molar tooth germs from mice | Recombination of epithelial and mesenchymal components of the tooth germ | Subcutaneous tissue at the dorsal portion of the torso or into the kidney capsule. | Transplants in the subcutaneous tissue are smaller than those transplanted into the kidney capsule and a normal tooth. However, the number and arrangement of cusps of the transplanted tooth were similar to those of a normal tooth. | The development of the transplanted tooth may be affected by the host organ. |
| (Palmer & Lumsden, 1987) | Mandibular first molar tooth germs were dissected from Mus musculus and Mus caroli mice (age range 15±19-day embryo) | Whole and interspecific recombinations of murine mandibular first molar molar-germ tissues | Orthotopic transplantation-alveolar crypt of extirpated first mandibular molars in neonatal M. musculus and M. caroli hosts | Transplants progressed from crown to incipient root formation | The role of interspecific recombinations in tooth TE |
| (Carlile et al., 1998a) | Mandibular first molar tooth germs were dissected from Mus musculus and Mus caroli (age range: 14- | Whole and interspecific recombinations of murine mandibular molar germ tissues. | The anterior chamber of the eye of adult mice for 2-4 week | Recombinations of M. caroli enamel-organ epithelium with M. musculus, dental papilla and follicle mesenchyme developed into normal teeth with | M. musculus/M. caroli chimera system |

| | | | | | |
|---|---|---|---|---|---|
| | day embryo to l-day postnatal). | | | advanced root, periodontal ligament and bone formation | |

**Table 1.** Strategies for tooth regeneration based on the recombination of the tissue of epithelial and mesenchymal origin and the subsequent transplantation.

Heterospecific or interspecific (Carlile et al., 1998b)(Palmer & Lumsden, 1987)(Lubbock et al., 1996) recombinations were also used to understand tooth development, leading to chimera tooth TE. Odontogenic epithelial cells/tissue were taken from one species, whereas mesenchyme was taken from another. They were then recombined and transplanted successfully, leading to the regeneration of chimeric teeth (Fig.9).

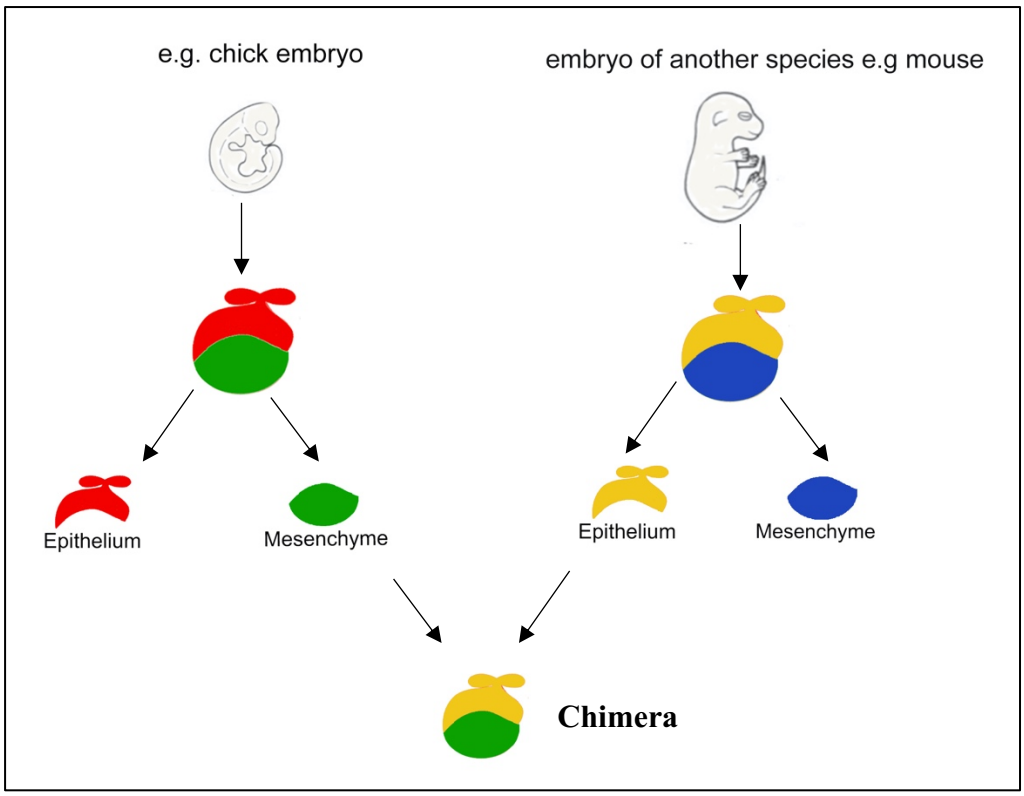


**Figure 9.** The diagram illustrates an example of a heterologous tissue recombination strategy for regenerating a whole tooth that was previously missing. Odontogenic epithelial cells/tissue were taken from one species, whereas mesenchyme was taken from another. They were then recombined and transplanted successfully, leading to the regeneration of chimeric teeth.

The table below shows some studies on heterospecific recombinations that lead to teeth regeneration after transplantation (Table 2).

| Author | Mesenchyme | Epithelium | Recombination resulted in |
|---|---|---|---|
| (Kollar & Fisher, 1980) | Mouse | Chick | A complete tooth with root development in proper relation to the crown, but the latter did not have the typical first-molar morphology |
| (Lemus et al., 1986) | Lizard | Quail | Advanced and relatively well-constructed teeth |
| (Fuenzalida et al., 1990) | Rabbit | Quail | Differentiated chimeric tooth structures |
| (Mitsiadis et al., 2006) | Mouse | Chick | Evidence of tooth formation |
| (Mitsiadis et al., 2006) | Mouse | Chick | Chimeric, toothlike structures |
| (Cai et al., 2009) | Mouse | Chick | Teeth were formed after 3 weeks with a single cusp pattern These recombinant teeth were smaller than mouse molars |
| (B. Wang et al., 2010) | Mouse | Human | Human–mouse chimeric tooth crown |

**Table 2.** In vivo heterospecific recombinations in chimeric tooth tissue engineering.

## *Cell-tissue recombination*

Instead of embryonic tissue recombinants, scientists attempted to regenerate a whole tooth using embryonic cells in combination with embryonic tissue. The mesenchyme of the embryonic tooth germ was separated from the epithelium. The mesenchyme was triturated into single cells, pelleted and combined with the embryonic epithelium. The method is known as cell pelleting or the reaggregation system (Fig.10). Using embryonic tissue from a mouse first molar, a tooth was generated, albeit with some irregularities with enamel and predentine, proving that reaggregated mesenchymal cells retain the odontogenic potential to produce a whole tooth(Yamamoto et al., 2003). Among other studies, Song et al. demonstrated the ability of mesenchymal cells to be reaggregated into a pellet which was subsequently recombined with epithelium and cultured using the Trowell-type organ culture (Trowell, 1954). This organ culture system used a metal grid supporting small filters to hold the recombinations at the gas medium interface. This approach resulted in tooth regeneration following subcutaneous implantation (Song et al., 2006). This scaffold-free approach allowed for direct cellular interactions without any obstruction from an exogenous material, thereby creating the necessary microenvironment in order to adapt to specific needs for whole tooth regeneration(Dissanayaka & Zhang, 2020). The scaffold-free approach relies on cell aggregation, for instance, by cell pelleting or hanging drop method(Contessi Negrini et al., 2021).

A landmark experiment went a step further whereby mesenchyme cells were replaced with an aggregation of cultured non-dental adult stem cells, neural stem cells and bone marrow cells which,

when combined with intact embryonic oral epithelium-induced tooth formation(Ohazama et al., 2004), representing a significant advance in creating artificial embryonic tooth primordia from cultured cells. The cultured cell populations were centrifuged to form a pellet, after which three or four pieces of epithelium were placed over the cell pellet, and the recombinant explants were incubated for 1-3 days resulting in the formation of tooth structures(Ohazama et al., 2004). Inversely, adult epithelial cells isolated from gingiva were injected onto the top of embryonic tooth mesenchyme tissue until the surface of the mouse mesenchyme was covered entirely with cells inside a 20-µL gel drop of Cellmatrix type I-A, the resulting constructs were transplanted in a renal capsule of mice which also successfully induced tooth formation including the root, albeit of aberrant size and shapes (Angelova Volponi et al., 2013). Taken together, these experiments proved that a tooth could be formed outside of the embryo, and cultured adult stem cells were odontogenic and could participate in tooth regeneration after receiving correct signals from an embryonic inductive source, albeit a very large number of embryonic inductive cells were required.

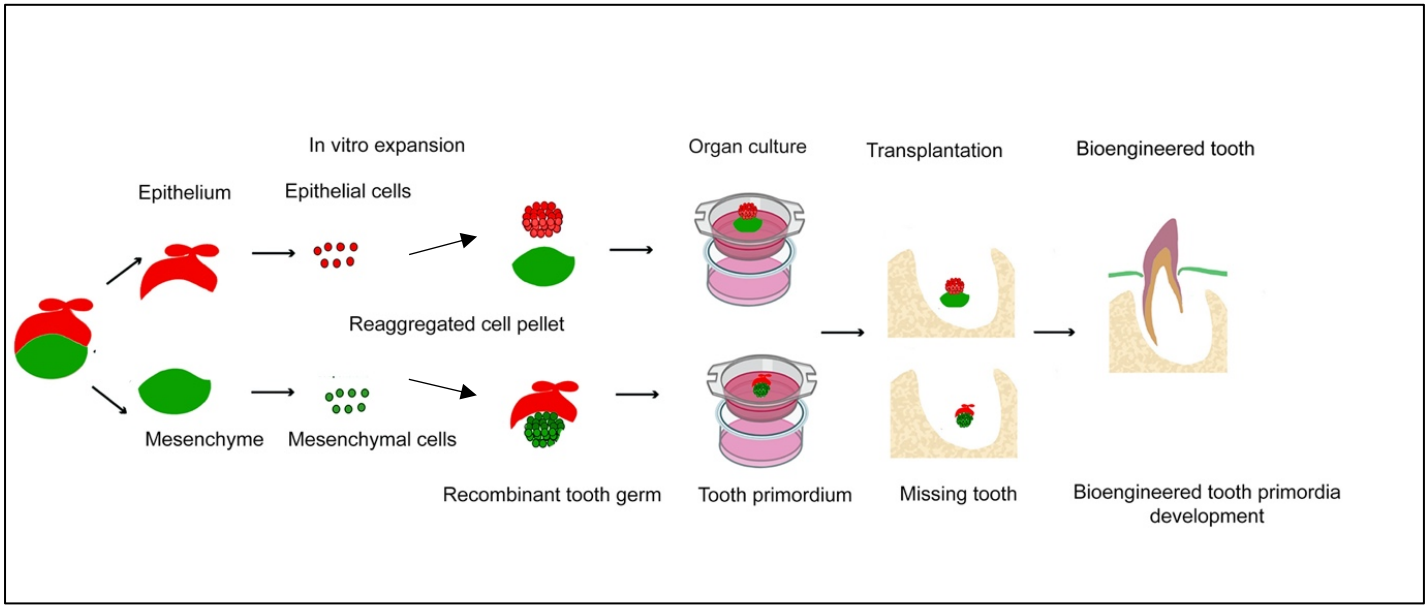


**Figure 10.** Diagram illustrates the cell-tissue recombination strategy for regenerating a whole tooth whereby previously missing.

*Cell-Cell recombination*

*Embryonic cells/organ germ or collagen drop method*

Investigations were further propelled to determine whether dental epithelium and mesenchymal cell suspensions could lead to whole-tooth regeneration. A three-dimensional organ-germ culture method was developed using completely dissociated single cells from epithelial and mesenchymal tissues of incisor tooth germ at the cap stage from the lower jaw in ED14.5 mice. The cells were injected in turn at high cell density into adjacent regions within a collagen gel drop, which was cultured and subsequently transplanted into the subrenal capsule in mice, resulting in a 100% success rate of structurally accurate tooth structure, showing penetration of blood vessels and nerve fibers(Nakao et al., 2007). This study was known as the collagen drop method, or the 'organ germ method' whereby cells are embedded into three-dimensional (3D) hydrogels during the scaffold fabrication, i.e., epithelial and mesenchymal cells are encapsulated into a collagen drop. The "organ germ method" attempts to mimic the tissue architecture and physiological properties of native tissues. This organ germ method was an early example of organoid technology. Since then, there has been a surge in studies in which stem cells are cultured using in vitro 3D culture systems that lead to the development of organoids. As the name suggests, organoids are organlike structures grown in a Petri dish; epithelial and mesenchymal stem cells ( ESCs, adults stem cells or iPSCs) self-organize to form the cellular organization of the organ itself, often involving the use of extracellular matrix (ECM) gels such as Matrigel(Lancaster & Knoblich, 2014). Not only does the organoid resemble a tooth, but it will, in general, contain multiple teeth-specific cell types, be capable of repeating a function of the tooth, be grouped and is, therefore, organized similarly to the tooth spatially(Lancaster & Knoblich, 2014).

Collagen is a type of hydrogel used because of its similarity to the native tissue ECM. Using molar tooth germ from E14.5 mice, dissociated cells were seeded at high density into collagen droplets and then implanted, leading to functional in vivo regeneration of teeth. Many subsequent studies would use the collagen drop method to optimize the shape, length, crown width, cusp position and tooth patterning of the regenerated teeth (Table 3). Ikeda et al. proved that a whole tooth, albeit with abnormal size and shape, could also be regenerated using the organ germ method if the bioengineered organ germ was transplanted into the alveolar bone in the maxilla of an 8-week-old adult murine lost tooth transplantation model(Ikeda et al., 2009). An experiment by Ishida et al. similarly seeded the dissociated cells into the collagen drop at different contact areas and found that the contact area between DE and DM cells determined the crown width and cusps number(Ishida et al., 2011). Oshima et al. addressed the regulation of the shape and length of the bio-tooth by inserting a bioengineered tooth germ into a ring-shaped size control device and then transplanting it into the subrenal capsule(Oshima et al., 2012).

Wang et al. utilized the collagen drop method to demonstrate that a tooth could be bioengineered in a large mammal model(F. Wang et al., 2018).

| Author | Cell source | Scaffold | Culture method | In vivo test | Main results | Histological analysis | Eruption (days) | Correct Morphology | Masticaticatory potential tested | Response to nociceptive stimuli tested |
|---|---|---|---|---|---|---|---|---|---|---|
| (Nakao et al., 2007) | Mice -Incisors and molars tooth germ at cap stage E14.5 | Type I-A collagen | Dissociated cells into single cell suspensions were injected at a high cell density into adjacent regions within a collagen gel drop (mixed or compartmentalized) | First transplanted into the subrenal capsule of mice. Bioengineered tooth germ was then transplanted in an incisor tooth cavity in an adult mouse | First evidence of successful reconstitution of an entire organ via the "organ germ method." | 14 days post-transplantation- correct tooth structure comprising enamel, dentin, root analog, dental pulp, blood vessels and bone | No | Small and multiple bioengineered teeth | - | - |
| (Nait Lechguer et al., 2008) | Mice-Mandibular first molars tooth germ ED14 | Type I-A collagen | Dissociated cells into single cell suspension in a collagen drop | Implanted between skin and muscles behind the ears and mandible of adult mice | Vascularization occurs readily | Full development of teeth, including vascularization. Blood vessels, dentine, dental pulp, enamel | - | Not a normal tooth | - | - |
| (Ikeda et al., 2009) | Mice-ED14.5 molar tooth germ | Type I-A collagen | Dissociated cells in a collagen drop | Hole in the alveolar bone of the upper first molar region in an 8-week-old adult murine- "lost tooth transplantation model". | The first report describes successful replacement with a fully functional bioengineered organ | Correct and complete tooth structure | 49.2 +/- 5.5 days after transplantation | Smaller compared to other normal teeth, plural cusps crown | Yes | Yes |
| (Ishida et al., 2011) | Mice-Molar tooth germ from E14.5 mice | Type I-A collagen | Dissociated cells in collagen drop (different contact areas) | Subrenal capsule transplant in mice | Crown width and cusps number are dependent on the contact area between DE and DM cells | At 21 days post-transplantation, the entire bioengineered tooth germ developed into a tooth unit with the correct | - | Single and width-controlled bioengineered tooth developed from one bioengineered tooth germ instead of multiple | - | - |
| (Oshima et al., 2012) | Mice -Molar tooth germ from E14.5 mice | Type I-A collagen | Dissociated cells in collagen drop | First, in the subrenal capsule, sing a spacer device. The bioengineered teeth unit was then transplanted into an edentulous region of the jaw of mice | Functional in vivo regeneration of size-controlled bioengineered tooth | Correct structure of a whole molar and the proper formation of periodontal tissue and surrounding alveolar bone | 60 days post-implantation | 40 days post-translation- a size-controlled tooth which integrated with the bone and erupted | Yes | Yes |
| (Wen et al., 2012) | Mice- Incisor tooth germ from E14.5 mice | Type I-A collagen | iPSCs mixed with DM cells in collagen drop; DE cells seeded adjacent in collagen | Subrenal capsule transplantation in mice | iPSCs do not affect the interaction between epi and mes cells. They can differentiate into a bone-like and dental pulp-like structure | Newly formed bone-like and dental pulp-like, and dentin-like structures | - | Mature toothlike structure similar to normal tooth | - | - |
| BF | Mice-A molar tooth from E12 (epithelial cells) and a molar | Type I-A collagen | Cells mixed and recombined with epithelium in collagen drop | Subcapsular transplantations of toothlike structures/tooth | Cell community effect in bioengineered teeth – in vitro cell expansion of embryonic tooth mes cells leaves them | - | - | Toothlike structures formed | - | - |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | from E14.5 mouse (mesenchymal cells) | | | primordia in mouse kidney | unable to induce tooth formation, however, they do not lose their ability to differentiate into odontoblasts. Postnatal pulp cells, however, lose all tooth-inducing and tooth-forming capacity following in vitro expansion, and at ratios >1:3 postnatal: embryonic cells, they inhibit the ability of embryonic dental mesenchyme cells to induce tooth formation." | | | | | |
| (F. Wang et al., 2018) | Mini-pigs- Forth deciduous molar germs E40 or E70 | Collagen gel drop | Dissociated tooth germ was reaggregated with a mixture of tooth epithelial and mesenchymal cells | Transplantation in mouse subrenal capsules and jawbones | The feasibility of whole tooth regeneration in large animal | Regenerated toothlike structures with crowns nearly completed in bony tissues after transplantation for 16 weeks with correctly arranged tooth components | 80% of explants erupted into the oral cavity 2 months post-implantation | Abnormal tooth structure, a bulge | - | - |

**Table 3.** Collagen drop method using entirely reassociated embryonic tooth cells

The successful regeneration of the teeth occurred in experiments that utilized cells harvested from an early stage, embryonic tooth buds. The ethical dilemma in destroying an embryo which some groups see as destroying life, the possibility of immune rejection after implantation as embryonic cells from embryos do not share the same genome with the patient, may give rise to teratocarcinomas and have genetic instability are all considerations that need to be taken when using embryonic cells or tissues (Q. Sun et al., 2014). The most significant limitation is obtaining suitable autologous human embryonic dental cells(W. Zhang et al., 2010). Hu et al. found that the minimum numbers of cells required to initiate tooth development were in the range of $4.0 \times 10^4$ to $4.0 \times 10^5$ cells, a number achievable only by using multiple embryos, an obvious limiting factor in humans(Hu et al., 2006). A further stumbling block in the use of embryonic cells was that they lost their inductive capacity within 24 to 48h of being cultured in vitro(Zheng et al., 2016). The focus on tooth regeneration shifted to devise reliable methods to regenerate teeth using postnatal adult cells (W. Zhang et al., 2010). Adult dental stem cells overcame some of the limitations of embryonic cells as it does not involve destroying an embryo and carries a more negligible risk of immune rejection as these adult stem cells would have the same genotype as the patients from which they were isolated(Q. Sun et al., 2014).

