Andria da Silva

GENOME EDITING TECHNOLOGY AND REGENERATIVE DENTISTRY – A NARRATIVE REVIEW

Universidade Fernando Pessoa
Faculdade de Ciências de Saúde

Porto, 2022-2023

Andria da Silva

GENOME EDITING TECHNOLOGY AND REGENERATIVE DENTISTRY – A NARRATIVE REVIEW

Universidade Fernando Pessoa
Faculdade de Ciências de Saúde

Porto, 2022-2023

Andria da Silva

GENOME EDITING TECHNOLOGY AND REGENERATIVE DENTISTRY – A NARRATIVE REVIEW

Trabalho apresentado à Universidade Fernando Pessoa
como parte dos requisitos para a obtenção do grau de
Mestre em Medicina Dentária.
Atesto a originalidade do trabalho,

_____

(Andria da Silva)

Porto, 2022-2023

ABSTRACT

The revolutionary prospects of regenerative dentistry and CRISPR genome editing in reshaping oral healthcare practices is such an exciting potential to discover. With a focus on addressing the challenges posed by poor oral hygiene and conventional tooth replacement methods, the research delves into the potential of regenerative dentistry through tissue engineering. Applications of this novel technique are investigated using a few examples from the world of dentistry. In addition, the revolutionary potential of CRISPR gene editing technology in dentistry will be introduced. Through a comprehensive discussion, the implications, challenges, and ethical considerations surrounding the implementation of these cutting-edge technologies are explored. The results highlight the enormous promise of CRISPR technology and regenerative dentistry while highlighting the need for more study and pragmatic issues.

Keywords: CRISPR; Genome editing; Regenerative Dentistry

RESUMO

As perspetivas revolucionárias da medicina dentária regenerativa e da edição do genoma por CRISPR na remodelação das práticas de cuidados de saúde oral são um potencial muito interessante a descobrir. Com o objetivo de enfrentar os desafios colocados pela má higiene oral e pelos métodos convencionais de substituição de dentes, a investigação averigua o potencial da medicina dentária regenerativa através da engenharia de tecidos. As aplicações desta nova técnica são investigadas utilizando alguns exemplos do mundo da medicina dentária. Além disso, será introduzido o potencial revolucionário da tecnologia de edição de genes CRISPR na medicina dentária. Através de uma discussão abrangente, são exploradas as implicações, os desafios e as considerações éticas que rodeiam a implementação destas tecnologias de ponta. Os resultados realçam a enorme promessa da tecnologia CRISPR e da medicina dentária regenerativa, ao mesmo tempo que destacam a necessidade de mais estudos e questões pragmáticas.

Palavras-chave: CRISPR; Edição de genoma; Medicina Dentária Regenerative

AGRADECIMENTOS

ABBREVIATIONS AND ACRONYMS

Acr: Anti-CRISPR proteins
AVV: Adeno-Associated Viruses
BMP: Bone Morphogenetic Protein
Cas: CRISPR Associated
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
DEFB1 gene: Beta-Defensin 1 gene
DFSC: Dental Follicle Stem Cell
DMFT: Decay-Missing-Filled Teeth
DPSC: Dental Pulp Stem Cell
dsDNAs: Double-stranded DNAs
EPS: Polysaccharides
FGF: Fibroblast Growth Factor
gRNA: Guide RNA
Gtfs: Glucotransferases
IAP gene: Inhibitor of Apoptosis gene
IGF2BP1: Insulin-like Growth Factor 2 mRNA-Binding Protein 1
JAK1: Janus Family Kinase 1
ODAM: Odontogenic Ameloblast-Associated Protein
25(OH)D3: 25-Hidroxyvitamin D3
OSCC: Oral Squamous Cell Carcinoma
PAM: Protospacer Adjacent Motif
PDLSC: Periodontal Ligament Stem Cell
PMN: Polymorphonuclear Neutrophil
PTPN2: Protein Tyrosine Phosphatase N2
RE: Regenerative Endodontics
SCAP: Stem Cell from the Apical Papilla
SG: Salivary Gland
sgRNA: Single Guide RNA
SHED: Stem Cell from Human Exfoliated Deciduous teeth
Shh: Sonic Hedgehog

TGFß: Transforming Growth Factor ß

TNF-a: Tumor Necrosis Factor a

VDR: Vitamin D Receptor

TABLE OF CONTENTS

I. Introduction

Poor oral hygiene is an extremely widespread scourge throughout the world, and has been since the dawn of mankind. Its consequences are the proliferation of dental biofilm, leading to the development of dental plaque or caries, which will inflame or cause irreparable loss of the dental organ (Bonnot & Pillon, 2013). The appearance of oral cancerous lesions may also be a result of poor oral hygiene combined with an inadequate lifestyle (Talamini *et al.,* 2000). There are currently many techniques which are available to counteract the loss of the dental organ, such as conventional dentures or implant replacement, each with its own advantages and disadvantages.

Advances in technology and biology have enabled new approaches, such as CRISPR that is based on genome editing. Studies using this strategy have shown an inhibitory role against plaque and caries formation (Gong *et al.,* 2018). Other studies have been used as a treatment against oral cancer (Bhattacharjee *et al.,* 2017). However, beside these approaches, the field of regenerative dentistry is challenging the preconceived notion of tooth replacement by traditional techniques, with developments that may lead to self-healing teeth or biological regenerative therapy for damaged teeth (Xiao & Nasu, 2014). These two examples are an armada of new technologies that will revolutionize dentistry and our overall approach to oral health in the future. This may have an enormous impact on how dentistry and oral hygiene will be practiced in years to come.

The aim of this narrative study is to discuss regenerative dentistry focusing on the role of stem cells and giving examples of this approach. Genome editing technology such as CRISPR will be presented.

1. Materials and Methods

For this narrative review, scientific articles indexed in databases such as PubMed, Medline, Biomed, Google Scholar and Science Direct were used. The chosen keywords used, in multiple combinations, were “oncology”; “regenerative dentistry”; “cell therapy”; “DNA editing” and “genetic therapy”. Papers published in the last 36 years (from 1987 to 2022), in English and Portuguese have been chosen.

The search included narrative and systematic review articles and clinical cases. Publications that did not obey to the goals of the study were excluded.

The bibliographic search allowed the selection of 112 articles that were in accordance with the specified subject and were relevant for the writing of this thesis.

II. Development

1. Regenerative Dentistry

Regenerative dentistry is a subset of regenerative medicine that focuses on oral and dental tissue regeneration (Groll *et al*., 2016). Tissue engineering was first spoken of in pediatric orthopedic research concerning the generation of a new cartilage in the 1970s (Vacanti, 2006). Initially tissue engineering was characterized as an interdisciplinary field that uses engineering and life science principles to build biological substitutes that restore, maintain, or improve tissue function. Presently a newer definition has been offered that combines tissue engineering and tissue science (Groll *et al*., 2016).

Regenerative dentistry allows the application of dental tissue engineering on the three main structures of the tooth: enamel, dentin and pulp. The enamel, is the toughest tissue in the human body and is a highly organized dental tissue, that covers the outer layer of the tooth crown (Bechtle *et al*., 2010). The significant hydroxyapatite concentration present in this tissue, the organization of apatite crystals into enamel prisms, and lastly the aligning of these prisms in a picket-fence form in a tissue of strong physical resistance and tremendous hardness, give distinctive mechanical and structural qualities (Szczes *et al*., 2017).

Enamel forming cells and ameloblasts are specialized epithelial cells that differentiate from the enamel organs inner cells (Mitsiadis & Graf, 2009). These cells use a polarized Golgi apparatus and an endoplasmatic reticulum to create and secrete enamel proteins, as well as influx phosphate and calcium ions into developing enamel matrix (Kirkham *et al*., 2017). Amelogin, ameloblastin, and enamelin are the three primary proteins found in developing teeth that are required for enamel production (Goldberg & Gaucher, 2011). Amelotin and odontogenic ameloblast-associated protein (ODAM), which were discovered in the junctional epithelium and during the maturation stage of amelogenesis, have recently been added to this list (Moffatt *et al*., 2006).

Through the maturation stage of amelogenesis, ameloblasts reabsorb water and breakdown enamel proteins after the enamel matrix has been formed (Klein *et al*., 2017).

These cells eventually suffer apoptosis, making the mature enamel acellular. As a result, unlike other biomineralized hard tissues such as dentin and bone, enamel cannot repair or regenerate on its own once damaged (Moradian-Oldak, 2012).

1.1. Tissue engineering

The conception of tissue engineering was first set in motion by Joseph Vacanti and Robert Langer in the mid-1980's. These scientists were able to make it feasible to regenerate a tissue by growing specific cells on a biocompatible material (Vacanti, 2006).

The purpose of tissue engineering is to create biological alternatives from cells or matrices that can replace, maintain, or enhance the function of human tissues. Regenerative medicine, another term for tissue engineering, has a wide range of intriguing therapeutic possibilities (Freyman *et al*., 2001). The strategy of tissue engineering is to reform or regenerate a tissue or organ as closely as possible by following the physiological development pathways in an extremely concise manner. For this, tissue engineering has three main components: cells, biomaterials such as scaffolds and bioactive molecules (Horst *et al.*, 2012).

1.1.1. Stem Cells

Stem cells are undifferentiated cells found in virtually all multicellular organisms that have the ability to divide and develop into different cell types, and can have as potential sources the embryo or fetus, the umbilical cord or a post-natal source (Weissman, 2000). First mentioned in the late 19th century by various studies (Ramalho-Santos & Willenbring, 2007), these cells are considered an internal repair system because they have the ability to self-renew and produce other stem cells in vivo or in vitro. Stem cells can be used to repair damaged tissues or recreate them to replenish the cell pool and promote healing (cell therapy) (Keller, 2005).