## *Cell-laden strategy*

### *Adult stem cells /organ germ or collagen drop method*

In this coculture strategy, cells are encapsulated within a hydrogel. As aforementioned in Table 3, many experiments were performed using the collagen drop method using embryonic cells. Subsequently, even adult stem cells were used to construct bioengineered tooth germs using the collagen drop method (Table 4). For example, an experiment in a large animal model used the premolar tooth germ from a 30-day-old beagle dog as the cell source of epithelial and mesenchymal postnatal cells, which, when embedded into a collagen drop, led to the functional regeneration of the bioengineered tooth after autologous transplantation of the organoid into the alveolar bone socket of the canine in the mandible(Ono et al., 2017). Other hydrogels were experimented with to improve the results of these dental bioengineering experiments: Type I collagen/ chitosan hydrogel blend with Matrigel (Ravindran et al., 2010), Collagen and Growth Factor Reduced (GFR) Matrigel(W. Zhang et al., 2010), gelatin

methacrylate (GelMA) hydrogel(E. E. Smith et al., 2017a)(E. E. Smith et al., 2018)(E. E. Smith et al., 2017b).

| Author | Donor | Culture method | Scaffold | Implantation | Significance | Histological analysis | Vascularization | Morphology |
|---|---|---|---|---|---|---|---|---|
| (Ravindran et al., 2010) | HAT-7 dental epithelial cells and mesenchymal hDPSCs | Cells were premixed with the collagen and chitosan monomer solutions | Type I collagen/chitosan hydrogel blend and Matrigel. | Subcutaneous implant in mice | The first successful 3D multilayered coculture system was established that could be exploited to study the interactions between dental epithelial and mesenchymal cells. | 4 weeks post-implantation - blood vessels with red blood corpuscles. | Neovascularization in vivo | - |
| (W. Zhang et al., 2010, p. 20) | Pig tooth buds from 6-month-old minipig (epi) and pulp of wisdom tooth from16-year-old Caucasian male (mes) | Single cells suspension of epi and mes cells. hDPSCs - seeded Collagen gel layers were cocultured with porcine DE cells suspended in Growth Factor Reduced (GFR) Matrigel | Collagen and Matrigel | Subcutaneous into a 4-week-old nude rat | Implanted constructs maintain predetermined shape and size | 4 weeks post-implantation-irregular mineralized tissue | Highly vascularised tissues | Formed calcified tissues did not resemble that of naturally formed dental hard tissues |
| (E. E. Smith et al., 2014) | DE and DM cells were isolated from unerupted porcine tooth buds and 5-month-old porcine jaws. | Single-cell suspensions were plated in DE, or DM cell selective media- GelMA hydrogels --human umbilical vein endothelial cells (HUVEC) encapsulated within GelMA hydrogels | GelMA | Subcutaneously (back of immunocompromised 5-month-old female Rowett Nude rats) | GelMA is a suitable scaffold material for the development of biomimetic, 3D tooth models | 6 weeks of subcutaneous implantation revealed mineralized tissue | - | Small structures, not similar in shape or size to natural teeth |
| (Ono et al., 2017) | Premolar tooth germ from 30-day-old beagle dogs | Epithelial tissue and mesenchymal cells in collagen drop | Type I-A collagen | Autologous transplantation into the alveolar bone socket of the canine mandible | Functional tooth restoration through the autologous transplantation of bioengineered tooth germ in a postnatal canine model, i.e. large animal model. | Correct tooth tissue structure and a single root shape composed of enamel dentin, cementum and periodontal ligament. Erupted into Jaw | . | Smaller in size compared to the natural tooth. Not similar in shape to natural teeth ( from photo), single-rooted instead of two roots |
| (E. E. Smith et al., 2017b) | Unerupted tooth buds extracted from 5-month-old porcine jaws. | Single-cell suspensions were plated in DE or DM cell selective media. Culture method:(GelMA DE-HUVEC loaded hydrogel layered on top of DM-HUVEC hydrogel) | GelMA | Implanted subcutaneously in nude rats | First to demonstrate the use of GelMA hydrogels to support the formation of postnatal dental progenitor cell-derived mineralized and functionally vascularized tissues of specified size and shape | Mineralized tissues were present in 9/13 (69%) of 6-week explants. No distinct enamel or dentin layers were observed | Vascularization occurs readily in bioengineered teeth | In many cases, mineralized tissue formation appeared to adopt the size and shape of the GelMA construct. |
| (E. E. Smith et al., 2018, p. 20) | DE and DM cells were isolated from unerupted porcine tooth buds and 5-month-old porcine jaws. | Single-cell suspensions were plated in DE, or DM cell selective media- GelMA hydrogels – HUVEC encapsulated within GelMA hydrogels | GelMA | Implanted subcutaneously in nude rats | The first report using postnatal dental cells to create bioengineered tooth buds that exhibit features similar to natural tooth development: dental epithelial stem cell niche, enamel knot signalling centres, transient amplifying cells, and mineralized dental tissue formation | 4W in vivo implanted constructs contained mineralized tissue. Bioengineered tooth buds exhibited many features characteristic of natural tooth buds, although in a less reproducible and less organized manner | Vascularization occurs readily in bioengineered teeth | Not similar in size or shape to natural teeth |

**Table 4.** Whole tooth regeneration studies utilizing the cell-laden strategy whereby adult stem cells are encapsulated in a hydrogel (epi) epithelial (mes)mesenchymal

### *Cell-seeded scaffold*

The scaffold-based strategy utilizes biomaterials fabricated beforehand and serves as a structural base onto which cells are seeded(Dissanayaka & Zhang, 2020). In 2002, dissociated postnatal cells from third molar tooth buds of six-month-old pig jaws successfully created a bioengineered tooth crown(Young et al., 2002). The single cell suspensions of dental epithelial and mesenchymal cells were seeded onto a biodegradable tooth-shaped polyglycolate/poly-L-lactate (PGA/PLLA), and poly-L-lactate-co-glycolate (PLGA) scaffold and subsequently the cell/ polymer construct was implanted into the omentum of athymic rats(Young et al., 2002). 20-30 weeks post-implantation analysis revealed a bioengineered crown in vivo, which, albeit contained accurately formed enamel, dentin and a well-defined pulp chamber, was very small(Young et al., 2002).In the acellular scaffold controls, there was no regeneration of mineralized tissue. This study highlighted that cultured postnatal DE and DM cell suspensions retain the ability to differentiate into dental tissue. Since 2002, numerous studies using the cell scaffold complex have been performed to optimize the cell-scaffold construct that would regenerate a bioengineered tooth of the correct size and shape that is vital, innervated and functional.

Studies have become increasingly more elaborate, with each aspect of the methodology being finetuned or changed to get closer to creating a bioengineered tooth with the correct form and function. Different sources have been used; postnatal cells such as premolar tooth bud cells, molar tooth bud cells, human dental pulp stem cells, dental epithelial cells, bone marrow stem cells etc. These tooth buds were either dissociated into heterogenous cell suspensions or epithelial and mesenchymal components first separated before dissociation into single cell suspensions. Porcine, canine and ovine models were developed for cell-based regeneration. Cells were either premixed, sequentially seeded onto the scaffold or seeded onto the scaffold using different configurations. Different scaffolds have been used to determine which would work best in TE: PGA, PLLA, PLGA, collagen sponges, gelatin-chondroitin-hyaluronan tri-copolymer scaffold (GCHT), hexafluoroisopropanol (HFIP)-derived silk fibroin scaffolds, collagen, matrigel, fibrin gel, β-tricalcium phosphate(β-TCP), platelet-rich fibrin (PRF), GelMA and decellularized tooth buds to name a few (Table 5).

| Reference | Cell origin | Culture method | Biomaterial | In vivo tests (Implantation) | Main findings | Histological Findings | Vascularization | Eruption | Morphology |
|---|---|---|---|---|---|---|---|---|---|
| (Young et al., 2002) | Third molars tooth bud from 6-month-old pig jaws | Buds dissociated into single-cell suspensions. A mixture of epithelial and mesenchymal cells seeded on scaffolds (in vitro culture for 6 days) | PGA fibre mesh with 3% w/ w PLLA. PLGA is prepared by NaCl crystals leaching. Scaffolds are collagen coated | Omentum of athymic rats | Bioengineered toothlike crown structure in vivo.1st bioengineered adult DSC Pig tooth | 30 weeks post-implantation, anatomically correct tooth crowns consisting of the dental pulp, dentin, and enamel. Recognizable cusps and root tips. No periodontium | Vascularization occurs readily | - | Bioengineered teeth didn't conform to the shape of the scaffold- multiple small tooth crowns (2 by 2mm) in bioengineered tooth constructs |
| (M. T. Duailibi et al., 2004) | 3- to 7-day-newborn Lewis rat molar tooth buds | Buds dissociated into single-cell suspensions. A mix of epi and mes cells statistically seeded on scaffolds (in vitro culture for 6 days) | PGA fibre mesh with 3% w/ PLLA. PLGA is prepared by NaCl crystals leaching. | Omentum of 6 to 12 months old Lewis rats | 4-day-newborn cells generated bioengineered tooth tissues .1st bioengineered adult DSC Rat tooth | 12-week post-implantation PGA and PLGA scaffold cell-seeded implants demonstrated the presence of dentin, enamel, and pulp tissues | Vascularization occurs readily | - | Multiple small tooth crowns per implanted construct |
| (Young, Abukawa, et al., 2005b) | Pig third molar tooth bud cells | Dissociated DE and DM cells seeded onto PGA AND PLGA scaffolds. Bone implants were generated from osteoblasts induced from bone marrow progenitor cells obtained from the same pig, seeded onto PLGA fused wafer scaffolds | PGA and PLGA scaffolds | Implanted in the omenta of adult rats | Coordinated formation of functional bioengineered tooth and bone tissues | 12 weeks post-implantation: Bioengineered tissue contained predentin, dentin, and enamel. Bioengineered bone tissue consisting of loosely formed alveolar bone, with smaller amounts of compact bone and immature osteoid tissue formed | - | - | Roughly conformed to the size and shape of the spherical scaffold |
| (Young, Kim, et al., 2005) | Six-month porcine third molar tooth buds | Dissociated DE and DM cells seeded onto PGA /PLGA scaffolds. | PGA/PLLA tooth scaffolds | Omentum of athymic rats | Bioengineered tooth structures possessing both natural and anomalous features, | 18 weeks or longer post-transplantation- complex clusters of developing heterogeneous tooth tissues. Approximately 15% of tooth structures present in any given implant exhibited correctly organized pulp, dentin and enamel layers | - | - | Irregular in shape |

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|---|---|---|---|---|---|---|---|---|---|
| (Honda et al., 2006) | First molar tooth buds from 8 male dogs aged 8 to 12 weeks | Tooth buds were dissociated into heterogeneous single cells suspension | PGA fibre mesh precoated with type I collagen | Tooth extraction sockets (from which the tooth buds were extracted) | The first report for the formation of dentin and bone from dissociated odontogenic cells | 24 weeks post transplantation- Heterogeneous composite of bone, dentin, and connective tissue; however, dental pulp, crown, and root formation were not observed | - | No | Abnormal circular-shaped structure |
| (Sumita et al., 2006) | Third molar teeth from a 6-month-old pig | Tissue was dissociated into single cells, and the heterogeneous cells were seeded onto the scaffolds | Collagen sponge or the polyglycolic acid fibre mesh | Omentum of immunocompromised rats | Collagen sponge resulted in the first report of complete tooth morphology, including the root structure (less than 5%) | 25 weeks post-implantation- Enamel and dentin were identified in the circular-shaped TE teeth. The stick-shaped TE-teeth obtained from the collagen sponge scaffold revealed the dentin, cementum-like tissue and pulp and resembled a root-like structure | Vascularization occurs readily | - | Irregular crown morphology- circular and stick-shaped irregular TE teeth |
| (Honda et al., 2007) | Third molar teeth from a 6-month-old pig | Tissue separated and dissociated into single cell suspensions. Different configurations of cells seeded on the collagen sponge were tested | Collagen sponge (75% type I . 25% type III collagen, dry weight) Initial high-density seeding of isolated mesenchymal cells onto the scaffold, followed by the placing of isolated epithelial cells to adhere on top of mesenchymal cells directly | Omentum of 5- to 6-week-old rat | Direct cell-cell contacts are necessary for tooth regeneration, | 20 weeks post-implantation, the regenerated teeth were made up of dentinal tissue- an abundance of mineralized dentin along with a small amount of enamel, with only one sequence per scaffold | - | - | Single regenerated teeth in each scaffold that were more uniform in shape |
| (S. E. Duailibi et al., 2008) | 4-day-post natal Lewis rat molar tooth bud | Rat tooth bud cells dissociated into single cell suspensions. A mix of epi and mes cells statistically seeded on scaffolds (in vitro culture for 6 days) | PGA fibre mesh with 3% w/ w PLLA. PLGA prepared by NaCl crystals leaching | Adult rat M1 mandibular tooth extraction sockets | 1st bioengineered tooth grown in a rat jaw | Bioengineered dental tissues were not as organized as those grown in the omentum; distinct dentin, enamel, and alveolar bone tissues were present. | - | No | Small tooth structures throughout the scaffold implant, as opposed to a single large tooth |

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|---|---|---|---|---|---|---|---|---|---|
| (Kuo et al., 2008) | Swine dental bud cells (DBCs) were isolated from the developing mandibular teeth | Dissociated into single cell suspensions | GCHT scaffold | Autografted back into the original alveolar socket | DBCs/GCHT scaffold is a suitable scaffold for tooth regeneration | 36-week post-transplantation- toothlike structures, including well-organized dentin-pulp complex, cementum, and periodontal ligament (2 in 6) | Vascularization occurs readily | - | Regenerated teeth were smaller in size than normal but appeared to be dictated by the size of the GCHT scaffold |
| (Xu et al., 2008) | Molar tooth buds were isolated from 4-day postnatal Lewis rat pups | Dissociated into single cell suspensions | HFIP-derived silk fibroin scaffolds | Implanted into the omenta of syngeneic Lewis rat hosts | First to use HFIP silk- not conducive to dental epithelial cell survival and differentiation | 20 weeks post-implantation: formation of mineralized osteodentin-like tissue but no enamel | Vascularization occurs readily in bioengineered teeth | - | Mineralized osteodentin adopted the size and shape of the silk scaffold pores |
| (Abukawa et al., 2009) | Unerupted third molar tooth buds from 3.6-month-old Yucatan minipig (for tooth constructs). For bone constructs, porcine bone marrow progenitor cells (BMPCs) were isolated from iliac-crest aspirates (same pigs). | Osteoinduced BMPCs were statically seeded onto bone scaffolds. The PGA/PLLA scaffolds were statically seeded with pulp organ-derived dental mes cells. Gelfoam-absorbable gelatin sponge strips were statically seeded with enamel organ-dental epi cells. PGA/PLLA spheres were wrapped with Gelfoam strips and were sutured together to generate tooth constructs. Subsequently, the hybrid tooth and bone construct were fabricated | PGA/PLLA spheres coated with type I collagen wrapped with gelatin sponge | Full-thickness segmental bone defects in hemimandible of Yucatan minipigs | The feasibility of coordinated autologous tooth and mandible regeneration | 20 weeks post-implantation- (1 of 6) implants contained dentin, pulp, cementum, and surrounding PDL | Vascularization occurs readily in bioengineered teeth | No | Small toothlike structures |
| (W. Zhang et al., 2009) | | Hybrid tooth and bone construct: tooth bud cell-seeded scaffolds combined with autologous iliac crest bone marrow-derived stem cell-seeded scaffolds | PGA/PLLA scaffolds and prefabricated Gelfoam absorbable gelatin sponge strip scaffold | Transplanted into surgically prepared mandibular defects in the same minipig | The feasibility of regeneration of teeth and associated alveolar bone in a single procedure | 20 weeks post-implantation: organized dentin, enamel, pulp, cementum, periodontal ligament, and surrounded by regenerated alveolar bone | - | No | Small toothlike structures |
| (Ohara et al., 2010) | Freshly isolated tooth germ from three 6-month-old miniature pigs | Dissociated tooth bud cells were seeded under static conditions | PGA, collagen gel, fibrin gel, β-TCP | Transplanted in the subcutaneous space on the backs of twelve 4- | Comparison of commonly used scaffold for dental | 8 weeks post-implantation- dentin and enamel-like structures were observed in both | - | No eruption | Irregular tooth-like structures |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | onto different scaffolds | | week-old KSN nude mice | regeneration. Collagen and fibrin gel support the initial regeneration process but not the maturation of tooth buds | the PGA and fibrin gel groups, while the amount of hard tissue was less in the fibrin gel group. Enamel-like structures were not observed in the β-TCP group, and hard tissue was generated along with the inner wall of the porous scaffold, which resembled a root | | | |
| (Kuo et al., 2010) | Unerupted second molar buds harvested from miniature pigs | DBCs suspended in bone marrow fluid were seeded onto the scaffold | GCHT scaffold | Autografted into the original alveolar sockets of the pigs | Porcine DBCs combined with bone, marrow fluid is a suitable scaffold that can regenerate teeth in a porcine model with autogenic cell transplantation | 40 weeks post-implantation. The present results showed that 1 in 8 pigs developed a complete tooth with crown, root, pulp, enamel, dentin, odontoblast, cementum, blood vessel, and the periodontal ligament in indiscriminate shape | Vascularization occurs readily | Erupted ( 1 of 8) | Irregular in shape |
| (W. Zhang et al., 2011) | hDPSCs harvested from a 3rd molar tooth of a 16-year-old African American female. Bone aspirate from an 18-year-old African American male for BMCs | hDPSCs and BMCs were seeded onto HFIP-based silk scaffolds | HFIP-based silk-based hydrogel and aqueous-based silk scaffolds | Transplanted subcutaneously into four-week-old nude rat hosts | Silk scaffold materials and hDPSCs pre-seeding cell treatments suitable for tooth regeneration | No distinct hard tissue regeneration was found in any implants. HFIP-based silk scaffolds supported the soft dental pulp formation better than the aqueous-based silk scaffolds | Vascularization occurs readily in bioengineered teeth | - | - |
| (Yang et al., 2012) | Unerupted second molar tooth buds from miniature swine | DBCs detached from culture dishes were resuspended in 500 ml of fibrinogen solution to form the DBC fibrinogen solution. 500 ml of thrombin solution was then added to form the fibrin glue | Fibrin enriched with PRF | Autografted back into the original alveolar sockets | Addition of PRF in fibrin glue is not sufficient to promote organized tooth regeneration | 36 weeks after implantation -1 in 3 pigs developed a complete tooth with crown, root, pulp, enamel, dentin, odontoblast, cementum, blood vessels, and periodontal ligament | Vascularization occurs readily in bioengineered teeth | Erupted | The shape of the bioengineered tooth does not conform to that of the natural tooth |
| (Monteiro et al., 2016a) | Unerupted tooth buds extracted from 5-month-old porcine jaws | Dissociated into single cell suspensions. DE and DM cell sheets combined with biomimetic enamel organ and pulp organ layers | GelMA hydrogels | Implanted subcutaneously onto the backs of immunocompromised 5-month-old female Rowett Nude rats | Biomimetic 3D tooth buds as models for tooth engineering | 3 weeks post-implantation, exhibited osteodentin-like tissue formation | Vascularization occurs readily in bioengineered teeth | - | Small, irregular structure |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| (Yang et al., 2016) | Dental pulp from upper incisors, canines, premolars, and molars was extracted from sexually mature miniature pigs- DPSCs. Epithelial cells were isolated from the gingival epithelium | Differentiation of DPSCs into odontoblasts and osteoblasts. The epithelial cells, odontoblasts, and osteoblasts were seeded onto the surface, upper, and lower layers, respectively, of a scaffold | GCHT bioactive scaffold | Transplanted into the mandibular alveolar socket of a pig | | 13.5 months post-implantation, seven of eight pigs developed two teeth with crown, root, and pulp structures. Enamel-like tissues, dentin, cementum, odontoblasts, and periodontal tissues | Vascularization | Erupted | All regenerated teeth showed a molar morphology, and the lengths of roots were varied. |
| (W. Zhang, Vazquez, et al., 2017) | hDPSCs (mes), porcine dental epithelial cells (pDE) (epi) cells, and third molar 6-month-old pig tooth buds to create decellularized tooth buds | 1) acellular decellularized tooth buds(dTBs 2) recellularized dTBs seeded with pDE, hDPSCs, and HUVEC recell-dTBs 3) dTBs seeded with BMP-2 and 4) freshly isolated nondecellularized natural TBs (nTBs) | Decellularized tooth bud scaffold | Tooth extraction sockets of 6-month-old Yucatan mini pigs | Regenerated teeth adopt the size of the decellularized scaffold in vivo. First report of the use of decellularized tooth buds which regenerated close to full-sized teeth have been bioengineered and grown in the jaws of mini pig hosts | 6-month post-implantation-bioengineered whole teeth, consisting of mature enamel, pulp, dentin, periodontal ligament, and tooth roots, which adopted the size and shape of the scaffold | Vascularization | Eruption | 6-mo nTB construct consisting of a well-developed tooth crown and root similar to the size and shape of a natural tooth |

**Table 5.** Cell-seeded scaffold strategies to create bioengineered teeth by seeding dissociated postnatal odontogenic cells onto scaffolds and implanting them in vivo.

To date, multiple cell-scaffold constructs have been used to bioengineer tooth tissue; however, the regenerated teeth using postnatal tooth cells still need finetuning to get the size and shape of the tooth right. Compared to the result of tooth engineering experiments with embryonic cells, postnatal tooth cells may have already lost essential factors involved in the regulation of tooth shape and size. Further experiments are needed to address this.

## *Scaffold-free approach*

### *Cell sheet engineering*

Cell sheet engineering (CSE) is another example of a scaffold-free approach. Cell sheet technology, as the name implies, involves cells being cultured on the usually thermosensitive surface resulting in a single, contiguous aggregation of cells in the form of a sheet that preserves cell-to-cell interaction and deposited ECM (Yamato & Okano, 2004). These viable cell sheets can be subsequently detached and transferred to other culture dishes in vitro or implanted into tissue surfaces in vivo(Yamato & Okano, 2004). This approach improves the limitation of the classic scaffold triad for tooth engineering in that it produces a high density of cells, unlike the sparse number of cells that can be found when seeded onto scaffolds. As the cell sheets contain secreted ECM, cell–matrix connections, intercellular connections and specific shapes provided by ECM are preserved, which will inevitably benefit tissue regeneration. CSE has been widely used in regenerative medicine and applied in the heart, liver, cornea, bladder, oesophagus and bone (Moschouris et al., 2016). In tooth TE, CSE has mainly been used for the regeneration of individual dental tissues such as pulp, PDL and bone; however, it has not been used widely in whole tooth regeneration. In a recent series of studies performed by Monteiro and colleagues, DE and DM cell sheets (CSs) combined with biomimetic enamel organ and pulp organ layers created using GelMA hydrogels were used in whole tooth regeneration with promising results (Monteiro et al., 2016b)(Monteiro & Yelick, 2017).

### *Spheroids production*

Spheroids are spherical cellular units generally cultured as free-floating aggregates (Gunti et al., 2021). A recent review on the strategies used for developing 3D stem cell spheroids and organoids for tissue repair and regeneration details the many strategies used for creating spheroids; the hanging drop, hydrogel scaffold, microwell, spinner and rotational methods, microfluidic, low attachment culture plates and magnetic field(Kim et al., 2022). The hanging drop method uses gravitational forces, which

cause single cells to aggregate at the tip of small to medium droplets that are hung on the cell culture substrate. This three-dimensional micro-culture system involved mixed epithelial-mesenchymal cell reorganization in a liquid instead of semisolid collagen gel, leading to spheroids forming coherent 3D cell aggregates. Implantation of these microtissues leads to the formation of well-formed crowns containing dentin and enamel and the initiation of root formation(Kuchler-Bopp et al., 2016).

*iPSCs- Induced pluripotent stem cells*

Autologous cell sources are limited in availability, and harvesting these cells does not come without some level of harm to the individual(Yelick & Sharpe, 2019). A method to circumnavigate this stumbling block was published in 2006, ultimately leading to John B. Gurdon and Shinya Yamanaka winning the Nobel prize in 2012. Through a series of work, intact mature cells in mice were reprogrammed to become immature stem cells with an embryonic-like state, i.e. they possessed the ability to develop in every type of cell in the human body and were therefore named iPSCs (Takahashi & Yamanaka, 2006). iPSCs have been used in studies aimed at the regeneration of individualized dental tissues but few for whole tooth regeneration. iPSCs cells were mixed with mesenchymal cells at a ratio of 1:4; the mixture was then injected into a collagen hemisphere, followed by the injection of epithelial cells into the adjacent area to promote direct cell contact(Wen et al., 2012). The reconstituted tooth germs were transplanted into the subrenal capsule of mice. This study confirmed the possibility of using iPSCs in whole tooth regeneration as the use of iPSCs did not affect the interaction between epithelial and mesenchymal cells, and they possessed the ability to differentiate into a bone-like, dentin-like and dental pulp-like structure. In 2013, using integration-free human urine-iPSCs were differentiated into an epithelial sheet. This epithelial component was recombined with embryonic mesenchyme from mice, leading to the generation of the tooth containing dental pulp, dentin, enamel space, and enamel organ structure after 3 weeks of subrenal capsule transplantation. (Cai et al., 2013).

### *3.2.3 Targeted molecular therapy – Stimulation of the third dentition*

Targeted molecular therapy is another feasible approach for tooth regeneration which involves the administration of drugs or other substances, such as small molecules, monoclonal antibodies, or siRNAs which target specific molecules involved in tooth regeneration. This therapy is already being used to treat specific types of cancer and rheumatoid arthritis(Zhong et al., 2021). Targeted molecular therapy aims to stimulate the formation of the "third dentition". To stimulate the so-called "third dentition"

through targeted molecular therapy, a detailed understanding of embryology, molecular and developmental biology has provided the scientific background to make this a possibility.

BMP and Wnt signalling orchestrate early tooth development(Yuan et al., 2015). However, they have also been involved in modulating the growth of multiple other organs and tissues. As a result, therapies that directly target them may have unwanted side effects, even death. Instead, to accomplish whole tooth regeneration, researchers target factors which antagonize BMP and Wnt specifically in tooth development, such as uterine sensitization-associated gene-1 (USAG-1), bone morphogenetic protein 7 (BMP-7) or CCAAT Enhancer Binding Protein Beta (CEBPB), for which activation or inhibition of these single candidate molecules can lead to supernumerary tooth formation (Takahashi et al., 2014).

The use of monoclonal antibodies for the recovery of tooth development in congenital tooth agenesis has demonstrated remarkable results. USAG-1 is expressed in odontogenic epithelial cells and is a BMP antagonist and modulator of the Wnt-signalling pathway. USAG-1 deficient mice have supernumerary teeth; USAG-1 abrogation rescued apoptotic elimination of odontogenic mesenchymal cells. USAG-1 controls the number of teeth by regulating apoptosis(Murashima-Suginami et al., 2007). Enhancing BMP signalling resulted in supernumerary teeth formation in the Usag-1-deficient mouse model(Murashima-Suginami et al., 2008). USAG-1 is an antagonist of BMP-7(Kiso et al., 2014). These findings led to the hypothesis that a single candidate molecule such as Usag-1 could, in essence, regenerate a whole tooth.

A single systemic administration of a USAG-neutralizing antibody in an ectodysplasin A1 (EDA-19-deficient mouse, a model for congenital tooth agenesis, led to the recovery of tooth development and the formation of a whole tooth by stimulating arrested tooth germs. The systemic administration of USAG-neutralizing antibody also led to the regeneration of a whole tooth in ferrets, which like humans, have diphyodont dentition. Further analysis was performed to determine whether BMP signalling or Wnt signalling was dominant during tooth development(Murashima-Suginami et al., 2021). This study revealed that the anti–USAG-1 antibody affected BMP signalling rather than Wnt Signalling. The author did note, however, that Wnt signalling involvement could not be eliminated as several mice in the experiment did not survive. As the USAG-neutralizing antibody did not recover tooth development in all the cases of congenital tooth agenesis, this therapy could only be utilized in congenital cases of missing teeth with mutations of specific causative genes.

Another recent study postulated the idea of topical treatments for congenital tooth agenesis using siRNA for targeted molecular therapy instead of monoclonal antibodies(Mishima et al., 2021). Runt-related transcription factor 2 (*Runx2)* has been identified as one of the several different causative genes in

patients with congenitally inherited tooth agenesis. *Usag-1* deficient mice have supernumerary teeth(Murashima-Suginami et al., 2007). (*Runx2)−/−* mice exhibit arrested tooth development. RUNX2 and USAG-1 act in an antagonistic manner, and therefore by crossing *Runx2−/−* mice with *Usag-1−/−* arrested, tooth formation was restored(Togo et al., 2016). Local administration with Usag-1 Stealth siRNA leads to tooth regeneration in *Runx2−/−* mice which normally exhibit arrested tooth development, both in vivo and in vitro(Mishima et al., 2021).

A recent report has attributed the etiology of supernumerary teeth formation to the third dentition(Kiso et al., 2019). The study also reports that some supernumerary teeth not attributable to the third dentition were derived from express SRY (sex determining region Y)-box 2 (Sox2) SOX2-positive OESCs, where SOX2 is a molecular marker of OESCs in mice(Kiso et al., 2019). There were markedly fewer SOX2-positive cells in the labial cervical loop epithelium of adult CEBPB−/− mouse incisors than in wild-type animals(Saito et al., 2018). The suggestion is that CEBPB maintains SOX 2-positive OESCs in the labial cervical loop epithelium during postnatal life.

For tooth formation to occur, odontogenic epithelial cells and mesenchymal cells are necessary. Odontogenic epithelial stem cells are differentiated from one of the tissue stem cells, enamel epithelial stem cells. Meanwhile, odontogenic mesenchymal cells are differentiated from odontogenic epithelial cells by epithelial-mesenchymal transition(Takahashi et al., 2020). CEBPB is, therefore, involved in maintaining the stemness of odontogenic epithelial stem cells and suppressing epithelial-mesenchymal transition (EMT)(Saito et al., 2018).

One plausible mechanism for de novo whole tooth formation involves targeting odontogenic epithelial stem cells by administering, for instance, a small molecule CEBPB. Ectomesenchymal abrogation of CEBPB can lead OESCs to differentiate into odontoblasts by loss of stemness and EMT and regenerate supernumerary teeth(Takahashi et al., 2020).

Targeted molecular therapy to stimulate the third is attractive for several reasons: the third dentition appears to be after birth which in essence means that it would be formed directly in the mouth, it takes that same amount of time to develop as a permanent tooth and can therefore erupt after permanent teeth eruption.

## 3.3 Conclusion

The "holy grail" of regenerative dentistry is the ability of a missing tooth to be replaced by a whole new tooth(Sharpe, 2020). Clues from polyphyodont research may soon narrow the gap in understanding why

humans no longer have continuous tooth replacement capacity. Dental TE has certainly come a long way over the decades, but despite the advancements made in tooth bioengineering, researchers need to identify a way to restore embryonic-like properties to more accessible adult stem cells(Sharpe, 2020). The progress in TE research is primarily based on in vitro and in vivo studies. Future clinical translation is needed to move this research forward. Important considerations remain, such as creating a tooth of a predetermined size, shape and shade that is fully vascularized and innervated and which, like natural teeth, provides masticatory function. Targeted molecular therapy is promising; however, further studies investigating its safety for use in humans will need to be thoroughly investigated. It's time for dentistry to evolve using biology-based therapies, much like medicine. While this thesis chapter focuses on whole tooth regeneration, undoubtedly of equal importance, the need to regenerate individual dental tissues that can be clinically translated is even more urgent.

# 4. EXPERIMENTAL REJUVENATION STUDY

## 4.1 Introduction

Cell-based therapies using adult or postnatal stem cells are a promising research direction with optimistic results. Adult dental stem cells are obtained from mature tissue. Dental pulp stem cells taken from aged persons compared to younger individuals have shown a decline in regenerative potential. In fact, this reduced ability of the adult stem cell populations is one of the main contributors to ageing. However, is it, in fact, aged individuals who are in most urgent need of regenerative therapies, an obvious catch-22 situation.

Aged stem cells exhibit reduced responsiveness to tissue injury, reduced proliferative ability, and functional decline, leading to reduced cell replacement and regenerative abilities. This age-dependent decline in adult stem cell function results from telomere attrition, DNA damage, loss of proteostasis, intracellular communication, mitochondrial dysfunction, cellular senescence, nutrient sensing, epigenetic alteration, and stem cell exhaustion over time(López-Otín et al., 2013).

A recent review eloquently details the strategies to rejuvenate aged stem cells(Neves et al., 2017a). Strategies to rejuvenate aged stem cells can be mainly divided into those that aim to modify their intrinsic mechanisms or extrinsic mechanisms. Intrinsic mechanisms in aged stem cells include targeting signalling pathways, repressing cell cycle inhibitors, reversing DNA damage or intervening with epigenetic modifications. Extrinsic mechanisms modify the stem cell niche or its systemic microenvironment. The niche describes any cell, tissue or ECM that resides in close proximity or direct contact with the stem cell population(Neves et al., 2017b). Altering the systemic environment may involve the use of soluble factors such as signalling ligands, hormones, growth factors etc.

One rejuvenation strategy is derived from a growing body of evidence suggesting that it's not the actual stem cells, but in fact, the paracrine factors released by the stem cell into the microenvironment are largely responsible for its regenerative properties(Conboy et al., 2005)(Fu et al., 2017). Paracrine factors or the secretome are the collective terms used for the stem cell's release of growth factors, cytokines, exosomes, and other micro-vesicles (Conboy et al., 2005). The conditioned medium (CM) derived from mesenchymal stem cells (MSCs) can be used both in vitro and in vivo to test specific paracrine effects(Danieli et al., 2016).

Numerous studies have shown the beneficial effects of CM from dental MSCs on many diseases affecting the aged population, such as cardiac lesions, diabetic disorders, dental diseases, hair loss, liver diseases, neurological disorders, pulmonary lesions, and immunity disorders(Chouaib et al., 2022). More pertinently, this systematic review identified that the donor age range in most studies was between

14 to 30 years old, i.e., most of the studies utilized CM from young mesenchymal stem cells(Chouaib et al., 2022).

Only a few studies have shown that aged MSCs can be rejuvenated following exposure to the CM of young mesenchymal stem cells(Bhandi et al., 2021)(Ma et al., 2009)(Conboy et al., 2005). In this study, we aim to investigate a rejuvenation strategy for improving the suboptimal performance of aged adult human dental stem cells by using the CM of young dental pulp stem cells, thereby enhancing stem cell tissue repair and regeneration potential.

## 4.2 Material and Methods

### 4.2.1 Isolation and culture of hDPSCs

In collaboration with the Odontostomatological and Special Surgery Unit, Ospedali Riuniti of Ancona, dental pulp (DP) was isolated from the extracted intact third molars of 12 systemically healthy individuals gender matching (mean age, 43 years; range, 20–64 years) by following approval by the Local Ethics Committee. All patients voluntarily agreed to participate in the trial and provided verbal informed consent. Samples were divided into two: group young (21 years; 20–23 years) and group old (64 years; 62–66 years). Extracted molars were stored in Dulbecco's Modified Eagle's Medium F12 (DMEM/F12) supplemented with 10% Fetal Bovine Serum (FBS), 2% penicillin/streptomycin (p/s) for 24 hrs before the removal of the pulp. The tooth was gently wiped with a tissue soaked in 70% ethanol at the pulp extraction stage. All attached soft tissues were scraped off with a scalpel. The tooth was wrapped into previously sterilized aluminum foil, fixed in place screw clamp and mechanically broken. With careful manipulation, the pulp was gently isolated with a dental hand instrument called an excavator and minced into smaller pieces.

Cells were isolated from the dental pulp of the extracted teeth, as described in the previous study(Gronthos et al., 2000). Briefly, tissue was removed and immersed in the digestive solution (3 mg/ml type I collagenase and 4 mg/ml dispase) for 1 hr at 37°C, then filtered by 70-μm cell strainers to exclude the non-digested tissue. Following centrifugation at 1500rpm for 10 mins, hDPSCs suspension was obtained. Cells were plated in T25 flasks and cultured in a complete culture medium DMEM/F12 with 10% fetal bovine serum (FBS) and 1% p/s at 37°C and 5% $CO_2$. The medium was refreshed every 2 or 3 days. hDPSCs from young donors (YDPSCs) and hDPSCs from old donors (ODPSCs) between passages 3–5 were used to avoid cell behavioural changes related to prolonged culture, such as senescence in vitro. For each experiment, the same passages of YDPSCs and ODPSCs

were used. hDPSCs were observed under Nikon Eclipse E600 light microscope to evaluate their morphologic appearance.

### *4.2.2 Characterization of hDPSCs*

Surface marker profiles of hDPSCs were evaluated using flow cytometry. $2.5 \times 10^4$ cells of each YDPSCs and ODPSCs were stained with antibodies to CD90, CD105, CD34, CD73, and HLA-DR (Immuno Tools Friesoythe, Germany) and appropriate isotype controls as per the manufacturer's instructions. For isotype controls, the primary antibodies were used instead of FITC- or PE-coupled nonspecific mouse immunoglobulin G. Analysis was performed using FACSCalibur flow cytometry system (BD Italia, Milan, Italy) and the FCS Express 6 Plus software (De Novo Software, Glendale, USA).

### *4.2.3 Alizarin Red Staining, Oil Red O staining and Alcian Blue Stain Assays*

For adipogenic differentiation, $10^4$ cells/cm$^2$ were seeded in two-well Lab-Tek® Chamber Slide and induced using the STEM- PRO® Adipogenesis Kit (Gibco life technologies, Grand Island, NY) for 2 weeks. The medium was changed twice a week. Next, cells were fixed in 4% PFA and exposed to Oil Red O solution (0.5% in 100% isopropyl alcohol) for 30 min at room temperature. Oil Red O dye was eluted with 60%, isopropanol further washed in $dH_2O$, and counterstained with haematoxylin. Immediately after, the cells were observed under a light microscope and counted.

For osteogenesis, cells were seeded at a density of $1.5 \times 10^3$ cells/cm$^2$ in two-well Lab-Tek® II Chamber Slide and grown in STEMPRO® osteogenesis medium for 3 weeks, changing the medium twice a week. Alkaline phosphatase (ALP) staining was performed after 7 days by incubating cells with a solution of 5- bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) alkaline-phosphate substrate (Sigma-Aldrich) in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 10 mM $MgCl_2$ buffer for 1 hr at RT, and rinsed in $dH_2O$. After 3 weeks, mineralization was assessed by Alizarin Red S (ARS) staining (Sigma-Aldrich). Briefly, cells were fixed with 4% PFA in PBS for 10 min, incubated with ARS for 30 min at RT, and washed in $dH_2O$. The reaction was observed under Nikon Eclipse 600 light microscope. ARS was destained with 10% cetylpyridinium chloride in 10 mM sodium phosphate for 60 min at RT to quantify calcium mineral content. The concentration was quantified using a spectrophotometer at 540 nm (Secoman, Anthelie light, version 3.8; Contardi, Cesano Maderno, Italy). For chondrogenic differentiation, aliquots of $10^6$ hDPSCs cells were suspended in 1 ml chondrogenic medium

(STEMPRO® Chondrogenesis Kit. Gibco life technologies, Grand Island, NY). The cells were spun down at 1200 rpm for 5 minutes, and the pellets were cultured for 2 weeks. The medium was changed twice a week. Pellets were then fixed in 4% PFA, paraffin-embedded, and sectioned. Sections were exposed to a solution of Alcian Blue (pH 1; Bio-Optica, Milano, Italy) for 30 min at RT and observed under Nikon Eclipse 600 light microscope.

### *4.2.4 Production of DPSC-conditioned medium (DPSC-CM)*

DPSCs (passages 3-5) were cultured in 25 $cm^2$ culture flasks in culture medium DMEM/F12 with 10% FBS and 1% p/s at 37°C and 5% $CO_2$. As previously described (Ma et al., 2009), the culture medium of YDPSCs and ODPSCs, was first collected at 70% confluence, then subsequently replenished every 24 h until full confluence was reached. The experiment was repeated 20 times for collecting the medium, and the average period for culture was 7 days. The collected supernatant was filtered through a 0.22-μm Millipore filter (Carrigtwohill Co, Cork, Ireland), centrifuged at 2000g for 10 minutes and finally mixed with an equal volume of fresh basal medium (dil. 1:1) and used as the conditioned medium (CM) for DPSC culture. The basal medium was used as a control medium. The experiment was divided into 4 groups: YDPSCs treated with CM of ODPSCs (YDPSCs-OCM), ODPSCs treated with CM of YDPSCs (ODPSCs-YCM), YDPSCs replenished with basal medium (YDPSCs-DMEM/F12) and ODPSCs replenished with basal medium (ODPSCs-DMEM/F12). Each group were observed under a phase contrast microscope (Olympus Optical Co Ltd, Tokyo, Japan) to evaluate their morphologic appearance.

### *4.2.5 Morphology and Intracellular structure in 4D live cell imaging*

The morphology and subcellular structure of hDPSCs were observed in real-time through 4D live-cell imaging with holotomographic microscopy (HTM) using the 3D Cell Explorer-Fluo (Nanolive, Ecublens, Switzerland). 5 $x10^3$ hDPSCs at passage 3 were seeded onto μ-Dishes 35 mm (Ibidi, Gräfelfing, Germany) and the cells were cultured until reaching 70% confluence. The test groups were treated with the corresponding conditioned medium until reaching 50% confluence (generally 3 days of treatment with conditioned medium) and incubated at 37°C in a $CO_2$. Following treatment, the cells were incubated for 24 hrs with a top-stage incubator (Okolab, Pozzuoli, Italy) at a temperature of 37 °C, humidity and $CO_2$ of 5% were maintained throughout image acquisitions. The specifications of the 3D Cell Explorer-Fluo were set as follows: 60× air objective (NA = 0.8), medium Refractive Index 1.345, and images captured every 3 mins for 24 hrs. Live Cell Imaging Software STEVE (Nanolive,

Ecublens, Switzerland), which controls the HTM microscope, was used to obtain RI volumes and convert them into .tiff format.

The 3D RI volumes in .tiff format were then processed in batches within FIJI (https://imagej.net/software/fiji/downloads) for performance purposes. Then 3D RI volumes were transformed into 2D RI maps using maximum intensity projections and saved as .tiff files.

### *4.2.6 Western blotting analysis*

The immunoblot determined expression levels of p21, caspase-3, p53 and iNOS analysis in all 4 groups. Cells ($3\times10^5$) were cultured in a 6-well plate and treated with either conditioned or basal medium as described above. Afterwards, all cells were pelleted and stored at -80°C. For Western Blotting analysis, protein extraction from the cell pellets was performed by adding RIPA lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA). The protein quantification was determined using DC protein assay (Bio-Rad, Hercules, CA, USA). Electroblotting was performed using Wet Blotting System (XCell II Blot Module; Invitrogen, Waltham, MA, USA). Supernatant proteins (10 μg) were separated by using Bolt™ 4-12% SDS–PAGE gel (Invitrogen) and transferred onto a 0.2 μm nitrocellulose membrane. Membranes were incubated overnight with primary antibodies anti-p21 (1:500, Santa Cruz Biotechnology), anti-caspase-3 (1:500; Santa Cruz Biotechnology), anti-p53 (1:500; Santa Cruz Biotechnology), anti-iNOS (1:500; Invitrogen) followed by incubation with a secondary antibody conjugated to Horseradish peroxidase (HRP) (1:15,000; Bethyl Laboratories Inc., Montgomery, AL, USA). The signals were captured using an Alliance Mini system (UVITEC, Cambridge, UK); the p21, caspase-3, p51 and iNOS bands were quantified using FIJI software. All protein expression levels were normalized to the levels of the GAPDH (1:10000; Proteintech Group, Rosemont, IL, USA) protein expression.

### *4.2.7 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)*

Total RNA was extracted from the cells using the RNeasy Mini kit used according to the manufacturer's protocol (Qiagen GmbH). Total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis kit used according to the manufacturer's protocol (Roche Diagnostics GmbH). qRT-PCR reactions were carried out in triplicate. Threshold cycle values were quantified, and the expression of each gene was normalized relative to that of reference genes.

Real-time assays were performed by the Mastercycler RealPlex2 (Eppendorf GmbH) using SsoFastTM EvaGreen Supermix 1x(Bio-Rad) in a final volume of 10 µl. All PCRs contained 1 µl of complementary DNA. Primer sequences were designed by Primer 3 web (Primer3Web version 4.1.0, https:// primer3.ut.ee/), and their specificity was tested by BLAST to avoid appreciable homology to pseudogenes or other unexpected targets. Every primer was used at 10 µM final concentration. In each assay, the mRNA of both reference genes and each gene of interest were measured simultaneously under identical conditions. The specificity of the PCR reactions was confirmed by melt curve analysis. Each PCR assay comprised a single cycle at 95°C for 30 s for enzyme activation and 40 cycles of denaturation at 95°C for 5 s, annealing, and extension at 60°C for 20 s.

Each assay was performed in triplicate, and threshold cycle ($C_t$) values for reference genes were used to normalize cellular mRNA data. In this instance, normalization involved the ratio of mRNA concentrations for specific genes of interest (as mentioned above) to that corresponding to $C_t$ medium values for GAPDH and GUSB (Ragni et al., 2013). The amount of each mRNA was calculated using the comparative $C_t$ method with $\Delta C_t = C_t$ (mRNA) − $C_t$ (GAPDH) or $C_t$(GUSB), and data were expressed as gene relative expression ($2^{-\Delta Ct}$). Moreover, to highlight the effect of ageing or material on cell behaviour, the $\Delta\Delta C_t$ method for the evaluation of fold-change ($2^{-\Delta\Delta Ct}$) was used to compare values obtained in cells of test groups with those of control groups (Livak and Schmittgen 2001). The qPCR efficiency in all our experiments was more than 90%. The difference between the actual and theoretical (100%) efficiencies would result in underestimating the mRNA concentration of all the analysed samples. The oligonucleotide sequences for target genes are reported in Table 6.