We can distinguish four types of stem cells depending on their differentiation potential:

Totipotent stem cells, found only at the beginning of the embryogenesis, have the highest differentiation potential, being able to originate any type of differentiated cells (Hemmat *et al*., 2010); Pluripotent stem cells, on the other hand, are present at the blastocyst stage

and can produce almost all of an organism's cell types, with the exception of the embryonic appendages (Liu *et al.*, 2020); Multipotent stem cells are already englobed in the process of differentiating pathways, and can produce a variety of differentiated cell types (Sobhani *et al*., 2017); Finally, unipotent stem cells can only produce one type of differentiated cell that continues to have self-renewal capacity common to all stem cells (Slack, 2000).

There are a total of five main sources of stem cells encountered in dental origin:

1- Stem Cells from Human Exfoliated Deciduous teeth (SHED). These are multipotent stem cells, with the capability of differentiating into a number of cell types, such as, adipocytes and odontoblasts. SHEDs were also found to be able to induce bone formation and generate dentin (Miura *et al*., 2003).
2- Stem Cells from the Apical Papilla (SCAP). These are mesenchymal stem cells with the capacity to differentiate into chondrocytes, adipocytes, osteoblasts and odontoblasts. These cells have potential applications towards bone generation, periodontal tissue regeneration and pulp-dentin regeneration (Kang *et al*., 2019).
3- Periodontal Ligament Stem Cells (PDLSC). These include several types of cells that can differentiate into osteoblasts and cementoblasts (Ivanovski *et al*., 2006). Progenitor cells in the periodontal ligament can be stimulated to replenish and regenerate tissues such as cement and alveolar bone (Prateeptongkum *et al*., 2016).
4- Dental Follicle Stem Cells (DFSC). These cells are a source for mesenchymal stem cells, that are believed to be multipotent due to the presence of progenitor cells for periodontal ligament cell, osteoblasts and cementoblasts, resulting in its capacity to produce periodontal ligament, bone and cementum (Honda *et al*., 2010).
5- Dental Pulp Stem Cells (DPSC). These cells have the potential to differentiate into several cell types (Casagrande *et al*., 2011). DPSCs contain mesenchymal stem cells that can develop and generate new dentin to repair damage (Dimitrova-Nakov *et al*., 2014).

1.1.2. Scaffolds

Scaffolds are three-dimensional 3D patterns made of natural or synthetic biomaterials that are required to support all the steps allowing the generation of desired

tissues. These steps include cells adhesion, proliferation, differentiation and delivery of bioactive molecules (Chevallay & Herbage, 2000).

The scaffolds also mimic the extracellular matrix acting as a barrier, a reserve of nutrients, oxygen and molecules. They act as a temporary structure that gradually degrades over time, being replaced by an extracellular matrix and the formed tissue (Badylak, 2002).

Scaffolds must be biocompatible and biodegradable, in order to promote and facilitate the development of new tissues and organs without compromising their integrity (Asghari *et al*., 2017).

There are two major types of scaffolds used in tissue engineering: biological polymer scaffolds, which have good biocompatibility but are not very malleable (Galler & D'Souza, 2011); and synthetic polymer scaffolds which are easily controllable but, have biocompatibility gaps (Place *et al*., 2009).

1.1.3. Bioactive Molecules

Bioactive molecules, also known as signaling molecules can result from several sources, including natural sources and synthetic sources. These last ones are created to copy the activity of natural molecules. These sources include proteins, growth factors, transcription factors and genes. When selecting bioactive molecules, it is vital to know their properties, as some of them, such as growth factors, are quickly excreted by the organism (Kačarević *et al*., 2019).

The different biological stages of cell division or molecular differentiation of the cell can result from the activation of a set of growth factors that participate in a cascade of signaling pathways, intervening by playing a stimulating role on the different biological constituents, with a variable specificity according to the influenced cells or tissues (Couly *et al.*, 2015).

Transforming Growth Factor ß (TGFß) molecules represent a group of growth factors that are active in tooth development. Their signaling pathway is critical for cell proliferation and differentiation in dental pulp formation (Yu Bai *et al.*, 2022).

Bone Morphogenetic Proteins (BMPs) are signaling molecules that belong to the TGFß superfamily. These are important molecules in cell signaling for odontoblast (and ameloblast) differentiation and stimulation of reactive dentin formation (Nakashima, 2005) and have the ability to induce bone formation (Lademann *et al.*, 2020).

Fibroblast Growth Factors (FGFs) make up a large family of 24 growth factors, that play a role in embryonic development by affecting cellular functions such as proliferation, differentiation, division or migration and adhesion (Goldberg & Gaucher, 2011).

1.2. Application of Regenerative Dentistry

Regenerative Dentistry is an emerging field that offers promising treatment options for numerous cases in dentistry with the primary goal to repair, restore and regenerate dental tissues to its initial form (Sharpe, 2020). In regenerative dentistry, many techniques are being used in areas regarding endodontics and periodontics, which will be presented below.

1.2.1. Regenerative Endodontics

Regenerative Endodontics (RE) was first introduced by Ostby in the early 1960's, when he concluded that the pulp had the ability to recover itself when aided by the presence of a blood clot within the root canal (Dash *et al*., 2020). The pulp-dentin complex should be mentioned when discussing regenerative endodontics in order to emphasize that because of their specific interdependence, they should be thought of as a single entity (Kim *et al*., 2018). A traditional procedure of RE, known as apexification, has the aim of treating immature permanent teeth with necrotic pulp/apical periodontitis, by utilizing calcium hydroxide or mineral trioxide aggregate with the goal of apical closure, but with no possibility to revive injured tissue (Chala *et al*., 2011).