### *4.2.8 Intracellular Reactive Oxygen Species (ROS) Generation*

The generation of intracellular ROS in YDPSCs and ODPSCs after exposure to conditioned medium was measured using DCFDA / H2DCFDA - Cellular ROS Assay Kit (Abcam, Cambridge, UK). Briefly, $1 \times 10^4$ hDPSCs cells were seeded into each well in a black bottom culture plate (96 well) and incubated at 37°C in a $CO_2$. As described in section 2.2, the cells were cultured until reaching 70% confluence. Then the test groups were treated with the corresponding conditioned medium until reading full confluence (generally 3 days of treatment with conditioned medium). The culture plate was then washed with PBS. 10-µM DCFH-DA was added to each well and incubated at 37°C. After 40 min of incubation, the plate was washed, and the fluorescence intensity of dichlorofluorescein (DCF) was measured at 485-nm excitation and 520-nm emissions using the microplate reader Infinite 200 PRO (Tecan, Männedorf, Switzerland). Data were represented as the percent of fluorescence intensity relative to the

young control (YDPSCs-DMEM/F12). The experiment was performed at least three times and used triplicate for each assay.

### *4.2.9 Immunofluorescence*

hDPSCs were seeded at the density of $2.5 \times 10^4$ cells per well on two-well Lab-Tek® Chamber Slides The TUNEL immunofluorescence assay was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. To detect cell proliferation, Ki67 antibody (Abcam, Cambridge, MA) was used on hDPSCs and countered with goat anti-mouse FITC-conjugated secondary antibody (Bethyl Laboratories Inc.; dilution, 1:100) to locate the primary Ab. The TUNEL and Ki67 data were quantified by using FIJI software on images captured at 40X magnification, which counts the nuclei (DAPI staining) and the TUNEL and Ki67 positive cells and calculates the percentage of proliferating cells to the total number of cells.

In all studies, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and samples were mounted using Vectashield® mounting medium (Vector Laboratories Inc, Burlingame, CA). For negative controls, the primary antibody was replaced with nonimmune serum. Cells were observed with a Nikon E600 fluorescence microscope (Milan, Italy).

### *4.2.10 Mitochondrial superoxide measurement*

Mitochondrial superoxide production was evaluated by the mitosox red compound (Invitrogen). A 5 mM stock solution of Mitosox in DMSO was prepared. $3 \times 10^4$ hDPSCs/well of passage 3 were seeded in a 96-well plate and incubated at 37°C in a $CO_2$. As described in section 2.2, the cells were cultured until reaching 70% confluence; the test groups were then treated with the corresponding conditioned medium until reaching full confluence (generally 3 days of treatment with conditioned medium). 5 mM stock solution was diluted in sterile Hanks' Balanced Salt Solution (HBSS) at a final concentration of 5 μM. All cells were washed with PBS and incubated with 5 μM Mitosox red for 10 min at 37 °C. After incubation, the plate was washed, and the fluorescence was measured at 510 nm excitation and 580 nm emissions using the microplate reader Infinite 200 PRO (Tecan, Männedorf, Switzerland). The experiment was performed at least three times and used triplicate for each assay.

### *4.2.11 Statistical analysis*

Statistical analysis was performed by Prism 6 Software (GraphPad Software, La Jolla, CA). Mean, and SD of all experiments was reported. Data were analysed by analysis of variance and Bonferroni's T-test. Statistical significance was tested at $p < 0.05$.

## 4.3 Results

### *4.3.1 Characterization of hDPSCs*

hDPSCs at passage 3 were characterized by surface marker profiling. Both young hDPSCs and old hDPSCs displayed positive expression of MSC markers CD90, CD105, and CD73 and negative expression of the common hematopoietic markers CD14, CD34, CD19 and CD45 and HLA-DR (Fig.11).

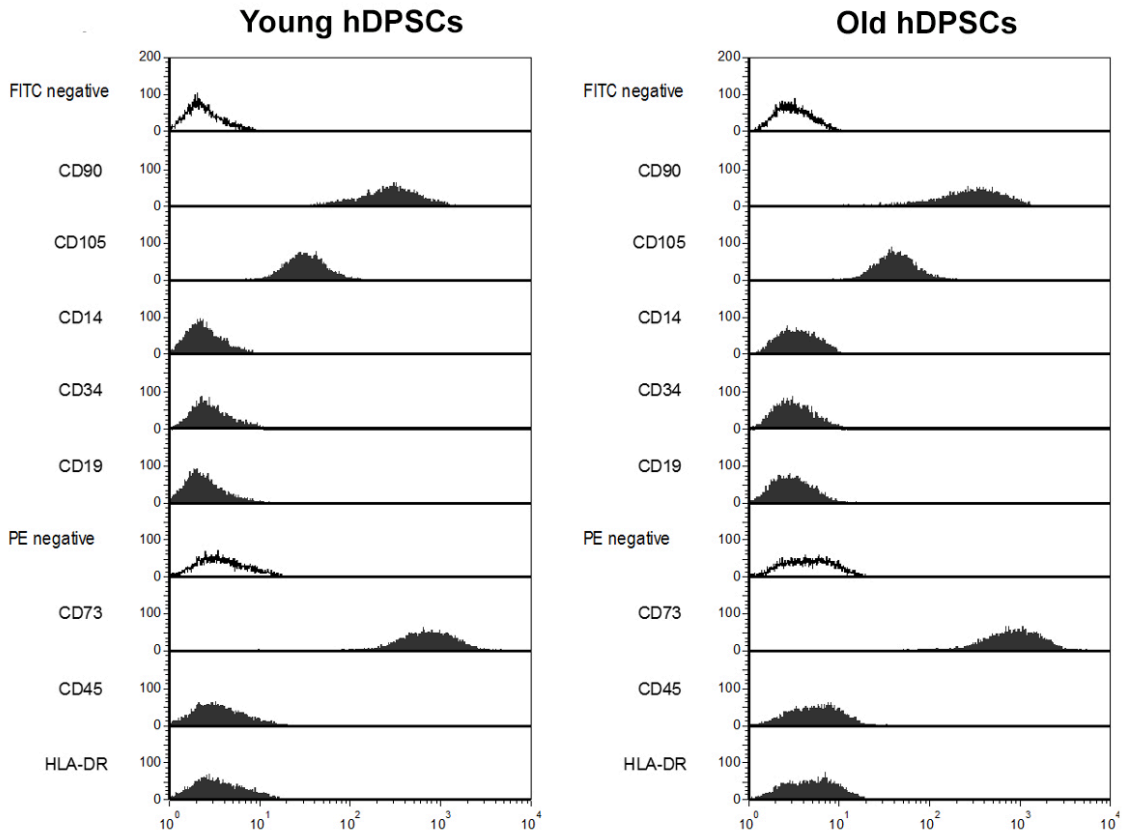


**Figure 11.** Surface Marker Profiling determined by flow cytometry. (White plots indicate FITC and PE negative controls).

### *4.3.2 Effect of donor age on differentiation ability of hDPSCs*

After culturing DPSCs in an osteogenic-inducing medium for 3 weeks, alizarin red staining and calcium quantitation revealed that mineralization was more robust in the young hDPSCs than in the old hDPSCs (Fig.12). We also compared the adipogenic differentiation potentials of DPSCs from different donors. After induction with adipogenic medium for 2 weeks, the oil red staining and its quantitative analysis revealed that young had more lipid deposits than old hDPSCs (Fig.12). Finally, after induction with chondrogenic medium for 2 weeks, the quantitation analysis of Alcian blue staining revealed similar amounts of proteoglycan production in both young hDPSCs and old hDPSCs (Fig.12).

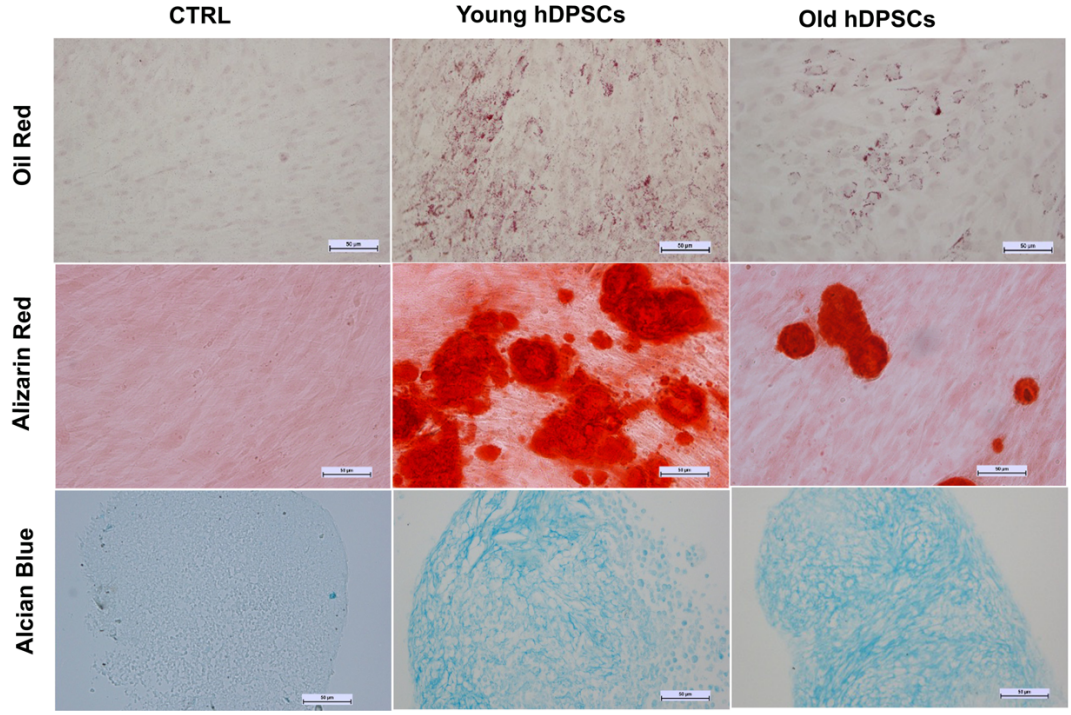


**Figure 12.** Following exposure to differentiation medium young and old hDPSCs showed adipogenic (right panels) differentiation as detected by oil red O staining (scale bars = 10 µm); osteoblastic differentiation of hDPSCs as detected by ARS staining (scale bars = 50 µm); chondrogenic differentiation as detected by Alcian blue staining (scale bars = 50 µm).

### *4.3.3 Effect of conditioned medium on the morphology of hDPSCs*

Both young hDPSCs and old hDPSCs showed a spindle fibroblast-like shape typical of mesenchymal stem cells. Old hDPSCs appeared slightly enlarged compared to young hDPSCs under the light microscope (Fig.13). Cells incubated with CM did not differ in morphology compared to cells cultivated in basal medium DMEM/ F12, i.e., treatment with CM does not alter the morphology of the stem cells.

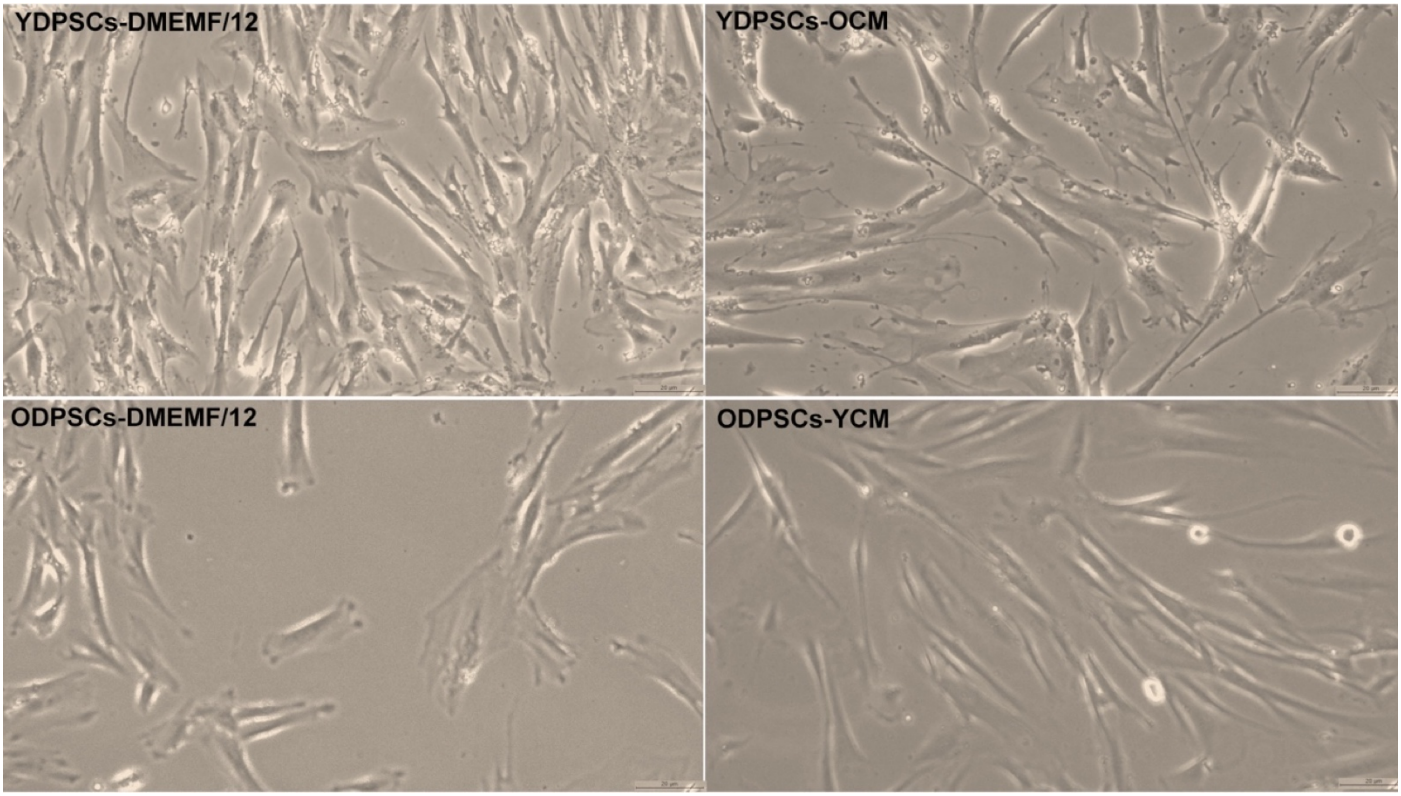


**Figure 13.** Phase contrast representative images displaying the morphology of a dental pulp stem cells' colony passage 3 of each group (scale bar 20μm).

The morphology and intracellular structures of each group were observed by HTM microscopy. Here 4 videos are presented, which were used to observe, in 4D live cell imaging, the effect of conditioned medium on the morphological changes (Fig.14). HTM microscopy revealed hDPSCs adhered to the substrate with many thin plasmalemma processes, i.e., filopodia. Each cell contained spherical or irregularly shaped large pale nuclei, which were frequently eccentrically located and with a large amount of euchromatin. Each nucleus had an evident nucleolus or sometimes two or more nucleoli. Numerous coated matrix vesicles (or scattered lysosome-like organelles) and granules of glycogen and lipid droplets were observed. Nearby the nucleus, small, elongated mitochondria were positioned. The hDPSCs in each of the 4 groups created and secreted vesicles approximately 0.5 μm in diameter. There were no major differences in morphology and intracellular structure between the 4 groups. The old hDPSCs did not appear more enlarged compared to the young hDPSCs. Cultivating old DPSCs in young CM did not alter its morphology and vice versa. One observation was that the young ctrl group (YDPSCs-DMEM/F12) had a higher lipid droplet presence than the other 3 groups. DPSCs from young donors also displayed greater mitotic activity compared to the old donors, regardless of whether they

were treated with CM. 2 mitotic divisions can be seen during the 24 hrs period in either YDPSCs-DMEM/F12 and YDPSCs-OCM group, whereas 1 mitotic division can be seen in the same period in both ODPSCs-DMEM/F12 and ODPSCs-YCM group.

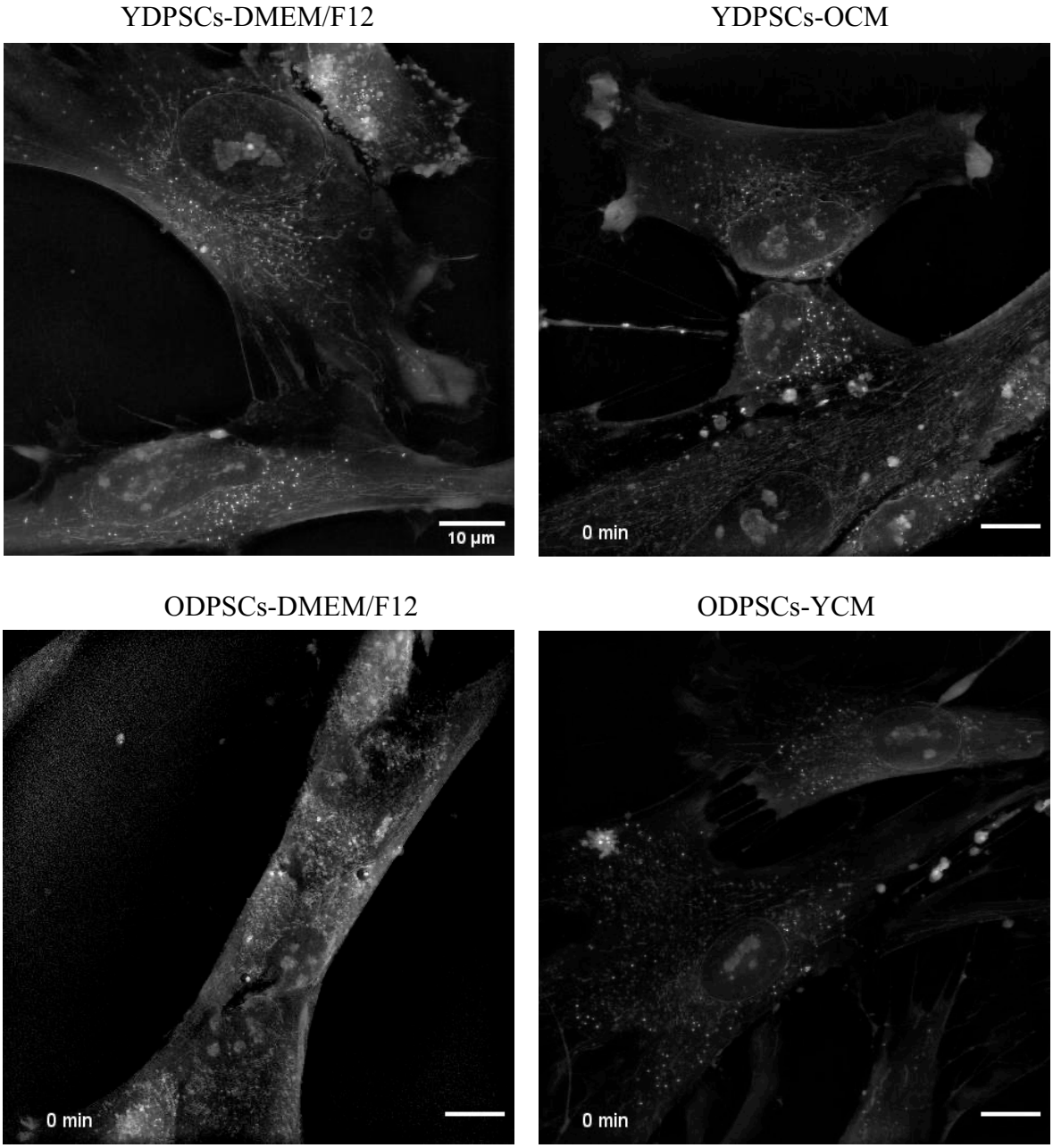


**Figure 14.** Captures extrapolated from long-term (24hrs) live imaging of human dental MSCs (scale bars = 10 µm).

### *4.3.4 Effect of conditioned medium on the proliferative capacity of aged hDPSCs*

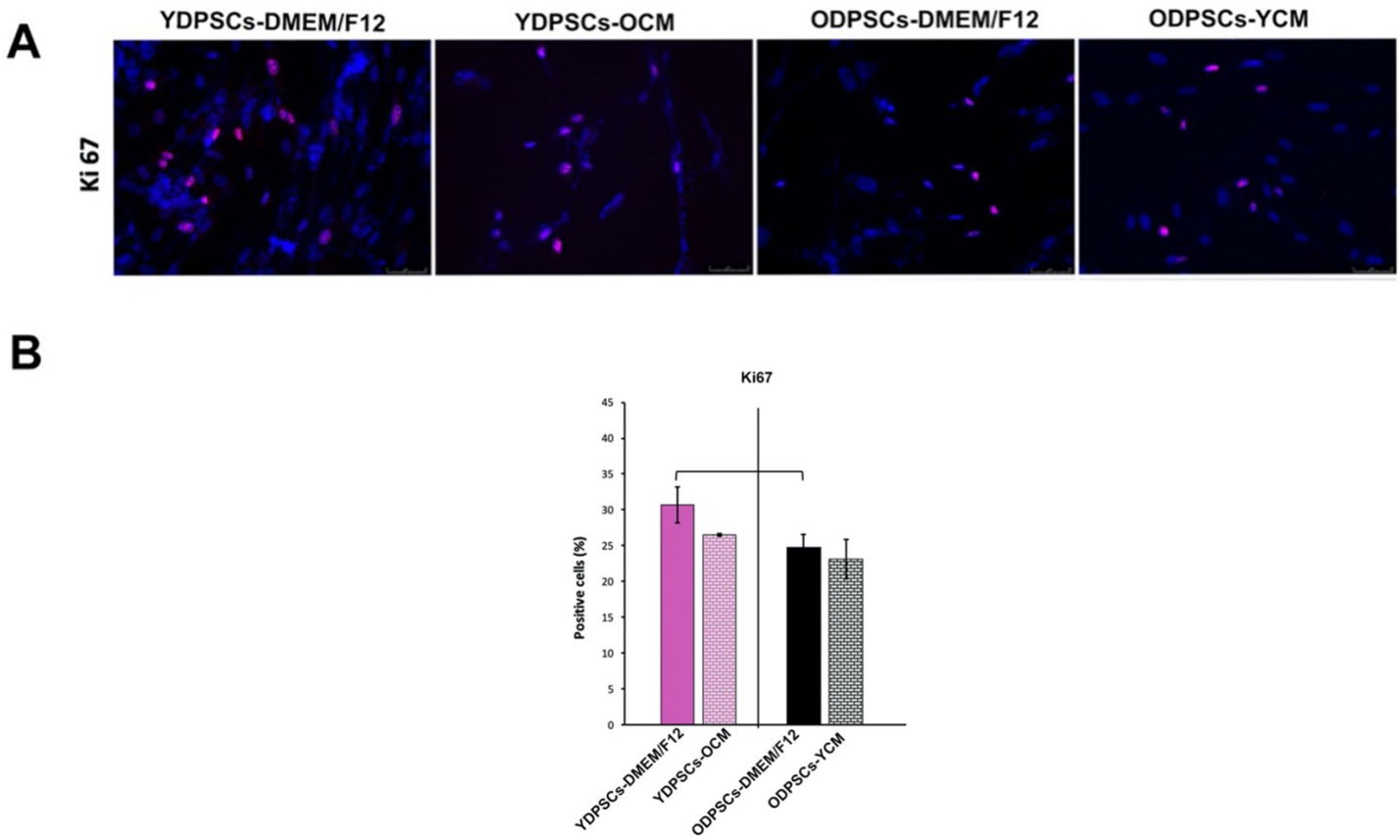


**Figure 15.** (A)Immunofluorescence staining of Ki67 to test the effect of conditioned medium on differently aged hDPSCs (scale bars = 25 μm). (B) Quantification of Ki67-positive cell percentage (%). *p < 0.05 versus group ctrl.

Aged hDPSCs exhibited a reduced proliferation ability compared to young hDPSCs (Fig.15). Cultivating young cells in old CM caused a significant decrease in Ki67-positive cells. Conversely, the addition of young conditioned medium slightly decreased the proliferative ability of old hDPSCs; however, this finding was not significant.

*4.3.5 Effect of conditioned medium on apoptosis of aged hDPSCs*

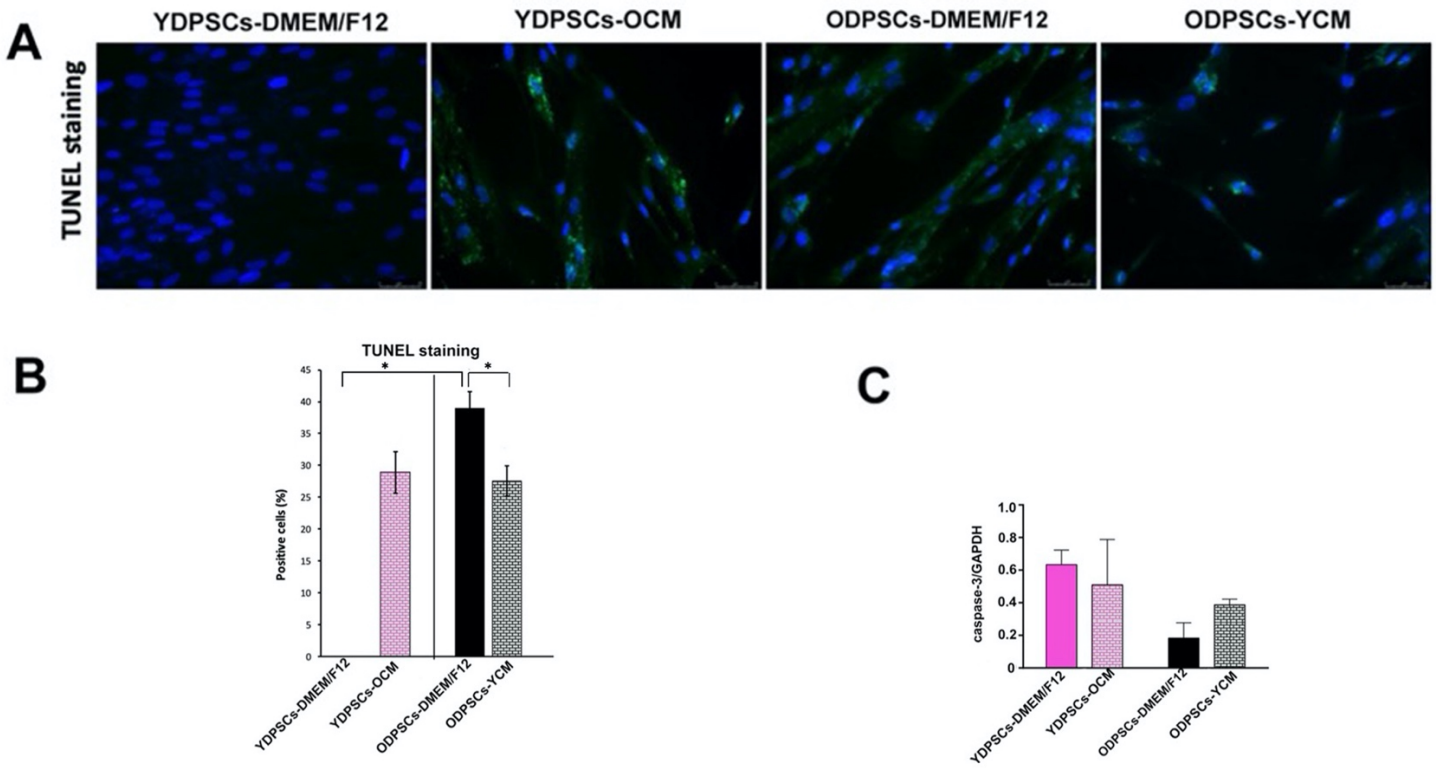


**Figure 16.** (A) TUNEL staining normal and conditioned medium-treated hDPSCs (scale bars = 25μm) (B) Quantification of TUNEL-positive cell percentage (%) (C) Caspase-3 expression assessed by Western blotting analysis. *p < 0.05 versus group ctrl.

Young hDPSCs exhibited reduced apoptosis compared to young hDPSCs (Fig.16 A, B). Cultivating YDPSCs in OCM significantly increased the number of cells positive for TUNEL staining. Conversely, cultivating ODPSCs in YCM significantly decreased the number of cells positive for TUNEL staining. The TUNEL staining results suggested that conditioned medium from YDPSCs could inhibit apoptosis in ODPSCs. An abnormality in the TUNEL staining results was that 0% of cells were positive for TUNEL staining, albeit a low percentage was expected. (Fig.16 A, B).

To further confirm apoptosis results, Western Blotting analysis for caspase-3 was performed (Fig.16 C); however, the effects of conditioned medium on apoptosis via TUNEL staining were inconsistent with that of the caspase-3 Western Blotting analysis. The expression of proapoptotic marker caspase-3 was higher in the young control group (YDPSCs-DMEM/F12) compared to the young treatment group (YDPSCs-OCM). The old control group displayed the lowest expression of caspase-3. Additionally,

the expression of caspase-3 in old hDPSCs increased with the addition of conditioned medium from young hDPSCs. None of these results was statistically significant.

### *4.3.6 Effect of conditioned medium on senescence of hDPSCs*

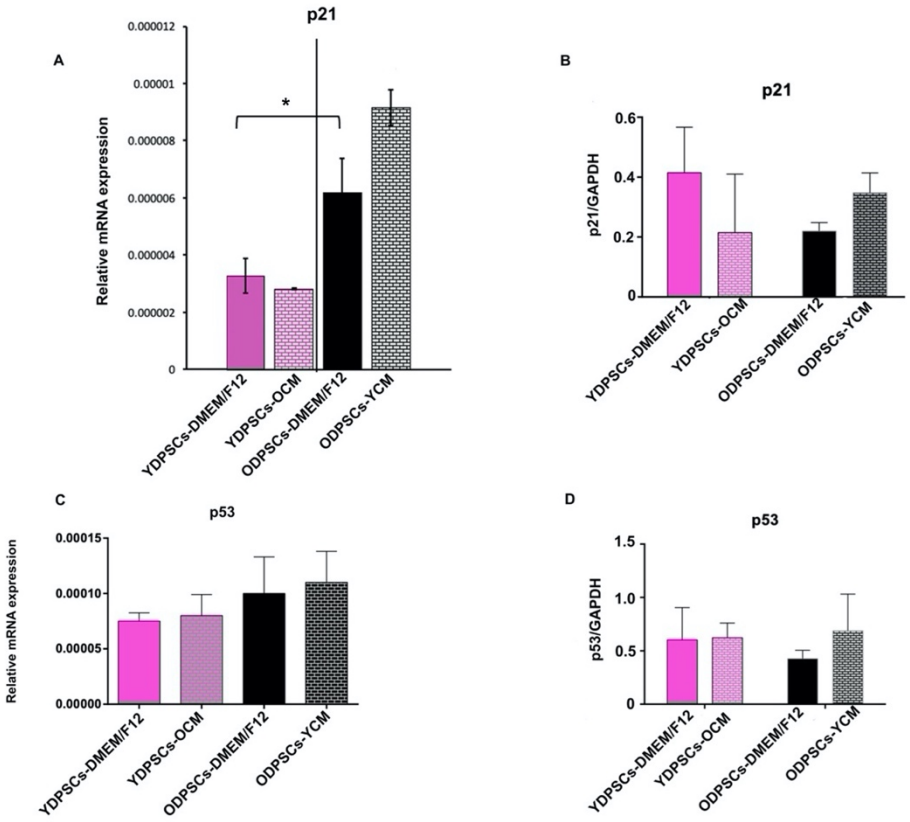


**Figure 17.** (A) Relative gene expression of p21 (B) Western blot quantification of p21 (C) Relative gene expression of p53 (D) Western blot quantification of p53. *p < 0.05 versus group ctrl.

The qRT-PCR analysis showed that p21 mRNA expression was upregulated with ageing (Fig. 17 A). Similarly, p21 mRNA expression was upregulated in ODPSCs-YCM compared to its control group ODPSCs-DMEM/F12(Fig. 17 A). No changes were found in p53 mRNA expression levels, for which the results were not significant (Fig. 17 C). mRNA relative expression in hDPSCs for all 4 groups is reported in Table 7.

On the contrary, Western Blotting analysis revealed that the expression of $p21^{cip1}$ and p53 was higher in young hDPSCs than in old hDPSCs (Fig. 17 B, D). Treatment with young CM increased the

expression of $p21^{cip1}$ and p53 in old hDPSCs. The results from both Western Blotting analyses were not statistically significant.

| Genes | Primer Forward (5'->3') | Primer Reverse (5'->3') |
|---|---|---|
| **p21** | AGGTGGACCTGGAGACTCTCAG | TCCTCTTGGAGAAGATCAGCCG |
| **p53** | CCTCAGCATCTTATCCGAGTGG | TGGATGGTGGTACAGTCAGAGC |
| **Gapdh*** | AAACGATTGCAGGGTTTCAC | CTCTCGTCGGTGACTGTTCA |

p21= Cyclin Dependent Kinase Inhibitor 1A; p53= tumor protein p53; Gapdh= glyceraldehyde-3-phosphatedehydrogenase. * Reference gene

**Table 6.** Analysed gene description.

| | YDPSCs-DMEM/F12 | | YDPSCs-OCM | | ODPSCs-DMEM/F12 | | ODPSCs-YCM | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **p21** | 3.4E-06 | 6.2E-07 | 2.9E-06 | 3.4E-08 | 6.3E-06 | 1.2E-06 | 9.4E-06 | 6.4E-07 |
| **p53** | 7.5E-05 | 7.6E-06 | 8.0E-05 | 1.9E-05 | 1.0E-04 | 3.3E-05 | 1.1E-04 | 2.8E-05 |

**Table 7.** mRNA relative expression in hDPSCs for all 4 groups

### *4.3.7 Effect of conditioned medium on oxidative stress of aged hDPSCs*

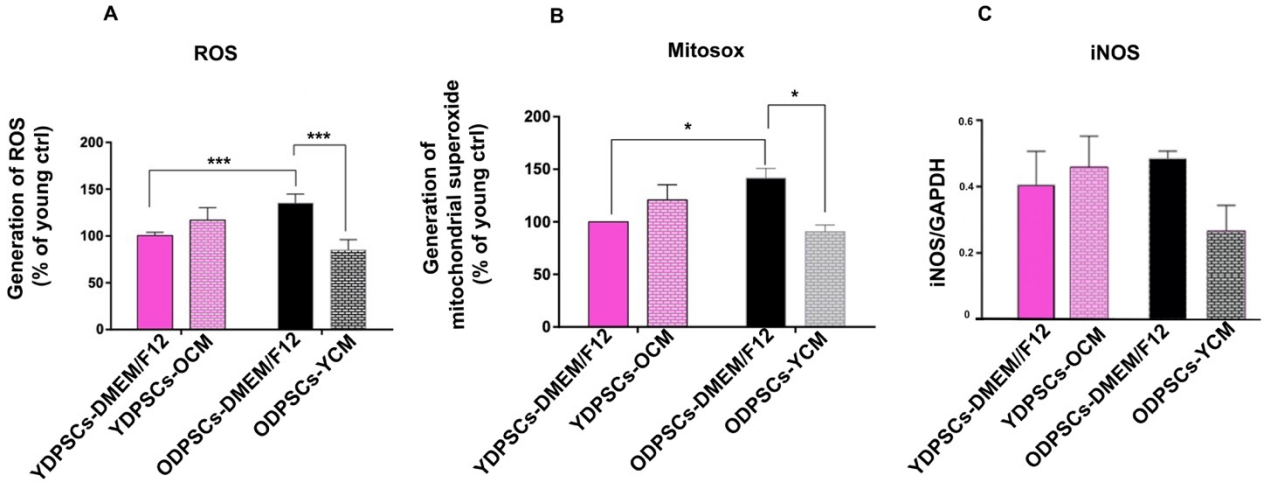


**Figure 18.** DPSCs-CM inhibits oxidative in aged DPSCs. (A) Intracellular ROS levels were evaluated by DCFH-DA (B) mitochondrial ROS levels by MitoSOX (C) inducible Nitric Oxide Synthase (iNOS) levels measured by Western blotting. Values indicate the mean ± SEM; $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

The intracellular and mitochondrial ROS levels were evaluated by DCFH-DA and MitoSOX, respectively (Fig. 18 A, B). The hDPSCs of young donors generated a significantly lower percentage of ROS and mitochondrial superoxide compared to old donors. ROS and mitochondrial superoxide production was significantly suppressed by the treatment of old hDPSCs with young CM, strongly suggesting that the CM's factors can suppress oxidative stresses accumulated in aged hDPSCs.
We further performed a Western Blotting analysis of iNOS (Fig. 18 C); the results were consistent with the results of DCFH-DA and MitoSOX but were also not statistically significant. hDPSCs of old donors expressed higher levels of iNOS than young donors, and the treatment with young CM decreased the expression of iNOS in old hDPSCs.

## 4.4 Discussion and Conclusion

To improve the suboptimal performance of aged adult dental stem cells, we attempted to cultivate aged adult human dental pulp stem cells in the conditioned medium of young human dental pulp stem cells and, in doing so, rejuvenate the aged adult stem cells using the factors and cytokines released into the extrinsic microenvironment of the old human dental pulp stem cells.
A very recent article performed a comparative analysis of cytokines and growth factors found in the conditioned media of stem cells from the pulp of deciduous, young, and old permanent tooth found the conditioned medium of SHEDs and young DPSCs exhibit more growth factors and lesser pro-inflammatory cytokines than old DPSCs (Bhandi et al., 2021). The influence of microenvironment factors on different ages of DPSCs was previously investigated, whereby adult rat DPSCs were exposed to the conditioned medium of juvenile DPSCs, and juvenile DPSCs were exposed to adult DPSC-CM (Ma et al., 2009). In another study, parabiotic pairings between young and old mice were performed, which enabled the skeletal muscle stem cells from old mice to be exposed to the serum of young mice (Conboy et al., 2005). Taken together, these previous experiments suggest that there is the possibility that aged MSCs can revert to a younger phenotype using the conditioned medium of the young MSCs; however, no experiment has investigated aged adult human dental pulp cells rejuvenation potential after exposure to the factors present in the conditioned medium of young adult human dental pulp cells.
The International Society for Cellular Therapy ( ISCT) position statement set out minimum criteria for defining multipotent mesenchymal stromal cells in 2006: 1) Adherence to plastic in standard culture conditions 2) Specific Surface Antigen expression Positive (≥ 95%) CD105, CD 73, CD 90 and negative ( ≤ 2%) CD45 CD34 CD14 or CD11b, CD79α or CD19 and HLA-DR 3 In vitro differentiation: osteoblasts, adipocytes, chondroblasts (demonstrated by staining of in vitro cell culture)(Dominici et

al., 2006). In 2013 and 2019, respectively, the ISCT updated the MSC definition to include (1) a bioassay of immunosuppressive properties, (2) the tissue origin of cells, (3) use of stromal cell nomenclature unless rigorous evidence for stemness is shown, and (4) including functional assays to define the therapeutic mechanism of action (Viswanathan et al., 2019). By meeting the three minimum criteria of the first position statement, we confirmed the successful isolation of a population of young hDPSCs and old hDPSCs.

We found no differences in surface marker expression between old and young hDPSCs, both equally displaying a positive expression of MSC markers and negative expression of hematopoietic markers, which was in agreement with other studies which found no significant difference in surface marker expression when comparing young and aged MSCs (Hung et al., 2022).

Under the light microscope, aged hDPSCs appear slightly enlarged compared to young donors. However, more detailed 4D imaging using HTM microscopy revealed no marked differences in morphology and intracellular structure. There appeared to be more lipid droplets in the young control group ( YDPSCs-DMEMF/12) which was an anomaly in our findings as ageing is associated with dysregulated lipid metabolism (Mutlu et al., 2021), and in general, senescent cells tend to have a higher accumulation of lipid droplets in compared to proliferating cells (Chee et al., 2021).

There was a higher percentage of Ki67-positive cells in young hDPSCs than in aged hDPSCs implying that hDPSCs isolated from the young donors showed a higher proliferation rate compared with those from aged donors. This was consistent with age-related changes in other MSCs (Kretlow et al., 2008)(Zaim et al., 2012)(Choudhery et al., 2014).

Our study also found that hDPSCs isolated from old donors displayed reduced osteogenic and adipogenic differentiation potentials but similar chondrogenic potential compared to young donors. There seem to be variations in the results on the effect of age on the differentiation ability of MSCs. Differentiation of aged adipose-derived stromal stem cells becomes skewed, resulting in a tendency towards enhanced adipogenesis and decreased osteogenesis (Kornicka et al., 2015). Investigation of the effect of donor age on the differentiation capacities of periodontal ligament stem cells revealed a decline in osteogenic and adipogenic differentiation with increasing age (J. Zhang et al., 2012). Another study investigating dental pulp stem cells similarly found that increased donor age impaired osteogenic and adipogenic differentiation potential but had no impact on chondrogenic differentiation potential (Yi et al., 2017).

In investigating the effect of the conditioned medium of young hDPSCs on aged hDPSCs, we investigated its effect on morphology, proliferation, apoptosis, cellular senescence and oxidative stress.

Under the light microscope and more detailed 4D imaging using HTM microscopy revealed no marked differences in morphology and intracellular structure between the groups treated with CM compared with the groups where cells were cultivated only in basal medium DMEM/F12.

There was a higher percentage of Ki67-positive cells in young hDPSCs than in the aged, which was coherent with the findings of HTM microscopy, whereby young hDPSCs showed a higher mitotic rate than old hDPSCs in a defined period. Cultivating young cells in old CM caused a significant decrease in the number of Ki67-positive cells. Conversely, the addition of young CM slightly decreased the proliferative ability of old hDPSCs, but the difference was not significant. With respect to the proliferative ability, the old CM seemed to contribute to the young cells acquiring a senescence phenotype more than the young CM being able to rejuvenate the old DPSCs. YCM did not promote cell proliferation compared to numerous studies which investigated the effects of CM on proliferation (Pan et al., 2019)(S. Li et al., 2017)(Noverina et al., 2019). CM also displayed this proliferative effect in studies investigating aged mesenchymal cells (Widowati et al., 2022).

We evaluated the influence of the CM on apoptosis in differently aged donor hDPSCs. Apoptosis can occur through two major cell pathways: a receptor-mediated (extrinsic pathway) and a mitochondrial (intrinsic) pathway. Caspase-3 is a downstream effector caspase in the intrinsic pathway. Overwhelming evidence has demonstrated that CM from human MSCs inhibits apoptosis in various in vivo and in vitro disease and injury models by suppressing the expression of caspase-3 via inhibiting the mitochondria-dependent caspase-3 pathway (Ohta et al., 1997)(Timmers et al., 2008). It has already been shown that human gingival mesenchymal stem cells-CM significantly decreased the expression of pro-apoptotic protein caspase-3, inhibiting apoptosis in injured motor-neuron-like NSC-34 cells (Rajan et al., 2017). Furthermore, Rajan et al. showed that the CM of human periodontal ligament stem cells from multiple sclerosis patients inhibited apoptosis by significantly preventing caspase-3 expression (Rajan et al., 2016). However, in our study, the addition of the CM of the young donors increased the expression of caspase-3 in the aged donors, thereby increasing apoptosis.

On the other hand, aged hDPSCs displayed a higher percentage of TUNEL-positive apoptotic cells than young hDPSCs. The addition of young CM to aged hDPSCs indicated a significant decrease in the number of apoptotic cells. In summary, the young conditioned medium increased caspase-3 expression but conversely decreased the number of TUNEL-positive cells. The data on the effect of CM from young hDPSCs on aged hDPSCs on apoptosis seemed contradictory.

One of the most common hallmarks of apoptosis is DNA fragmentation analyzed by TUNEL assay. Our findings indicated that the addition of young CM to aged hDPSCs showed a significant decrease

in the number of apoptotic bodies via TUNEL staining. However, the differences in caspase-3 activation between the treatment and control groups were not significant. Despite caspases being crucial mediators of apoptosis, there have now been several published reports which provide evidence that caspases may not control the cellular commitment to programmed cell death in all cells(Okuno et al., 1998)(Ohta et al., 1997). A recent study found that conditioned medium from MSCs improves graft contractility after transplantation in brain-dead donor hearts (Korkmaz-Icöz et al., 2020). This study similarly found that CM from MSCs was able to decrease the DNA strands present in the donor's heart, but there were no differences in caspase-3 expression among the experimental groups, postulating that CM worked through a caspase-independent pathway. Our results demonstrated that young CM decreased caspase-independent nuclear DNA fragmentation in aged hDPSCs.