An alternative treatment to apexification was acknowledged in the early 2000's, and it is known as revascularization or revitalization. This treatment option has the advantage of thickening the walls of the root and increasing its length instead of only apical closure plus reducing the probability of fracturing the tooth (Wigler *et al.,* 2013).

One of the most recent procedures in regenerative endodontics is the combined use of DPSC and a silk fibroin scaffold with a specific FGF (b-FGF), resulting in the formation of a pulp-like tissue. The b-FGF is a growth factor and signaling molecule encoded by the *FGF-2* gene (Yang *et al*., 2015). This growth factor and signaling molecule plays a critical role in cell proliferation, angiogenesis (formation of new blood

vessels), and tissue regeneration. By incorporating b-FGF into the silk fibroin scaffold, it can stimulate the growth and differentiation of DPSCs, enhancing the regenerative potential of the treatment (Nakashima *et al.*, 2003).

The combined approach of DPSCs and a silk fibroin scaffold with b-FGF has shown promising results in the formation of pulp-like tissue. DPSCs are a type of stem cells found in dental pulp, that have the ability to differentiate into various cell types, including odontoblasts, which are responsible for dentin formation. The silk fibroin scaffold provides a three-dimensional structure that mimics the extracellular matrix, creating a suitable environment for cell attachment, proliferation, and differentiation. The scaffold offers mechanical support and promotes the organization of cells into tissue-like structures (Wang *et al.*, 2010).

1.2.2. Regeneration of the Alveolar Bone

At the craniomaxillofacial level, there is an important balance resulting from the contribution of different elements composing the oral zone, the teeth, the soft and hard support tissues. This sensitive balance is disturbed when using a bone or non-bone implant structure, which will change the rigidity of the bone structure, resulting in irreparable long-term resorption of the alveolar ridge (Hansson & Halldin, 2012). Therefore, the goal of regenerative therapy must be to achieve both morphological and functional tissue replacement.

The alveolar bone regeneration procedure involves the placement of autogenous bone, which has the advantage of providing osteogenic cells (osteoblasts and osteoclasts), osteoinductive growth factors and osteoconductive scaffold. Nowadays, this is the gold standard procedure for bone reconstruction (Chiarello *et al.*, 2013).

Moreover, injection of bone marrow mesenchymal stem cells into the bone regeneration site promotes faster bone formation, especially bone marrow derived CD90+ and CD14+ cells (Kaigler *et al.*, 2013). These stem cells act in bone regeneration through multiple interactions between several signaling molecules such as BMPs, FGF or Sonic Hedgehog (Shh) (Ma *et al.*, 2013).

Regenerative dentistry finds its place entirely in alveolar bone regeneration, involving different mechanisms of tissue engineering, where stem cells are activated in order to obtain an optimal result both functionally and morphologically.

Innovative methods to boost alveolar bone repair have recently been presented thanks to developments in regenerative dentistry. One of these methods uses tissue engineering techniques to mix biomaterials, growth factors, and stem cells to produce an environment that is favorable for bone repair. Scaffold materials may be created to mimic the natural architecture of bone and offer a framework for cell attachment and proliferation. Examples of such materials include biocompatible polymers (Xiao *et al.*, 2003).

1.2.3. Regenerative Approach in Salivary Gland Disfunction

Regenerative approach in salivary gland disfunction is based on the loss of salivary gland (SG) function. This can occur due to various reasons such as, radiation therapy which the SG are very sensitive to, or xerostomia that might be a side effect induced by certain medications or also caused by an autoimmune pathology such as Gougerot-Sjögren's Syndrome (Qin *et al.*, 2014).

Many methods have come to be utilized in SG regeneration research, with promising developments in this area of research. For example, salisphere derived c-Kit (+) cells injected into irradiated submandibular glands of a mouse model, demonstrated a remarkable regeneration of the SG, restoring tissue homeostasis and rescuing hypo-salivation (Nanduri *et al*., 2013).

Knowing that the embryological origin of SG is epithelial and mesenchymal, another example would be the use of DPSC as a mesenchyme inducer for SG regeneration (Janebodin & Reyes, 2012).

Effective reconstitution of the SG has also been announced via transplantation of a bioengineered SG germ (Ogawa & Tsuji, 2015).

According to Ogawa & Tsuji, the transplantation of a bioengineered salivary gland germ has the potential to successfully reconstitute the salivary gland. This method entails the transplanting of SG germ-like structures, that have been artificially produced and have the capacity to differentiate and mature into functioning SG. The goal could be the SG function restoration or treating symptoms associated with dysfunctional SG by using the regenerative capacity of bioengineering structures (Ogawa & Tsuji, 2015).

1.3. CRISPR Genome Editing

1.3.1. Mechanism

CRISPR for Clustered Regularly Interspaced Short Palindromic Repeats was discovered by Japanese scientists in 1987 in a region of the *IAP* (inhibitor of apoptosis) gene (Ishino *et al*., 1987).