One of the most widely studied pathways involved in regulating cellular senescence is the p53/$p21^{cip1}$ pathway. Several internal or external stress factors initiate the DNA-damage response (DDR) pathway, triggering the pathway. These stressors trigger DNA damage and transactivate p53 and $p21^{cip1}$. Moreover, $p21^{cip1}$ protein levels may lead to the inhibition of cyclin D kinase Cdk4/6 activity, contributing to the G1 arrest or senescence (Hobson et al., 2019). In our study, we also aimed to investigate the effect of the CM on senescence in aged hDPSCs.

The qRT-PCR analysis showed that p21 mRNA expression was upregulated with ageing. However, p21 mRNA expression was upregulated in ODPSCs-YCM compared to its control group ODPSCs-DMEM/F12. No significant difference was found in p53 mRNA expression levels. Western Blotting analysis revealed that the expression of both p21 and p53 was higher in young hDPSCs than in old hDPSCs. Interestingly, similar to qRT-PCR analysis, treatment with young CM increased the expression of p21 and p53 in old hDPSCs, but no significant difference in expression between either control or treatment groups was found. Our findings were inconsistent with other studies whereby treatment of cells with MSC-CM significantly reduced the expression of p53 and p21. (S. Li et al., 2017). In summary, our findings revealed the factors secreted by the young CM could not reverse the old hDPSCs aged phenotype in relation to its expression of markers p53 and p21.

Reactive oxygen species (ROS) play an important role in ageing and age-related diseases. CM-H2DCFDA has been used extensively to measure cellular $H_2O_2$ and other ROS, but it does not measure superoxide directly [(Rajan et al., 2016)]. MitoSOX red is a selective indicator of mitochondrial superoxide. MitoSOX red is selectively targeted to mitochondria and can compete efficiently with superoxide dismutase (SOD) for superoxide (Liu & Schubert, 2009). Treatment of aged hDPSCS with YCM protected aged hDPSCS from oxidative stress, as evidenced by significantly reduced ROS and

superoxide generation. This result can be corroborated by numerous previous studies which looked at the antiaging effect of CM on ROS generation(B. Sun et al., 2023)(Sohn et al., 2018)(Khanh et al., 2020)(S. Li et al., 2017). Notably, using the CM derived from young hDPSCs can be useful for the improvement of elderly stem cell functions by inhibiting ROS elevation.

We attempted to corroborate our results with a Western Blotting analysis of the oxidative stress marker, inducible Nitric Oxide Synthase (iNOS). The results were consistent with the results of H2DCFDA and Mitosox. hDPSCs of old donors expressed higher levels of iNOS than young donors, and the treatment with YCM decreased the expression of iNOS in old hDPSCs. However, there was no significant difference in expression between the control and treatment groups. Several studies investigating the effect of CM from MSCs on inflammation and disease are in agreement with our findings (Chen et al., 2018)(Cheng et al., 2017) (Rajan et al., 2017).

In conclusion, our data demonstrate that the CM of young hDPSCs may not rejuvenate aged hDPSCs fully. The trophic factors secreted by the young CM exerted their rejuvenating effect via its anti-oxidative potential in this study. YCM protected aged hDPSCs from oxidative stress, as evidenced by significantly reduced ROS and superoxide generation. Our results demonstrated that young CM decreased nuclear DNA fragmentation in aged hDPSCs via a caspase-independent pathway. The antiproliferative and antisenescence effect of the conditioned medium from young hDPSCs was not fully demonstrated in aged hDPSCs and will require future investigation.

# 5. CONCLUSIONS AND FUTURE PERSPECTIVES

Losing one or more natural teeth have been acknowledged as a serious life event. Medicine is grounded in biology-based treatment options; however, dentistry remained stagnated, offering treatment options that are inert synthetic and, above all, not based on biology. Regenerative dentistry is a branch of research investigating the potential of replacing a missing tooth with a biologically created whole new tooth(Sharpe, 2020). Clues from polyphyodont research may help us understand why humans can no longer have continuous tooth replacement capacity. Dental TE has progressed over the decades, but despite the advancements made, researchers still need to identify how to restore embryonic-like properties to more accessible adult stem cells(Sharpe, 2020). Targeted molecular therapy is promising; however, further studies investigating its safety for use in humans will need to be thoroughly investigated. Most current strategies for whole tooth regeneration are based on stem cell therapy using adult MSCs. It was previously believed that the regenerative effect of MSCs was due to the stem cell's ability to implant itself into diseased or damaged tissue. Research has changed gears, and the current belief is that the secretome of MSCs is mainly responsible for their regenerative potential.

Given that the market for whole tooth regeneration will be majorly focused on aged adults who, throughout their lifetime, have lost teeth for various reasons as opposed to children and young adults who are less prone to having missing teeth, our study focused on the rejuvenation potential of the aged adult human dental pulp stem cells. Our data demonstrated that the CM of young hDPSCs might not be able to rejuvenate aged hDPSCs fully. In this study, the trophic factors secreted by the young CM exerted their rejuvenating effect via its anti-oxidative and anti-apoptotic effect; however, it did not demonstrate an antiproliferative or antisenescence effect of the conditioned medium from young donors was not fully demonstrated. Future studies are needed to investigate the concentration of the trophic factors present in the conditioned medium of aged hDPSCs and determine if pre-conditioning of the conditioned medium would increase the rejuvenation potential of young hDPSCs.

# References


Abukawa, H., Zhang, W., Young, C. S., Asrican, R., Vacanti, J. P., Kaban, L. B., Troulis, M. J., & Yelick, P. C. (2009). Reconstructing Mandibular Defects Using Autologous Tissue-Engineered Tooth and Bone Constructs. *Journal of Oral and Maxillofacial Surgery*, *67*(2), 335–347. https://doi.org/10.1016/j.joms.2008.09.002

Al Madhoun, A., Sindhu, S., Haddad, D., Atari, M., Ahmad, R., & Al-Mulla, F. (2021). Dental Pulp Stem Cells Derived From Adult Human Third Molar Tooth: A Brief Review. *Frontiers in Cell and Developmental Biology*, *9*. https://www.frontiersin.org/articles/10.3389/fcell.2021.717624

Aljafar, A., Alibrahim, H., Alahmed, A., AbuAli, A., Nazir, M., Alakel, A., & Almas, K. (2021). Reasons for Permanent Teeth Extractions and Related Factors among Adult Patients in the Eastern Province of Saudi Arabia. *The Scientific World Journal*, *2021*, e5534455. https://doi.org/10.1155/2021/5534455

Al-Rafee, M. A. (2020). The epidemiology of edentulism and the associated factors: A literature Review. *Journal of Family Medicine and Primary Care*, *9*(4), 1841–1843. https://doi.org/10.4103/jfmpc.jfmpc_1181_19

Al-Talabani, N. G., & Smith, C. J. (1978). Continued development of 5-day old tooth-germs transplanted to syngeneic hamster (Mesocricetus auratus) cheek pouch. *Archives of Oral Biology*, *23*(12), 1069–1076. https://doi.org/10.1016/0003-9969(78)90110-3

Al-Talabani, N. G., & Smith, C. J. (1979). A histological study of the effect of developmental stage of tooth-germ on transplantation to hamster cheek pouch. *Archives of Oral Biology*, *24*(12), 933–937. https://doi.org/10.1016/0003-9969(79)90220-6

Angelova Volponi, A., Kawasaki, M., & Sharpe, P. T. (2013). Adult Human Gingival Epithelial Cells as a Source for Whole-tooth Bioengineering. *Journal of Dental Research*, *92*(4), 329–334. https://doi.org/10.1177/0022034513481041

Anthonappa, R. P., King, N. M., & Rabie, A. B. M. (2013). Aetiology of supernumerary teeth: A literature review. *European Archives of Paediatric Dentistry*, *14*(5), 279–288. https://doi.org/10.1007/s40368-013-0082-z

Bergendal, B. (1989). The relative importance of tooth loss and denture wearing in Swedish adults. *Community Dental Health*, *6*(2), 103–111.

Bergman, B. (1987). Periodontal reactions related to removable partial dentures: A literature review. *The Journal of Prosthetic Dentistry*, *58*(4), 454–458. https://doi.org/10.1016/0022-3913(87)90275-7

Bhandi, S., Al Khatani, A., Abdulaziz Sumayli, H., Yahya Sabyei, M., Mohammed Al Zailai, A., Ali Sumayli, M., Ibrahim Hakami, H., Abdurabu Jafer, M., Vyas, N., Ali Baeshen, H., Testarelli, L., & Patil, S. (2021). Comparative analysis of cytokines and growth factors in the conditioned media of stem cells from the pulp of deciduous, young, and old permanent tooth. *Saudi Journal of Biological Sciences*, *28*(6), 3559–3565. https://doi.org/10.1016/j.sjbs.2021.03.031

Bikash, D., Bishnu, S. P., & Rajani, S. (2019). To Find Out the Causes of Extraction of Teeth in the Patients Coming to the Department of Oral and Maxillofacial Surgery at Kantipur Dental College Teaching Hospital and Research Center from January 2014 to December 2018. *American Journal of Biomedical Science & Research*, *2*(2), 93–97.

Buchtová, M., Štembírek, J., Glocová, K., Matalová, E., & Tucker, A. S. (2012). Early Regression of the Dental Lamina Underlies the Development of Diphyodont Dentitions. *Journal of Dental Research*, *91*(5), 491–498. https://doi.org/10.1177/0022034512442896

Cai, J., Cho, S.-W., Ishiyama, M., Mikami, M., Hosoya, A., Kozawa, Y., Ohshima, H., & Jung, H.-S. (2009). Chick tooth induction revisited. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, *312B*(5), 465–472. https://doi.org/10.1002/jez.b.21265

Cai, J., Zhang, Y., Liu, P., Chen, S., Wu, X., Sun, Y., Li, A., Huang, K., Luo, R., Wang, L., Liu, Y., Zhou, T., Wei, S., Pan, G., & Pei, D. (2013). Generation of tooth-like structures from integration-free human urine induced pluripotent stem cells. *Cell Regeneration*, *2*, 6. https://doi.org/10.1186/2045-9769-2-6

Carlile, M. J., Harrison, V. T., Lumsden, A. G. S., & Palmer, R. M. (1998a). Development and cell fate in interspeci®c (Mus musculus/ Mus caroli) orthotopic transplants of mouse molar tooth germs detected by in situ hybridizationp. *Archives of Oral Biology*, 12.

Carlile, M. J., Harrison, V. T., Lumsden, A. G. S., & Palmer, R. M. (1998b). Development and cell fate in interspecific (Mus musculus/Mus caroli) orthotopic transplants of mouse molar tooth germs detected by in situ hybridization11Based on a thesis submitted to London University in partial fulfillment of the requirements for the PhD degree. *Archives of Oral Biology*, *43*(5), 395–406. https://doi.org/10.1016/S0003-9969(97)00118-0

Chee, W.-Y., Kurahashi, Y., Kim, J., Miura, K., Okuzaki, D., Ishitani, T., Kajiwara, K., Nada, S., Okano, H., & Okada, M. (2021). β-catenin-promoted cholesterol metabolism protects against cellular senescence in naked mole-rat cells. *Communications Biology*, *4*(1), Article 1. https://doi.org/10.1038/s42003-021-01879-8

Chen, Y.-C., Chang, Y.-W., Tan, K. P., Shen, Y.-S., Wang, Y.-H., & Chang, C.-H. (2018). Can mesenchymal stem cells and their conditioned medium assist inflammatory chondrocytes recovery? *PLOS ONE*, *13*(11), e0205563. https://doi.org/10.1371/journal.pone.0205563

Cheng, Z., Bosco, D. B., Sun, L., Chen, X., Xu, Y., Tai, W., Didier, R., Li, J., Fan, J., He, X., & Ren, Y. (2017). Neural Stem Cell-Conditioned Medium Suppresses Inflammation and Promotes Spinal Cord Injury Recovery. *Cell Transplantation*, *26*(3), 469–482. https://doi.org/10.3727/096368916X693473

Cho, S.-W. (2003). Lineage of non-cranial neural crest cell in the dental mesenchyme: Using a lacZ reporter gene during early tooth development. *Journal of Electron Microscopy*, *52*(6), 567–571. https://doi.org/10.1093/jmicro/52.6.567

Chouaib, B., Cuisinier, F., & Collart-Dutilleul, P.-Y. (2022). Dental stem cell-conditioned medium for tissue regeneration: Optimization of production and storage. *World Journal of Stem Cells*, *14*(4), 287–302. https://doi.org/10.4252/wjsc.v14.i4.287

Choudhery, M. S., Badowski, M., Muise, A., Pierce, J., & Harris, D. T. (2014). Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. *Journal of Translational Medicine*, *12*(1), 8. https://doi.org/10.1186/1479-5876-12-8

Conboy, I. M., Conboy, M. J., Wagers, A. J., Girma, E. R., Weissman, I. L., & Rando, T. A. (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature*, *433*(7027), 760–764. https://doi.org/10.1038/nature03260

Contessi Negrini, N., Angelova Volponi, A., Higgins, C. A., Sharpe, P. T., & Celiz, A. D. (2021). Scaffold-based developmental tissue engineering strategies for ectodermal organ regeneration. *Materials Today Bio*, *10*, 100107. https://doi.org/10.1016/j.mtbio.2021.100107

Danieli, P., Malpasso, G., Ciuffreda, M. C., & Gnecchi, M. (2016). Testing the Paracrine Properties of Human Mesenchymal Stem Cells Using Conditioned Medium. *Methods in Molecular Biology (Clifton, N.J.)*, *1416*, 445–456. https://doi.org/10.1007/978-1-4939-3584-0_26

Davis, D. M., Fiske, J., Scott, B., & Radford, D. R. (2000). *The emotional effects of tooth loss: A preliminary quantitative study*. *188*, 4.

Debnath, S., Yallowitz, A. R., McCormick, J., Lalani, S., Zhang, T., Xu, R., Li, N., Liu, Y., Yang, Y. S., Eiseman, M., Shim, J.-H., Hameed, M., Healey, J. H., Bostrom, M. P., Landau, D. A., & Greenblatt, M. B. (2018). Discovery of a periosteal stem cell mediating intramembranous bone formation. *Nature*, *562*(7725), 133–139. https://doi.org/10.1038/s41586-018-0554-8

*Dental Morphology—1st Edition*. (n.d.). Retrieved 4 January 2023, from https://www.elsevier.com/books/dental-morphology/978-0-7236-0666-6

Dissanayaka, W. L., & Zhang, C. (2020). Scaffold-based and Scaffold-free Strategies in Dental Pulp Regeneration. *Journal of Endodontics*, *46*(9, Supplement), S81–S89. https://doi.org/10.1016/j.joen.2020.06.022

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Krause, D. S., Deans, R. J., Keating, A., Prockop, D. J., & Horwitz, E. M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, *8*(4), 315–317. https://doi.org/10.1080/14653240600855905

Dosedělová, H., Dumková, J., Lesot, H., Glocová, K., Kunová, M., Tucker, A. S., Veselá, I., Krejčí, P., Tichý, F., Hampl, A., & Buchtová, M. (2015). Fate of the Molar Dental Lamina in the Monophyodont Mouse. *PLOS ONE*, *10*(5), e0127543. https://doi.org/10.1371/journal.pone.0127543

Dosumu, O. O., Ogunrinde, J. T., & Bamigboye, S. A. (2014). KNOWLEDGE OF CONSEQUENCES OF MISSING TEETH IN PATIENTS ATTENDING PROSTHETIC CLINIC IN U.C.H. IBADAN. *Annals of Ibadan Postgraduate Medicine*, *12*(1), 42–48.

Duailibi, M. T., Duailibi, S. E., Young, C. S., Bartlett, J. D., Vacanti, J. P., & Yelick, P. C. (2004). Bioengineered Teeth from Cultured Rat Tooth Bud Cells. *Journal of Dental Research*, *83*(7), 523–528. https://doi.org/10.1177/154405910408300703

Duailibi, S. E., Duailibi, M. T., Zhang, W., Asrican, R., Vacanti, J. P., & Yelick, P. C. (2008). Bioengineered Dental Tissues Grown in the Rat Jaw. *Journal of Dental Research*, *87*(8), 745–750. https://doi.org/10.1177/154405910808700811

Egusa, H., Sonoyama, W., Nishimura, M., Atsuta, I., & Akiyama, K. (2012). Stem cells in dentistry – Part I: Stem cell sources. *Journal of Prosthodontic Research*, *56*(3), 151–165. https://doi.org/10.1016/j.jpor.2012.06.001

Feng, J., van der Zwaag, M., Stokman, M. A., van Os, R., & Coppes, R. P. (2009). Isolation and characterization of human salivary gland cells for stem cell transplantation to reduce radiation-induced hyposalivation. *Radiotherapy and Oncology*, *92*(3), 466–471. https://doi.org/10.1016/j.radonc.2009.06.023

Fraser, G. J., Bloomquist, R. F., & Streelman, J. T. (2013). Common developmental pathways link tooth shape to regeneration. *Developmental Biology*, *377*(2), 399–414. https://doi.org/10.1016/j.ydbio.2013.02.007

Fu, Y., Karbaat, L., Wu, L., Leijten, J., Both, S. K., & Karperien, M. (2017). Trophic Effects of Mesenchymal Stem Cells in Tissue Regeneration. *Tissue Engineering Part B: Reviews*, *23*(6), 515–528. https://doi.org/10.1089/ten.teb.2016.0365

Fuenzalida, M., Lemus, R., Romero, S., Fernandez-Valencia, R., & Lemus, D. (1990). Behavior of rabbit dental tissues in heterospecific association with embryonic quail ectoderm. *The Journal of Experimental Zoology*, *256*(3), 264–272. https://doi.org/10.1002/jez.1402560305

Gaete, M., & Tucker, A. S. (2013). Organized Emergence of Multiple-Generations of Teeth in Snakes Is Dysregulated by Activation of Wnt/Beta-Catenin Signalling. *PLOS ONE*, *8*(9), e74484. https://doi.org/10.1371/journal.pone.0074484

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., & Robertson, M. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. *Cell*, *66*(3), 589–600. https://doi.org/10.1016/0092-8674(81)90021-0

Gronthos, S., Mankani, M., Brahim, J., Robey, P. G., & Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(25), 13625–13630.

Gunti, S., Hoke, A. T. K., Vu, K. P., & London, N. R. (2021). Organoid and Spheroid Tumor Models: Techniques and Applications. *Cancers*, *13*(4), 874. https://doi.org/10.3390/cancers13040874

Handa, K., Saito, M., Tsunoda, A., Yamauchi, M., Hattori, S., Sato, S., Toyoda, M., Teranaka, T., & Narayanan, A. S. (2002). Progenitor Cells From Dental Follicle Are Able to Form Cementum Matrix In Vivo. *Connective Tissue Research*, *43*(2–3), 406–408. https://doi.org/10.1080/03008200290001023

Hassani, S.-N., Moradi, S., Taleahmad, S., Braun, T., & Baharvand, H. (2019). Transition of inner cell mass to embryonic stem cells: Mechanisms, facts, and hypotheses. *Cellular and Molecular Life Sciences: CMLS*, *76*(5), 873–892. https://doi.org/10.1007/s00018-018-2965-y

Higuchi, Y., Yamamoto, H., Cai, J., Suzuki, K., Jung, H.-S., & Kozawa, Y. (2008). Comparison of the Structures of Tooth Germs Transplanted into the Subcutaneous Tissue or the Kidney Capsule of Mice. *International Journal of Oral-Medical Sciences*, *7*(1), 27–34. https://doi.org/10.5466/ijoms.7.27

Hobson, S., Arefin, S., Kublickiene, K., Shiels, P. G., & Stenvinkel, P. (2019). Senescent Cells in Early Vascular Ageing and Bone Disease of Chronic Kidney Disease—A Novel Target for Treatment. *Toxins*, *11*(2), Article 2. https://doi.org/10.3390/toxins11020082

Honda, M. J., Ohara, T., Sumita, Y., Ogaeri, T., Kagami, H., & Ueda, M. (2006). Preliminary Study of Tissue-Engineered Odontogenesis in the Canine Jaw. *Journal of Oral and Maxillofacial Surgery*, *64*(2), 283–289. https://doi.org/10.1016/j.joms.2005.10.020

Honda, M. J., Tsuchiya, S., Sumita, Y., Sagara, H., & Ueda, M. (2007). The sequential seeding of epithelial and mesenchymal cells for tissue-engineered tooth regeneration. *Biomaterials*, *28*(4), 680–689. https://doi.org/10.1016/j.biomaterials.2006.09.039

Hovorakova, M., Lesot, H., Peterka, M., & Peterkova, R. (2018). Early development of the human dentition revisited. *Journal of Anatomy*, *233*(2), 135–145. https://doi.org/10.1111/joa.12825

Howes, R. I. (1977). Root formation in ectopically transplanted teeth of the frog, Rana pipiens I. Tooth morphogenesis. *Acta Anatomica*, *97*(2), 151–165.

Howes, R. I. (1979). Root morphogenesis in ectopically transplanted pleurodont teeth of the iguana. *Cells Tissues Organs*, *103*(4), 400–408. https://doi.org/10.1159/000145042

Hu, B., Nadiri, A., Kuchler-Bopp, S., Perrin-Schmitt, F., Peters, H., & Lesot, H. (2006). Tissue engineering of tooth crown, root, and periodontium. *Tissue Engineering*, *12*(8), 2069–2075. https://doi.org/10.1089/ten.2006.12.2069

Huggins, C. B., McCarroll, H. R., & Dahlberg, A. A. (1934). TRANSPLANTATION OF TOOTH GERM ELEMENTS AND THE EXPERIMENTAL HETEROTOPIC FORMATION OF DENTIN AND ENAMEL. *The Journal of Experimental Medicine*, *60*(2), 199–210. https://doi.org/10.1084/jem.60.2.199

Hung, G., Ashvetiya, T., Leszczynska, A., Yang, W., Hwang, C.-W., Gerstenblith, G., Barth, A. S., & Johnston, P. V. (2022). Paracrine-mediated rejuvenation of aged mesenchymal stem cells is associated with downregulation of the autophagy-lysosomal pathway. *Npj Aging*, *8*(1), Article 1. https://doi.org/10.1038/s41514-022-00091-0

Ikeda, E., Morita, R., Nakao, K., Ishida, K., Nakamura, T., Takano-Yamamoto, T., Ogawa, M., Mizuno, M., Kasugai, S., & Tsuji, T. (2009). Fully functional bioengineered tooth replacement as an organ replacement therapy. *Proceedings of the National Academy of Sciences*, *106*(32), 13475–13480. https://doi.org/10.1073/pnas.0902944106

Ikeda, E., Yagi, K., Kojima, M., Yagyuu, T., Ohshima, A., Sobajima, S., Tadokoro, M., Katsube, Y., Isoda, K., Kondoh, M., Kawase, M., Go, M. J., Adachi, H., Yokota, Y., Kirita, T., & Ohgushi, H. (2008). Multipotent cells from the human third molar: Feasibility of cell-based therapy for liver disease. *Differentiation*, *76*(5), 495–505. https://doi.org/10.1111/j.1432-0436.2007.00245.x

Ishida, K., Murofushi, M., Nakao, K., Morita, R., Ogawa, M., & Tsuji, T. (2011). The regulation of tooth morphogenesis is associated with epithelial cell proliferation and the expression of Sonic hedgehog through epithelial-mesenchymal interactions. *Biochemical and Biophysical Research Communications*, *405*(3), 455–461. https://doi.org/10.1016/j.bbrc.2011.01.052

Ishizeki, K., Fujiwara, N., & Nawa, T. (1989). Morphogenesis of mineralized tissues induced by neonatal mouse molar pulp isografts in the spleen. *Archives of Oral Biology*, *34*(6), 465–473. https://doi.org/10.1016/0003-9969(89)90126-X

Ishizeki, K., Fujiwara, N., Sakakura, Y., & Nawa, T. (1987). The development of mandibular molar tooth germs isografted in the mouse spleen. *Archives of Oral Biology*, *32*(10), 695–704. https://doi.org/10.1016/0003-9969(87)90112-9

Ishizeki, K., Nawa, T., & Sugawara, M. (1990). Calcification capacity of dental papilla mesenchymal cells transplanted in the isogenic mouse spleen. *The Anatomical Record*, *226*(3), 279–287. https://doi.org/10.1002/ar.1092260303

Isogawa, N., Terashima, T., Nakano, Y., Kindaichi, J., Takagi, Y., & Takano, Y. (2004). The induction of enamel and dentin complexes by subcutaneous implantation of reconstructed human and murine tooth germ elements. *Archives of Histology and Cytology*, *67*(1), 65–77. https://doi.org/10.1679/aohc.67.65

James, S. L., Abate, D., Abate, K. H., Abay, S. M., Abbafati, C., Abbasi, N., Abbastabar, H., Abd-Allah, F., Abdela, J., Abdelalim, A., Abdollahpour, I., Abdulkader, R. S., Abebe, Z., Abera, S.

F., Abil, O. Z., Abraha, H. N., Abu-Raddad, L. J., Abu-Rmeileh, N. M. E., Accrombessi, M. M. K., … Murray, C. J. L. (2018). Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: A systematic analysis for the Global Burden of Disease Study 2017. *The Lancet*, *392*(10159), 1789–1858. https://doi.org/10.1016/S0140-6736(18)32279-7

Katancik, J. A., Kumarswamy, A., Branch-Mays, G., & Califano, J. V. (2016). Infections of the Periodontal Apparatus. In *Head, Neck, and Orofacial Infections* (pp. 189–202). Elsevier. https://doi.org/10.1016/B978-0-323-28945-0.00011-9

Khanh, V. C., Yamashita, T., Ohneda, K., Tokunaga, C., Kato, H., Osaka, M., Hiramatsu, Y., & Ohneda, O. (2020). Rejuvenation of mesenchymal stem cells by extracellular vesicles inhibits the elevation of reactive oxygen species. *Scientific Reports*, *10*(1), Article 1. https://doi.org/10.1038/s41598-020-74444-8

Kielan-Jaworowska, Z., Cifelli, R. L., & Luo, Z.-X. (2004). *Mammals from the Age of Dinosaurs: Origins, Evolution, and Structure*. Columbia University Press. https://doi.org/10.7312/kiel11918

Kim, W., Gwon, Y., Park, S., Kim, H., & Kim, J. (2022). Therapeutic strategies of three-dimensional stem cell spheroids and organoids for tissue repair and regeneration. *Bioactive Materials*, *19*, 50–74. https://doi.org/10.1016/j.bioactmat.2022.03.039

Kiso, H., Takahashi, K., Mishima, S., Murashima-Suginami, A., Kakeno, A., Yamazaki, T., Asai, K., Tokita, Y., Uozumi, R., Sugai, M., Harada, H., Huang, B., MacDougall, M., & Bessho, K. (2019). Third Dentition Is the Main Cause of Premolar Supernumerary Tooth Formation. *Journal of Dental Research*, *98*(9), 968–974. https://doi.org/10.1177/0022034519858282

Kiso, H., Takahashi, K., Saito, K., Togo, Y., Tsukamoto, H., Huang, B., Sugai, M., Shimizu, A., Tabata, Y., Economides, A. N., Slavkin, H. C., & Bessho, K. (2014). Interactions between

BMP-7 and USAG-1 (uterine sensitization-associated gene-1) regulate supernumerary organ formations. *PloS One*, *9*(5), e96938. https://doi.org/10.1371/journal.pone.0096938

Kollar, E. J., & Baird, G. R. (1969). The influence of the dental papilla on the development of tooth shape in embryonic mouse tooth germs. *Journal of Embryology and Experimental Morphology*, *21*(1), 131–148.

Kollar, E. J., & Baird, G. R. (1970a). Tissue interactions in embryonic mouse tooth germs. I. Reorganization of the dental epithelium during tooth-germ reconstruction. *Journal of Embryology and Experimental Morphology*, *24*(1), 159–171.

Kollar, E. J., & Baird, G. R. (1970b). Tissue interactions in embryonic mouse tooth germs. II. The inductive role of the dental papilla. *Journal of Embryology and Experimental Morphology*, *24*(1), 173–186.

Kollar, E. J., & Fisher, C. (1980). Tooth induction in chick epithelium: Expression of quiescent genes for enamel synthesis. *Science (New York, N.Y.)*, *207*(4434), 993–995. https://doi.org/10.1126/science.7352302

Korkmaz-Icöz, S., Li, K., Loganathan, S., Ding, Q., Ruppert, M., Radovits, T., Brlecic, P., Sayour, A. A., Karck, M., & Szabó, G. (2020). Brain-dead donor heart conservation with a preservation solution supplemented by a conditioned medium from mesenchymal stem cells improves graft contractility after transplantation. *American Journal of Transplantation*, *20*(10), 2847–2856. https://doi.org/10.1111/ajt.15843

Kornicka, K., Marycz, K., Tomaszewski, K. A., Marędziak, M., & Śmieszek, A. (2015). The Effect of Age on Osteogenic and Adipogenic Differentiation Potential of Human Adipose Derived Stromal Stem Cells (hASCs) and the Impact of Stress Factors in the Course of the Differentiation Process. *Oxidative Medicine and Cellular Longevity*, *2015*, 309169. https://doi.org/10.1155/2015/309169

Kretlow, J. D., Jin, Y.-Q., Liu, W., Zhang, W. J., Hong, T.-H., Zhou, G., Baggett, L. S., Mikos, A. G., & Cao, Y. (2008). Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells. *BMC Cell Biology*, *9*(1), 60. https://doi.org/10.1186/1471-2121-9-60

Kuchler-Bopp, S., Bécavin, T., Kökten, T., Weickert, J. L., Keller, L., Lesot, H., Deveaux, E., & Benkirane-Jessel, N. (2016). Three-dimensional Micro-culture System for Tooth Tissue Engineering. *Journal of Dental Research*, *95*(6), 657–664. https://doi.org/10.1177/0022034516634334

Kuo, T.-F., Huang, A.-T., Chang, H.-H., Lin, F.-H., Chen, S.-T., Chen, R.-S., Chou, C.-H., Lin, H.-C., Chiang, H., & Chen, M.-H. (2008). Regeneration of dentin-pulp complex with cementum and periodontal ligament formation using dental bud cells in gelatin-chondroitin-hyaluronan tri-copolymer scaffold in swine. *Journal of Biomedical Materials Research Part A*, *86A*(4), 1062–1068. https://doi.org/10.1002/jbm.a.31746

Kuo, T.-F., Lin, H.-C., Yang, K.-C., Lin, F.-H., Chen, M.-H., Wu, C.-C., & Chang, H.-H. (2010). Bone Marrow Combined With Dental Bud Cells Promotes Tooth Regeneration in Miniature Pig Model: BMSC MAY PROMOTE TOOTH REGENERATION. *Artificial Organs*, no-no. https://doi.org/10.1111/j.1525-1594.2010.01064.x

Kwon, H.-J. E., & Jiang, R. (2018). Development of Teeth ☆. In *Reference Module in Biomedical Sciences* (p. B9780128012383642000). Elsevier. https://doi.org/10.1016/B978-0-12-801238-3.64113-2

Lancaster, M. A., & Knoblich, J. A. (2014). Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science*, *345*(6194), 1247125. https://doi.org/10.1126/science.1247125

Lee, A. (2015, March 20). *Nothing but the tooth: The history of dentistry in Britain*. Express.Co.Uk. https://www.express.co.uk/life-style/health/565139/teeth-dentistry-Drills-Dentures-And-Dentistry

Lemus, D., Coloma, L., Fuenzalida, M., Illanes, J., Paz de la Vega, Y., Ondarza, A., & Blanquez, M. J. (1986). Odontogenesis and amelogenesis in interacting lizard-quail tissue combinations. *Journal of Morphology*, *189*(2), 121–129. https://doi.org/10.1002/jmor.1051890203

Li, L., Tang, Q., Wang, A., & Chen, Y. (2019). Regrowing a tooth: In vitro and in vivo approaches. *Current Opinion in Cell Biology*, *61*, 126–131. https://doi.org/10.1016/j.ceb.2019.08.002

Li, S., Qi, Y., Hu, S., Piao, F., Guan, H., Wang, Z., Chen, R., & Liu, S. (2017). Mesenchymal stem cells-conditioned medium protects PC12 cells against 2,5-hexanedione-induced apoptosis via inhibiting mitochondria-dependent caspase 3 pathway. *Toxicology and Industrial Health*, *33*(2), 107–118. https://doi.org/10.1177/0748233715598267

Liu, Y., & Schubert, D. R. (2009). The specificity of neuroprotection by antioxidants. *Journal of Biomedical Science*, *16*(1), 98. https://doi.org/10.1186/1423-0127-16-98

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. (2013). The Hallmarks of Aging. *Cell*, *153*(6), 1194–1217. https://doi.org/10.1016/j.cell.2013.05.039

Lu, X., Yu, F., Liu, J., Cai, W., Zhao, Y., Zhao, S., & Liu, S. (2017). The epidemiology of supernumerary teeth and the associated molecular mechanism. *Organogenesis*, *13*(3), 71–82. https://doi.org/10.1080/15476278.2017.1332554

Lubbock, M. J., Harrison, V. T., Lumsden, A. G. S., & Palmer, R. M. (1996). Development and cell fate in interspecific (mus musculus/mus caroli) intraocular transplants of mouse molar tooth-germ tissues detected by in situ hybridization. *Archives of Oral Biology*, *41*(1), 77–84. https://doi.org/10.1016/0003-9969(95)00094-1

Ma, D., Ma, Z., Zhang, X., Wang, W., Yang, Z., Zhang, M., Wu, G., Lu, W., Deng, Z., & Jin, Y. (2009). Effect of age and extrinsic microenvironment on the proliferation and osteogenic differentiation of rat dental pulp stem cells in vitro. *Journal of Endodontics*, *35*(11), 1546–1553. https://doi.org/10.1016/j.joen.2009.07.016

Mina, M., & Kollar, E. J. (1987). The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. *Archives of Oral Biology*, *32*(2), 123–127. https://doi.org/10.1016/0003-9969(87)90055-0

Mishima, S., Takahashi, K., Kiso, H., Murashima-Suginami, A., Tokita, Y., Jo, J.-I., Uozumi, R., Nambu, Y., Huang, B., Harada, H., Komori, T., Sugai, M., Tabata, Y., & Bessho, K. (2021). Local application of Usag-1 siRNA can promote tooth regeneration in Runx2-deficient mice. *Scientific Reports*, *11*(1), 13674. https://doi.org/10.1038/s41598-021-93256-y

Mitsiadis, T. A., Caton, J., & Cobourne, M. (2006). Waking-up the sleeping beauty: Recovery of the ancestral bird odontogenic program. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *306B*(3), 227–233. https://doi.org/10.1002/jez.b.21094

Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, L. W., Robey, P. G., & Shi, S. (2003). SHED: Stem cells from human exfoliated deciduous teeth. *Proceedings of the National Academy of Sciences*, *100*(10), 5807–5812. https://doi.org/10.1073/pnas.0937635100

Monteiro, N., Smith, E. E., Angstadt, S., Zhang, W., Khademhosseini, A., & Yelick, P. C. (2016a). Dental cell sheet biomimetic tooth bud model. *Biomaterials*, *106*, 167–179. https://doi.org/10.1016/j.biomaterials.2016.08.024

Monteiro, N., Smith, E. E., Angstadt, S., Zhang, W., Khademhosseini, A., & Yelick, P. C. (2016b). Dental cell sheet biomimetic tooth bud model. *Biomaterials*, *106*, 167–179. https://doi.org/10.1016/j.biomaterials.2016.08.024

Monteiro, N., & Yelick, P. C. (2017). Advances and perspectives in tooth tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine*, *11*(9), 2443–2461. https://doi.org/10.1002/term.2134

Morio, I. (1985). Recombinant study of the mouse molar cervical loop and dental papilla by renal transplantation. *Archives of Oral Biology*, *30*(7), 557–561. https://doi.org/10.1016/0003-9969(85)90057-3

Moschouris, K., Firoozi, N., & Kang, Y. (2016). The application of cell sheet engineering in the vascularization of tissue regeneration. *Regenerative Medicine*, *11*(6), 559–570. https://doi.org/10.2217/rme-2016-0059

Murashima-Suginami, A., Kiso, H., Tokita, Y., Mihara, E., Nambu, Y., Uozumi, R., Tabata, Y., Bessho, K., Takagi, J., Sugai, M., & Takahashi, K. (2021). Anti-USAG-1 therapy for tooth regeneration through enhanced BMP signaling. *Science Advances*, *7*(7). https://doi.org/10.1126/sciadv.abf1798

Murashima-Suginami, A., Takahashi, K., Kawabata, T., Sakata, T., Tsukamoto, H., Sugai, M., Yanagita, M., Shimizu, A., Sakurai, T., Slavkin, H. C., & Bessho, K. (2007). Rudiment incisors survive and erupt as supernumerary teeth as a result of USAG-1 abrogation. *Biochemical and Biophysical Research Communications*, *359*(3), 549–555. https://doi.org/10.1016/j.bbrc.2007.05.148

Murashima-Suginami, A., Takahashi, K., Sakata, T., Tsukamoto, H., Sugai, M., Yanagita, M., Shimizu, A., Sakurai, T., Slavkin, H. C., & Bessho, K. (2008). Enhanced BMP signaling results in supernumerary tooth formation in USAG-1 deficient mouse. *Biochemical and Biophysical Research Communications*, *369*(4), 1012–1016. https://doi.org/10.1016/j.bbrc.2008.02.135

Mutlu, A. S., Duffy, J., & Wang, M. C. (2021). Lipid metabolism and lipid signals in aging and longevity. *Developmental Cell*, *56*(10), 1394–1407. https://doi.org/10.1016/j.devcel.2021.03.034

Nait Lechguer, A., Kuchler-Bopp, S., Hu, B., Haïkel, Y., & Lesot, H. (2008). Vascularization of Engineered Teeth. *Journal of Dental Research*, *87*(12), 1138–1143. https://doi.org/10.1177/154405910808701216

Nakamura, T., de Vega, S., Fukumoto, S., Jimenez, L., Unda, F., & Yamada, Y. (2008). Transcription factor epiprofin is essential for tooth morphogenesis by regulating epithelial cell fate and tooth number. *The Journal of Biological Chemistry*, *283*(8), 4825–4833. https://doi.org/10.1074/jbc.M708388200

Nakao, K., Morita, R., Saji, Y., Ishida, K., Tomita, Y., Ogawa, M., Saitoh, M., Tomooka, Y., & Tsuji, T. (2007). The development of a bioengineered organ germ method. *Nature Methods*, *4*(3), 227–230. https://doi.org/10.1038/nmeth1012

Nery, E. B., Kraus, B. S., & Croup, M. (1970). Timing and topography of early human tooth development. *Archives of Oral Biology*, *15*(12), 1315-IN36. https://doi.org/10.1016/0003-9969(70)90020-8

*Netter's Clinical Anatomy—5th Edition*. (n.d.). Retrieved 4 January 2023, from https://www.elsevier.com/books/netter's-clinical-anatomy/978-0-323-82662-4

Neves, J., Sousa-Victor, P., & Jasper, H. (2017a). Rejuvenating Strategies for Stem Cell-based Therapies in Aging. *Cell Stem Cell*, *20*(2), 161–175. https://doi.org/10.1016/j.stem.2017.01.008

Neves, J., Sousa-Victor, P., & Jasper, H. (2017b). Rejuvenating Strategies for Stem Cell-based Therapies in Aging. *Cell Stem Cell*, *20*(2), 161–175. https://doi.org/10.1016/j.stem.2017.01.008

Noverina, R., Widowati, W., Ayuningtyas, W., Kurniawan, D., Afifah, E., Laksmitawati, D. R., Rinendyaputri, R., Rilianawati, R., Faried, A., Bachtiar, I., & Wirakusumah, F. F. (2019). Growth factors profile in conditioned medium human adipose tissue-derived mesenchymal stem cells (CM-hATMSCs). *Clinical Nutrition Experimental*, *24*, 34–44. https://doi.org/10.1016/j.yclnex.2019.01.002

Ohara, T., Itaya, T., Usami, K., Ando, Y., Sakurai, H., Honda, M. J., Ueda, M., & Kagami, H. (2010). Evaluation of scaffold materials for tooth tissue engineering. *Journal of Biomedical Materials Research Part A*, *9999A*, NA-NA. https://doi.org/10.1002/jbm.a.32749

Ohazama, A., Modino, S. a. C., Miletich, I., & Sharpe, P. T. (2004). Stem-cell-based tissue engineering of murine teeth. *Journal of Dental Research*, *83*(7), 518–522. https://doi.org/10.1177/154405910408300702

Ohta, T., Kinoshita, T., Naito, M., Nozaki, T., Masutani, M., Tsuruo, T., & Miyajima, A. (1997). Requirement of the Caspase-3/CPP32 Protease Cascade for Apoptotic Death following Cytokine Deprivation in Hematopoietic Cells *. *Journal of Biological Chemistry*, *272*(37), 23111–23116. https://doi.org/10.1074/jbc.272.37.23111

Okuno, S., Shimizu, S., Ito, T., Nomura, M., Hamada, E., Tsujimoto, Y., & Matsuda, H. (1998). Bcl-2 Prevents Caspase-independent Cell Death *. *Journal of Biological Chemistry*, *273*(51), 34272–34277. https://doi.org/10.1074/jbc.273.51.34272

Ono, M., Oshima, M., Ogawa, M., Sonoyama, W., Hara, E. S., Oida, Y., Shinkawa, S., Nakajima, R., Mine, A., Hayano, S., Fukumoto, S., Kasugai, S., Yamaguchi, A., Tsuji, T., & Kuboki, T. (2017). Practical whole-tooth restoration utilizing autologous bioengineered tooth germ transplantation in a postnatal canine model. *Scientific Reports*, *7*, 44522. https://doi.org/10.1038/srep44522

Ooé, T. (1957). On the early development of human dental lamina. *Okajimas Folia Anatomica Japonica*, *30*(2–3), 197-210_1. https://doi.org/10.2535/ofaj1936.30.2-3_197

Oshima, M., Ogawa, M., Yasukawa, M., & Tsuji, T. (2012). Generation of a bioengineered tooth by using a three-dimensional cell manipulation method (organ germ method). *Methods in Molecular Biology (Clifton, N.J.)*, *887*, 149–165. https://doi.org/10.1007/978-1-61779-860-3_14

Palmer, R. M., & Lumsden, A. G. (1987). Development of periodontal ligament and alveolar bone in homografted recombinations of enamel organs and papillary, pulpal and follicular mesenchyme in the mouse. *Archives of Oral Biology*, *32*(4), 281–289. https://doi.org/10.1016/0003-9969(87)90022-7

Pan, C., Lang, H., Zhang, T., Wang, R., Lin, X., Shi, P., Zhao, F., & Pang, X. (2019). Conditioned medium derived from human amniotic stem cells delays H2O2-induced premature senescence in human dermal fibroblasts. *International Journal of Molecular Medicine*, *44*(5), 1629–1640. https://doi.org/10.3892/ijmm.2019.4346

Papagerakis, S., Pannone, G., Zheng, L., About, I., Taqi, N., Nguyen, N. P. T., Matossian, M., McAlpin, B., Santoro, A., McHugh, J., Prince, M. E., & Papagerakis, P. (2014). Oral epithelial stem cells—Implications in normal development and cancer metastasis. *Experimental Cell Research*, *325*(2), 111–129. https://doi.org/10.1016/j.yexcr.2014.04.021

Passarelli, P. C., Pagnoni, S., Piccirillo, G. B., Desantis, V., Benegiamo, M., Liguori, A., Papa, R., Papi, P., Pompa, G., & D'Addona, A. (2020). Reasons for Tooth Extractions and Related Risk Factors in Adult Patients: A Cohort Study. *International Journal of Environmental Research and Public Health*, *17*(7), 2575. https://doi.org/10.3390/ijerph17072575

Pergolizzi, J., Magnusson, P., Lequang, J. A., Gharibo, C., & Varrassi, G. (2020). The pharmacological management of dental pain. *Expert Opinion on Pharmacotherapy*, *21*, 1–11. https://doi.org/10.1080/14656566.2020.1718651

Petridis, H. P., Tsiggos, N., Michail, A., Kafantaris, S. N., Hatzikyriakos, A., & Kafantaris, N. M. (2010). Three-dimensional positional changes of teeth adjacent to posterior edentulous spaces in relation to age at time of tooth loss and elapsed time. *The European Journal of Prosthodontics and Restorative Dentistry*, *18*(2), 78–83.