Jansen and his team discovered that the CRISPR sequences were always accompanied by genes families that would be found only if there were CRISPR sequences nearby. Those genes would come to be named Cas for CRISPR Associated. The proximity of Cas sequences with CRISPR sequences led to believe that there is a link between the two. These two regions would constitute the CRISPR-Cas system (Jansen *et al*., 2002).

Subsequently, Mojica *et al.* (2005) showed that CRISPR spacers are derived from chromosomal or genetic elements from bacteriophages or plasmids. These researchers observed that these extrachromosomal elements are not able to infect strains carrying these spacers, suggesting a relationship between CRISPR and immunity against targeted DNA (Mojica *et al*., 2005).

CRISPR sequences provide bacterial adaptive immunity to viruses and plasmids. In fact, some bacteria, such as *Streptococcus pyogenes*, under the impulse of CRISPR sequences, detect foreign viral DNA and are able to cut it. Viral DNA sequences are integrated so that the bacteria can recognize it in the future and destroy it. Thanks to this mechanism, bacteria have an adaptive immunity against viral DNA (Heler *et al*., 2015). There are three adaptive immunity systems. The mechanisms of type I and III are very complex and are not used for genome editing. The mechanism of adaptive immunity type II is the simplest and involves the presence of Cas9 protein, CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA). This last one is involved in crRNA maturation and in the formation of the Cas9 complex (Hryhorowicz *et al*., 2016).

The type II system consists of 3 steps:

- The first step is acquisition, where the Cas1 and Cas2 proteins recognize the viral DNA, cut out a part of it and integrate it into the CRISPR network.
- The second step is biogenesis, where the CRISPR network, used as a template, generates a non-coding crRNA (CRISPR RNA) that will facilitate the recognition of the viral DNA. Once the crRNA becomes coding, it forms a protein.

- Finally, in the last step: interference, after the crRNA activates Cas9, the CRISPR network cleaves the viral DNA and can destroy it. The crRNA guides the CRISPR Cas9 protein complex through a trans-activating crRNA, which is essential to activate its maturation (Hryhorowicz *et al*., 2016).

The Cas9 protein associated with CRISPR is an endonuclease. This enzyme uses a guide sequence in a duplex RNA molecule (tracrRNA: crRNA) to anneal with target DNA sequences. In this way, Cas9 will break the DNA molecule in a specific site. The tracrRNA: crRNA, called single guide RNA (sgRNA) has two important characteristics: at the 5' end it has a sequence responsible for determining the target site by complementary base pairing; at the 3' end it has a duplex RNA structure that attaches to Cas9 (Doudna & Charpentier, 2014). When Cas9 binds to the sgRNA, the cleavage of other non-targeted DNA is prevented (Doudna & Charpentier, 2014).

The Protospacer Adjacent Motif (PAM) sequence is a 2 to 6 base-pair DNA sequence adjacent to the DNA sequence targeted by the Cas9 nuclease. PAM is another mechanism that makes up the guidance of the CRISPR-Cas9 complex. For the viral DNA to be correctly recognized, it is crucial that Cas9 recognizes the precise PAM sequence in order to attach to the DNA and cleave it. In addition, PAM protects the bacteria by preventing Cas9 from binding to the several sequences of the CRISPR array, thus preventing Cas9 from cutting its own DNA (Doudna & Charpentier, 2014).

The Cas9 endonuclease has six functional domains which are responsible for breaking the phosphodiesters bonds in the DNA backbone, being a key component of the CRISPR-Cas9 system. Among the 6 domains composing the Cas9 endonuclease is the bridge helix that makes up a conformational change of the DNA recognized by the sgRNA. This nuclease also contains Rec I, HNH and RuvC nuclease, involved in the cleavage of the DNA. Thus, Rec I, HNH and RuvC move into position: Rec I manages the recognition and binding of the crRNA complex, leaving room for HNH which cuts the complementary DNA strand and RuvC which cuts the other DNA strand, thus giving a double stranded DNA cut (Hryhorowicz *et al*., 2016). The double-strand breaks are introduced 3 basepairs upstream from the PAM sequence (Szczelkun *et al*., 2014). The PAM complex is used in the interference stage, to identify the PAM sequence, allowing to distinguish between the invading DNA and the host genome that does not contain PAM (Hryhorowicz *et al*., 2016).

In short, the CRISPR system is a remarkable tool in bacterial immunity, creating a memory of the target DNA, integrating it into its own network, consisting of alternating

spacers and palindromic regions, with help the binding of the Cas9 complex and the sgRNA. Thus, the target DNA can be recognized and destroyed by the Cas9 system (Charpentier & Marraffini, 2014).

In fact, this simple two-component system can be used to target any DNA sequence by changing the guide sequence of the sgRNA. In this way, it is possible to use this system to target, edit, modify, regulate and mark specifically and efficiently genomic loci of diverse cells and organisms (Doudna & Charpentier, 2014).

1.3.2. Applications

A completely new world of therapeutical approaches is made possible by CRISPR technology. Orofacial clefts, tooth agenesis, head and neck cancer, orofacial pain, temporomandibular disorders, and facial shape are only a few of the oral and craniofacial conditions that have been linked to specific genes in the human genome (Chavez-Granados *et al*., 2022).