Pippi, R. (2014). Odontomas and Supernumerary Teeth: Is There a Common Origin? *International Journal of Medical Sciences*, *11*(12), 1282–1297. https://doi.org/10.7150/ijms.10501

Popa, E. M., Buchtova, M., & Tucker, A. S. (2019). Revitalising the rudimentary replacement dentition in the mouse. *Development*, *146*(3), dev171363. https://doi.org/10.1242/dev.171363

Rajan, T. S., Diomede, F., Bramanti, P., Trubiani, O., & Mazzon, E. (2017). Conditioned medium from human gingival mesenchymal stem cells protects motor-neuron-like NSC-34 cells against scratch-injury-induced cell death. *International Journal of Immunopathology and Pharmacology*, *30*(4), 383–394. https://doi.org/10.1177/0394632017740976

Rajan, T. S., Giacoppo, S., Diomede, F., Ballerini, P., Paolantonio, M., Marchisio, M., Piattelli, A., Bramanti, P., Mazzon, E., & Trubiani, O. (2016). The secretome of periodontal ligament stem cells from MS patients protects against EAE. *Scientific Reports*, *6*(1), Article 1. https://doi.org/10.1038/srep38743

Rakhshan, V. (2015). Congenitally missing teeth (hypodontia): A review of the literature concerning the etiology, prevalence, risk factors, patterns and treatment. *Dental Research Journal*, *12*(1), 1–13. https://doi.org/10.4103/1735-3327.150286

Rasch, L. J., Cooper, R. L., Underwood, C., Dillard, W. A., Thiery, A. P., & Fraser, G. J. (2020). Development and regeneration of the crushing dentition in skates (Rajidae). *Developmental Biology*, *466*(1–2), 59–72. https://doi.org/10.1016/j.ydbio.2020.07.014

Ravindran, S., Song, Y., & George, A. (2010). Development of Three-Dimensional Biomimetic Scaffold to Study Epithelial–Mesenchymal Interactions. *Tissue Engineering Part A*, *16*(1), 327–342. https://doi.org/10.1089/ten.tea.2009.0110

Saito, K., Takahashi, K., Huang, B., Asahara, M., Kiso, H., Togo, Y., Tsukamoto, H., Mishima, S., Nagata, M., Iida, M., Tokita, Y., Asai, M., Shimizu, A., Komori, T., Harada, H., MacDougall, M., Sugai, M., & Bessho, K. (2018). Loss of Stemness, EMT, and Supernumerary Tooth Formation in Cebpb−/−Runx2+/− Murine Incisors. *Scientific Reports*, *8*(1), 5169. https://doi.org/10.1038/s41598-018-23515-y

Schwass, D. R., Lyons, K. M., & Purton, D. G. (2013). How long will it last? The expected longevity of prosthodontic and restorative treatment. *New Zealand Dental Journal*, 8.

Seo, B.-M., Miura, M., Gronthos, S., Mark Bartold, P., Batouli, S., Brahim, J., Young, M., Gehron Robey, P., Wang, C. Y., & Shi, S. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. *The Lancet*, *364*(9429), 149–155. https://doi.org/10.1016/S0140-6736(04)16627-0

Sharpe, P. (2020). Regenerative Dentistry. *Frontiers in Dental Medicine*, *1*. https://doi.org/10.3389/fdmed.2020.00003

Shi, Y., Inoue, H., Wu, J. C., & Yamanaka, S. (2017). Induced pluripotent stem cell technology: A decade of progress. *Nature Reviews. Drug Discovery*, *16*(2), 115–130. https://doi.org/10.1038/nrd.2016.245

Slavkin, H. C., Snead, M. L., Zeichner-David, M., Jaskoll, T. F., & Smith, B. T. (1984). Concepts of epithelial-mesenchymal interactions during development: Tooth and lung organogenesis. *Journal of Cellular Biochemistry*, *26*(2), 117–125. https://doi.org/10.1002/jcb.240260207

Smith, E. E., Angstadt, S., Monteiro, N., Zhang, W., Khademhosseini, A., & Yelick, P. C. (2018). Bioengineered Tooth Buds Exhibit Features of Natural Tooth Buds. *Journal of Dental Research*, *97*(10), 1144–1151. https://doi.org/10.1177/0022034518779075

Smith, E. E., Yelick, P. C., & Khademhosseini, A. (2014). Optimization of a biomimetic model for tooth regeneration. *2014 40th Annual Northeast Bioengineering Conference (NEBEC)*, 1–2. https://doi.org/10.1109/NEBEC.2014.6972943

Smith, E. E., Zhang, W., Schiele, N. R., Khademhosseini, A., Kuo, C. K., & Yelick, P. C. (2017a). Developing a biomimetic tooth bud model. *Journal of Tissue Engineering and Regenerative Medicine*, *11*(12), 3326–3336. https://doi.org/10.1002/term.2246

Smith, E. E., Zhang, W., Schiele, N. R., Khademhosseini, A., Kuo, C. K., & Yelick, P. C. (2017b). Developing a biomimetic tooth bud model: Developing a biomimetic tooth bud model. *Journal of Tissue Engineering and Regenerative Medicine*, *11*(12), 3326–3336. https://doi.org/10.1002/term.2246

Smith, M. M., Fraser, G. J., & Mitsiadis, T. A. (2009). Dental lamina as source of odontogenic stem cells: Evolutionary origins and developmental control of tooth generation in gnathostomes. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, *312B*(4), 260–280. https://doi.org/10.1002/jez.b.21272

Sohn, S. J., Yu, J. M., Lee, E. Y., Nam, Y. J., Kim, J., Kang, S., Kim, D. H., Kim, A., & Kang, S. (2018). Anti-aging Properties of Conditioned Media of Epidermal Progenitor Cells Derived from Mesenchymal Stem Cells. *Dermatology and Therapy*, *8*(2), 229–244. https://doi.org/10.1007/s13555-018-0229-2

Song, Y., Zhang, Z., Yu, X., Yan, M., Zhang, X., Gu, S., Stuart, T., Liu, C., Reiser, J., Zhang, Y., & Chen, Y. (2006). Application of lentivirus-mediated RNAi in studying gene function in mammalian tooth development. *Developmental Dynamics*, *235*(5), 1347–1357. https://doi.org/10.1002/dvdy.20706

Sonoyama, W., Liu, Y., Yamaza, T., Tuan, R. S., Wang, S., Shi, S., & Huang, G. T.-J. (2008). Characterization of the Apical Papilla and Its Residing Stem Cells from Human Immature Permanent Teeth: A Pilot Study. *Journal of Endodontics*, *34*(2), 166–171. https://doi.org/10.1016/j.joen.2007.11.021

Štembírek, J., Buchtová, M., Král, T., Matalová, E., Lozanoff, S., & Míšek, I. (2010). Early morphogenesis of heterodont dentition in minipigs. *European Journal of Oral Sciences*, *118*(6), 547–558. https://doi.org/10.1111/j.1600-0722.2010.00772.x

Sumita, Y., Honda, M., Ohara, T., Tsuchiya, S., Sagara, H., Kagami, H., & Ueda, M. (2006). Performance of collagen sponge as a 3-D scaffold for tooth-tissue engineering. *Biomaterials*, *27*(17), 3238–3248. https://doi.org/10.1016/j.biomaterials.2006.01.055

Sun, B., Meng, X., Li, Y., Li, Y., Liu, R., & Xiao, Z. (2023). Conditioned medium from human cord blood mesenchymal stem cells attenuates age-related immune dysfunctions. *Frontiers in Cell and Developmental Biology*, *10*. https://www.frontiersin.org/articles/10.3389/fcell.2022.1042609

Sun, Q., Zhang, Z., & Sun, Z. (2014). The potential and challenges of using stem cells for cardiovascular repair and regeneration. *Genes & Diseases*, *1*(1), 113–119. https://doi.org/10.1016/j.gendis.2014.07.003

Takahashi, K., Kiso, H., Murashima-Suginami, A., Tokita, Y., Sugai, M., Tabata, Y., & Bessho, K. (2020). Development of tooth regenerative medicine strategies by controlling the number of

teeth using targeted molecular therapy. *Inflammation and Regeneration*, *40*(1), 21. https://doi.org/10.1186/s41232-020-00130-x

Takahashi, K., Kiso, H., Saito, K., Togo, Y., Tsukamoto, H., Huang, B., & Bessho, K. (2014). Feasibility of Molecularly Targeted Therapy for Tooth Regeneration. In H. Hibi & M. Ueda (Eds.), *New Trends in Tissue Engineering and Regenerative Medicine—Official Book of the Japanese Society for Regenerative Medicine*. InTech. https://doi.org/10.5772/58904

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, *126*(4), 663–676. https://doi.org/10.1016/j.cell.2006.07.024

Takeo, M., & Tsuji, T. (2018). Organ regeneration based on developmental biology: Past and future. *Current Opinion in Genetics & Development*, *52*, 42–47. https://doi.org/10.1016/j.gde.2018.05.008

Tallgren, A., Lang, B. R., & Miller, R. L. (1991). Longitudinal study of soft-tissue profile changes in patients receiving immediate complete dentures. *The International Journal of Prosthodontics*, *4*(1), 9–16.

Thesleff, I. (2000). Genetic basis of tooth development and dental defects. *Acta Odontologica Scandinavica*, *58*(5), 191–194. https://doi.org/10.1080/000163500750051728

Thesleff, I., & Jernvall, J. (1997). The enamel knot: A putative signaling center regulating tooth development. *Cold Spring Harbor Symposia on Quantitative Biology*, *62*, 257–267.

Thesleff, I., & Juuri, E. (2015). Chapter 19—Dental Epithelial Stem Cells. In A. Vishwakarma, P. Sharpe, S. Shi, & M. Ramalingam (Eds.), *Stem Cell Biology and Tissue Engineering in Dental Sciences* (pp. 263–270). Academic Press. https://doi.org/10.1016/B978-0-12-397157-9.00021-7

Thesleff, I., & Tummers, M. (2008). Tooth organogenesis and regeneration. In *StemBook*. Harvard Stem Cell Institute. http://www.ncbi.nlm.nih.gov/books/NBK27071/

Timmers, L., Lim, S. K., Arslan, F., Armstrong, J. S., Hoefer, I. E., Doevendans, P. A., Piek, J. J., El Oakley, R. M., Choo, A., Lee, C. N., Pasterkamp, G., & de Kleijn, D. P. V. (2008). Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Research*, *1*(2), 129–137. https://doi.org/10.1016/j.scr.2008.02.002

Togo, Y., Takahashi, K., Saito, K., Kiso, H., Tsukamoto, H., Huang, B., Yanagita, M., Sugai, M., Harada, H., Komori, T., Shimizu, A., MacDougall, M., & Bessho, K. (2016). Antagonistic Functions of USAG-1 and RUNX2 during Tooth Development. *PLOS ONE*, *11*(8), e0161067. https://doi.org/10.1371/journal.pone.0161067

Trowell, O. A. (1954). A modified technique for organ culture in vitro. *Experimental Cell Research*, *6*(1), 246–248. https://doi.org/10.1016/0014-4827(54)90169-X

Tucker, A. S., & Fraser, G. J. (2014). Evolution and developmental diversity of tooth regeneration. *Seminars in Cell & Developmental Biology*, *25–26*, 71–80. https://doi.org/10.1016/j.semcdb.2013.12.013

Tummers, M., & Thesleff, I. (2009). The importance of signal pathway modulation in all aspects of tooth development. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, *312B*(4), 309–319. https://doi.org/10.1002/jez.b.21280

Valderhaug, J. (1991). A 15-year clinical evaluation of fixed prosthodontics. *Acta Odontologica Scandinavica*, *49*(1), 35–40. https://doi.org/10.3109/00016359109041138

Vidhya, J., & Krishnan, C. (2015). Partial Edentulism and its Correlation to Age, Gender, Socio-economic Status and Incidence of Various Kennedy's Classes– A Literature Review. *Journal of Clinical and Diagnostic Research : JCDR*, *9*, ZE14-7. https://doi.org/10.7860/JCDR/2015/13776.6124

Viswanathan, S., Shi, Y., Galipeau, J., Krampera, M., Leblanc, K., Martin, I., Nolta, J., Phinney, D. G., & Sensebe, L. (2019). Mesenchymal stem versus stromal cells: International Society for Cell & Gene Therapy (ISCT®) Mesenchymal Stromal Cell committee position statement on nomenclature. *Cytotherapy*, *21*(10), 1019–1024. https://doi.org/10.1016/j.jcyt.2019.08.002

Wang, B., Li, L., Du, S., Liu, C., Lin, X., Chen, Y., & Zhang, Y. (2010). Induction of human keratinocytes into enamel-secreting ameloblasts. *Developmental Biology*, *344*(2), 795–799. https://doi.org/10.1016/j.ydbio.2010.05.511

Wang, F., Wu, Z., Fan, Z., Wu, T., Wang, J., Zhang, C., & Wang, S. (2018). The cell re-association-based whole-tooth regeneration strategies in large animal, Sus scrofa. *Cell Proliferation*, *51*(4), e12479. https://doi.org/10.1111/cpr.12479

Wen, Y., Wang, F., Zhang, W., Li, Y., Yu, M., Nan, X., Chen, L., Yue, W., Xu, X., & Pei, X. (2012). Application of Induced Pluripotent Stem Cells in Generation of a Tissue-Engineered Tooth-Like Structure. *Tissue Engineering Part A*, *18*(15–16), 1677–1685. https://doi.org/10.1089/ten.tea.2011.0220

Widowati, W., Noverina, R., Ayuningtyas, W., Kurniawan, D., Arumwardana, S., Kusuma, H. S. W., Rizal, R., Laksmitawati, D. R., Rinendyaputri, R., Rilianawati, R., & Faried, A. (2022). *Potential of Conditioned Medium of hATMSCs in Aging Cells Model*. Bogor Agricultural University. https://doaj.org/article/5418680f6ef146afb9701082f5708b23

Wu, P., Wu, X., Jiang, T.-X., Elsey, R. M., Temple, B. L., Divers, S. J., Glenn, T. C., Yuan, K., Chen, M.-H., Widelitz, R. B., & Chuong, C.-M. (2013). Specialized stem cell niche enables repetitive renewal of alligator teeth. *Proceedings of the National Academy of Sciences*, *110*(22), E2009–E2018.

Xu, W.-P., Zhang, W., Asrican, R., Kim, H.-J., Kaplan, D. L., & Yelick, P. C. (2008). Accurately Shaped Tooth Bud Cell–Derived Mineralized Tissue Formation on Silk Scaffolds. *Tissue Engineering Part A*, *14*(4), 549–557. https://doi.org/10.1089/tea.2007.0227

Yamamoto, H., Chai, J., Suzuki, K., Yokota, R., Chisaka, H., Sakae, T., Jung, H.-S., & Kozawa, Y. (2005). Studies on the Enamel Structure of Transplanted Tooth Germ. *Journal of Hard Tissue Biology*, *14*(2), 218–220. https://doi.org/10.2485/jhtb.14.218

Yamamoto, H., Kim, E.-J., Cho, S.-W., & Jung, H.-S. (2003). Analysis of tooth formation by reaggregated dental mesenchyme from mouse embryo. *Journal of Electron Microscopy*, *52*(6), 559–566. https://doi.org/10.1093/jmicro/52.6.559

Yamato, M., & Okano, T. (2004). Cell sheet engineering. *Materials Today*, *7*(5), 42–47. https://doi.org/10.1016/S1369-7021(04)00234-2

Yang, K.-C., Kitamura, Y., Wu, C.-C., Chang, H.-H., Ling, T.-Y., & Kuo, T.-F. (2016). Tooth Germ-Like Construct Transplantation for Whole-Tooth Regeneration: An In Vivo Study in the Miniature Pig: Tooth Germ-like Construct for Whole-tooth Regeneration. *Artificial Organs*, *40*(4), E39–E50. https://doi.org/10.1111/aor.12630

Yang, K.-C., Wang, C.-H., Chang, H.-H., Chan, W. P., Chi, C.-H., & Kuo, T.-F. (2012). Fibrin glue mixed with platelet-rich fibrin as a scaffold seeded with dental bud cells for tooth regeneration: Scaffold seeded with dental bud cells for tooth regeneration. *Journal of Tissue Engineering and Regenerative Medicine*, *6*(10), 777–785. https://doi.org/10.1002/term.483

Yelick, P. C., & Sharpe, P. T. (2019). Tooth Bioengineering and Regenerative Dentistry. *Journal of Dental Research*, *98*(11), 1173–1182. https://doi.org/10.1177/0022034519861903

Yi, Q., Liu, O., Yan, F., Lin, X., Diao, S., Wang, L., Jin, L., Wang, S., Lu, Y., & Fan, Z. (2017). Analysis of Senescence-Related Differentiation Potentials and Gene Expression Profiles in

Human Dental Pulp Stem Cells. *Cells Tissues Organs*, *203*(1), 1–11. https://doi.org/10.1159/000448026

Young, C. S., Abukawa, H., Asrican, R., Ravens, M., Troulis, M. J., Kaban, L. B., Vacanti, J. P., & Yelick, P. C. (2005a). Tissue-Engineered Hybrid Tooth and Bone. *Tissue Engineering*, *11*(9–10), 1599–1610. https://doi.org/10.1089/ten.2005.11.1599

Young, C. S., Abukawa, H., Asrican, R., Ravens, M., Troulis, M. J., Kaban, L. B., Vacanti, J. P., & Yelick, P. C. (2005b). Tissue-Engineered Hybrid Tooth and Bone. *Tissue Engineering*, *11*(9–10), 1599–1610. https://doi.org/10.1089/ten.2005.11.1599

Young, C. S., Kim, S.-W., Qin, C., Baba, O., Butler, W. T., Taylor, R. R., Bartlett, J. D., Vacanti, J. P., & Yelick, P. C. (2005). Developmental analysis and computer modelling of bioengineered teeth. *Archives of Oral Biology*, *50*(2), 259–265. https://doi.org/10.1016/j.archoralbio.2004.11.020

Young, C. S., Terada, S., Vacanti, J. P., Honda, M., Bartlett, J. D., & Yelick, P. C. (2002). Tissue Engineering of Complex Tooth Structures on Biodegradable Polymer Scaffolds. *Journal of Dental Research*, *81*(10), 695–700. https://doi.org/10.1177/154405910208101008

Yuan, G., Yang, G., Zheng, Y., Zhu, X., Chen, Z., Zhang, Z., & Chen, Y. (2015). The non-canonical BMP and Wnt/β-catenin signaling pathways orchestrate early tooth development. *Development (Cambridge, England)*, *142*(1), 128–139. https://doi.org/10.1242/dev.117887

Zaim, M., Karaman, S., Cetin, G., & Isik, S. (2012). Donor age and long-term culture affect differentiation and proliferation of human bone marrow mesenchymal stem cells. *Annals of Hematology*, *91*(8), 1175–1186. https://doi.org/10.1007/s00277-012-1438-x

Zhang, J., An, Y., Gao, L.-N., Zhang, Y.-J., Jin, Y., & Chen, F.-M. (2012). The effect of aging on the pluripotential capacity and regenerative potential of human periodontal ligament stem cells. *Biomaterials*, *33*(29), 6974–6986. https://doi.org/10.1016/j.biomaterials.2012.06.032

Zhang, Q., Shi, S., Liu, Y., Uyanne, J., Shi, Y., Shi, S., & Le, A. D. (2009). Mesenchymal Stem Cells Derived from Human Gingiva Are Capable of Immunomodulatory Functions and Ameliorate Inflammation-Related Tissue Destruction in Experimental Colitis1. *The Journal of Immunology*, *183*(12), 7787–7798. https://doi.org/10.4049/jimmunol.0902318

Zhang, W., Abukawa, H., Troulis, M. J., Kaban, L. B., Vacanti, J. P., & Yelick, P. C. (2009). Tissue engineered hybrid tooth–bone constructs. *Methods*, *47*(2), 122–128. https://doi.org/10.1016/j.ymeth.2008.09.004

Zhang, W., Ahluwalia, I. P., Literman, R., Kaplan, D. L., & Yelick, P. C. (2011). Human dental pulp progenitor cell behavior on aqueous and hexafluoroisopropanol based silk scaffolds. *Journal of Biomedical Materials Research Part A*, *97A*(4), 414–422. https://doi.org/10.1002/jbm.a.33062

Zhang, W., Ahluwalia, I. P., & Yelick, P. C. (2010). Three dimensional dental epithelial-mesenchymal constructs of predetermined size and shape for tooth regeneration. *Biomaterials*, *31*(31), 7995–8003. https://doi.org/10.1016/j.biomaterials.2010.07.020

Zhang, W., Vazquez, B., Oreadi, D., & Yelick, P. C. (2017). Decellularized Tooth Bud Scaffolds for Tooth Regeneration. *Journal of Dental Research*, *96*(5), 516–523. https://doi.org/10.1177/0022034516689082

Zhang, W., Vázquez, B., & Yelick, P. C. (2017). Bioengineered post-natal recombinant tooth bud models. *Journal of Tissue Engineering and Regenerative Medicine*, *11*(3), 658–668. https://doi.org/10.1002/term.1962

Zheng, Y., Cai, J., Hutchins, A. P., Jia, L., Liu, P., Yang, D., Chen, S., Ge, L., Pei, D., & Wei, S. (2016). Remission for Loss of Odontogenic Potential in a New Micromilieu In Vitro. *PLoS ONE*, *11*(4), e0152893. https://doi.org/10.1371/journal.pone.0152893

Zhong, L., Li, Y., Xiong, L., Wang, W., Wu, M., Yuan, T., Yang, W., Tian, C., Miao, Z., Wang, T., & Yang, S. (2021). Small molecules in targeted cancer therapy: Advances, challenges, and future perspectives. *Signal Transduction and Targeted Therapy*, *6*(1), 1–48. https://doi.org/10.1038/s41392-021-00572-w

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