As previously referred, CRISPR offers the possibility of precise genome editing, which could lead to innovating therapeutic options for dental diseases as well as the opportunity to comprehend the genes that are responsible for these conditions, and at the same time offer an individualized treatment approach. This meaning, taking into consideration the patients genotype, environmental factors and other elements that may predispose or augment their predisposition to a specific condition (Barbour *et al*., 2021).

The following examples are some of the different applications of CRISPR in the dentistry field to date.

<u>Dental caries</u>

Streptococcus mutans is an important element of the human oral microbiota and is the main bacterial cause of dental caries. This bacterial strain produces extracellular polysaccharides that supply places for bacterial adherence and expansion, which then eventually produce biofilm (Mosterd & Moineau, 2020). Knowing that the two main virulence factors in *S. mutans* are Gtfs and polysaccharides (EPS), in a study of Gong *et al*. (2018), glucotransferases (Gtfs) were specifically targeted with CRISPR in an attempt to prevent the growth of biofilm. This study showed that for the standard operation of *S. mutans* CRISPR-Cas9 system, crucial elements were needed, such as a suitable PAM site,

tracrRNA, Cas9 and finally RNase III. Self-targeting CRISPR arrays were designed and later cloned into plasmids. Subsequently, the plasmids and editing templates were transformed into self-targeting to obtain the necessary mutants. The technology demonstrated to be capable of accomplishing the editing of specific genes, namely the *Gtfs* gene and with this the reduction in EPS synthesis and consequently the decrease in biofilm formation (Gong *et al*., 2018).

In addition to the use of CRISPR technology in understanding the main bacterial cause of dental caries, CRISPR can also be used to edit the genome of patients having a high predisposition for the development of dental caries. Knowing that genetics plays a major role in the sensibility to dental caries of each individual, the study of Ozturk *et al*. (2010) states that the G-20A variant of the beta-defensin 1 (*DEFB1*) gene is linked to a significant probability of developing dental caries, resulting in a high decay-missing-filled teeth index (DMFT) score, while carrying the (G-52A) variant allele for the *DEFB1* marker is related to lower DMFT score, thus concluding that both variants could be related to a different caries experience, meaning that these variants possess the potential to become clinical markers to identify caries risk in humans (Ozturk *et al*., 2010).

Periodontal disease

Polymorphonuclear neutrophils (PMNs) are cells from the innate immune system, which provide defense against bacteria present in the biofilm. In this study it was shown that diabetes mellitus is a direct risk factor for periodontal disease. These two conditions are strongly connected to the malfunction of inflammatory mediators. Knowing that 25-hidroxyvitamin D3 (25(OH)D3) plays a crucial role in inflammatory modulation, it was demonstrated that (25(OH)D3) improved experimental periodontitis in diabetic mice, an intraperitoneal administration of 25(OH)D3 into the diabetic mice also lowered fasting glucose and serum tumor necrosis factor type a (TNF-a) levels, and consequently leading to reduced alveolar bone loss. When analyzing the gingival epithelia, it was observed that with the application of (25(OH)D3), vitamin D receptor (VDR) and protein tyrosine phosphatase N2 (PTPN2) were increased, knowing that the lack of vitamin D could lead to an amplified inflammatory status and that the PTPN2 plays a role in the attenuation of diabetes. On the other hand, the expression of NF-jB and the phosphorylation of Janus family kinase 1 (JAK1), both being related to inflammatory mediators, decreased. The results of this study allowed to explain the therapeutic advantages and anti-inflammatory

properties of (25(OH)D3), leading to major impacts on the clinical management of diabetic periodontitis (Li *et al*., 2013).

In a second study on inflammatory response, CRISPR-Cas9 technology was utilized as a research tool to identify cellular pathways involved in periodontitis, constructing two cell lines of THP-1 lineage through lentiviral vectors, enabling them to examine the deletion of insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1). It was found that IGF2BP1 blocks LPS-induced manufacturing of pro-inflammatory cytokines. However, when this gene is upregulated contributes to LPS-induced pro-inflammatory cytokines, and when repressing IGF2BP1 minimizes inflammatory responses. These findings allowed to conclude that IGF2BP1 has a crucial responsibility in inflammatory responses (Xie *et al*., 2019).

Furthermore, over the years, CRISPR parallel genome editing systems have been under development. This system contains higher specificity in the control of gene expression and the power of not altering the DNA sequence. Among the new systems are CRISPRa, CRISPRi or Cas13 (Barbour *et al*., 2021).

Oral cancer

Oral Squamous Cell Carcinoma (OSCC) is the most prevalent mouth cancer. Studies on CRISPR-Cas9 brought to light the association of 918 genes with the cancer cell's potential to survive (Chai *et al*., 2020). At the University of Pennsylvania, in the year of 2019, an introductory CRISPR-made cancer treatment trial was set in motion, involving four genetic alterations to T cells. These cells are part of the immune system and aid in the elimination of tumor cells. CRISPR was utilized to build on a synthetic gene, allowing the T-cell to acknowledge a certain molecule on several cancer cells, and then withdraw three genes that suppressed the T-cell from deleting cancerous cells. In the beginning stages of the treatment trial, it was shown to be effective on three of the four individuals but over the course of time became unsuccessful. Although the treatment did not reach its full potential, it is wonderful to know that there is great potential for a CRISPR technique that in a near future will be successful (Stadtmauer *et al*., 2020).

1.3.3. Limitations

CRISPR as it exists today, has its limitations and risks, making it very difficult to use in clinical trials, mainly in humans.

Crucial concerns are presented below.

CRISPR/Cas9 delivery challenges

The selection of a safe delivery technique is fundamental to execute the CRISPR system. Distinct methods are used to deploy CRISPR/Cas9 technologies, including physical, viral and extracellular vesicle-based system delivery techniques (Yip, 2020).

The most difficult challenges when selecting the correct vector are related to packaging, delivery and correct site targeting (Mout *et al.*, 2017). Adeno-associated viruses (AAV), a viral delivery vector has the handicap of possessing a very small packaging size. In this way, multiple AAVs are required to execute the CRISPR systems (Martínez-Lage *et al*., 2018). One of most efficient approaches towards this limitation is the division of the Cas9 protein into two adeno-associated viruses (AAV-split-Cas9) vectors rather than merely one (Chew *et al*., 2016).

CRISPR/Cas9 ethical issues

Human genome editing has always been a topic of ethical discussion. The evolution of CRISPR-Cas9 technology has been great, and the ability to turn genes on and off, the possibility of understanding how these genes function or how certain mutations in genes alter cell function, are only a few of the unfolded advancements (Lino *et al*., 2018).

Another controversial approach would be the alteration of the human DNA to eradicate anomalies responsible for human diseases (Shinwari *et al*., 2018).

The appealing applications of these advancements arises moral, ethical and security measures (Bailey, 2019). The ethical questions result from three main reasons: its limitations (Peng *et al*., 2016), inadequate editing (Tu *et al*., 2017) and finally faulty on-target or off-target editing (Zischewski *et al*., 2017).

Thus, the ambiguity brought forth by these limitations make risk/benefit judgement very complicated, resulting in moral decision making more challenging (Brokowski & Adli, 2018).

A positive aspect of utilizing appropriately CRISPR technology is that it has the power to significantly impact health wise but not forgetting that the effectiveness of this technique will always depend on its application (Howard *et al*., 2018).

Off-targeting

Another limitation of the CRISPR/Cas9 system is the huge amount of off-targeting, which consequently leads guide RNA (gRNA) to an incorrect target, due to the resemblances within the genome (Kang *et al*., 2020).

Strategies to overcome this limitation have been divided into three groups. The first one is the use of bioinformatic tools to design precise gRNA and anticipate off-targeting by determining the accurate editing site (Barman *et al*., 2020). The second one is the use of Cas9 nickases, that have a distinct cutting mechanism than the standard Cas9 protein. These nickases only cut the phosphodiester bond in a unique strand of the DNA, using double adjacent gRNAs instead of sgRNAs, reduces additional damage in the target DNA and the amount of off-targeting (Shen *et al*., 2014). Finally, the use of anti-CRISPR proteins (Acr) which play a major role in the deactivation of the Cas9 protein after targeting a location, subsequently limiting the amount of off-targeting (Pawluk *et al*., 2016).

Gene polymorphism in cancer

Cancer results from the occurrence of various mutations that lead to dysregulation of numerous genes such as tumor suppressor genes (Baugh *et al*., 2018). Correcting these mutations of tumor suppressor genes via knocking-in is more precise than knocking out (Miura *et al*., 2018), which requires a bigger amount of gRNA, leading to a higher possibility of off-targeting. Due to factors such as the following, being one of the principal reasons related to the homology-directed repair (HDR) efficiency utilizing double-stranded DNAs (dsDNAs) is overall unsatisfactory. The improper design and construction of the donor template, the specificity of genome editing tools and finally the complexity of the genome itself (Miura et al., 2018).

In order to successfully knockout oncogenes, the gRNA has to be created to target the oncogenes binding site and avoid protein-protein communication (Yang *et al*., 2019). There are two strategies for overcoming polymorphism. The first one is performing Cas hybrid for multiplexed editing and screening applications (CHyMErA) method. This method possesses the ability of targeting numerous sites in a sole mammalian cell (Gonatopoulos-Pournatzis *et al*., 2020). The second one involves the detection of a protein interaction site. When intending to anticipate and achieve the intended knockout effects, bioinformatic tools can be quite useful. For instance, several databases can be applied to identify interaction sites of proteins, and with the usage of these platforms a gRNA can be created based on the amino acids encountered in the proteins active site (Johansson-Åkhe *et al*., 2019).

Autoimmune response against endogenous Cas9 protein

One of the main components of the CRISPR system is the Cas9 protein, which is crucial for binding double-stranded DNA and cutting it at an exact place (Makarova & Koonin, 2015). The Cas9 protein derives from *Streptococcus pyogenes* which is a microorganism that is responsible for several recurrent diseases in the human being. As soon as the protein is recognized by the immune system as unfamiliar it immediately triggers an immune response (Colque-Navarro *et al*., 2010). The activation of the immune response leads to a rapid deterioration of the Cas9 protein, making it impossible to carry out its gene-editing role (Crudele & Chamberlain, 2018).

There are also strategies to overcome immunogenicity. Knowing that numerous disorders can be identified before birth, one of such strategies is enforcing the CRISPR/Cas9 system for gene editing in an early lifetime. Another one is targeting immune-privileged organs. This means using a location where a transplanted tissue will not be rejected by the body. Examples of a privileged organ could be the placenta or fetus (Kanellopoulos-Langevin *et al*., 2003).

III. Discussion

Regenerative dentistry uses biological principles and methods to restore, maintain or improve damaged or lost dental organs. In terms of function, durability and aesthetics, traditional dentistry is showing limitations. With the current state of knowledge, new opportunities are opening up to regenerate the dental organ (Amrollahi *et al.*, 2016).

A promising strategy mentioned above is the use of stem cells in regenerative dentistry. Studies have shown very interesting results in using stem cells for dental pulp regeneration, periodontal tissue regeneration, and even tooth regeneration. But, as any other technologies, they present limits such as the stem cells conservation or ethical provenances (Zhai *et al.*, 2019).

Another emerging technology that has the potential to revolutionize dentistry is CRISPR-Cas9, which could potentially be used to treat genetic diseases or improve the properties of dental tissue (Chavez-Granados *et al*, 2022). In the last decade, more than a thousand papers have been published with CRISPR included in their title or abstract, but the quality of the results is difficult to analyze due to the difference on used experimental methods and their success rates. However, there is evidence of the popularity of this technology, leaving hope for ultra-fast and efficient development (Ma *et al.*, 2014).

CRISPR-Cas9 has the potential to revolutionize oral healthcare, holding significant importance in prevention: in fact, it could target precisely the apparition of dental caries or plaque formation, two major contributors to oral disease (Gong *et al*., 2018). This technology could demonstrate a more effective action than alternative pharmacological treatment such as iodopovidone, by example, used in the pediatric dentistry (Milgrom *et al.*, 2020). In addition, CRISPR-Cas9 being a potential for regenerative dentistry, aiding in the regeneration of damaged tissues.

Not forgetting to mention that oral cancer also occupies a rather important place in oral health care, presenting an opportunity for precise genome editing of specific genes responsible of the tumor (Yu *et al*., 2018).

As the nanotechnologist Paige Johnson says: “If you think of genetic modification today as slicing the genome with a scalpel, in the 1960s they were hitting it with a hammer” (Johnson, 2011).

Although the existence of a solution to CRISPR-Cas9 technical or ethical limitations, it should be always kept in mind the importance and necessity for further

research to be able to fully understand the safety, efficacy and mainly its long-term effects in the field of oral healthcare (Garhnayak *et al*., 2023).

IV. Conclusion

New opportunities are opening up in the field of dentistry through the understanding and development of CRISPR to optimize and improve oral treatment, potentially revolutionizing the art of dentistry. Advances in stem cell research and tissue engineering are paving the way for biological regeneration of dental tissue. Despite the challenges and discoveries that lie ahead, it is clear that these technologies have tremendous potential to transform the practice of dentistry and contribute to better patient outcomes.

On the other hand, there are countless challenges and ethical considerations that must be addressed. Indeed, the safety and effectiveness of these technologies need to be carefully evaluated through preclinical and clinical studies. Accessibility and affordability also need to be ensured.

Nevertheless, these challenges do not hide the bright future of CRISPR technology and regenerative dentistry. By regenerating dental tissue, and precisely and concisely modifying the genome, we can revolutionize the direction of medical and dental care.

Further research is needed to ensure that dentistry will one day focus exclusively on a regenerative approach and that therapeutics will evolve while addressing ethical and practical concerns.

V. Bibliography

Amrollahi P;, *et al*. (2016). Recent advancements in regenerative dentistry: a review. *Materials Science and Engineering*, 69, pp. 1383-1390.

Asghari F., *et al.* (2017). Biodegradable and biocompatible polymers for tissue engineering application: a review. *Artificial cells, Nanomedicine and Biotechnology*, 45(2), pp. 185-192.

Badylak S. F. (2002). The extracellular matrix as a scaffold for tissue reconstruction. *Seminar in Cell & Developmental Biology*, 13(5), pp. 377-383.

Bailey J. (2019). CRISPR-mediated gene editing: scientific and ethical issues. *Trends in Biotechnology*, 37(9), pp. 920-921.

Barbour A., *et al*. (2021). The role of CRISPR-Cas in advancing precision periodontics. *Journal of Periodontal Research*, 56(3), pp. 454-461.

Barman A., *et al*. (2020). A glance at genome editing with CRIPSR-Cas9 technology. *Current Genetics*, 66(3), pp. 447-462.

Baugh E. H., *et al*. (2018). Why are there hotspot mutations in the *TP53* gene in human cancers? *Cell Death and Differentiation*, 25, pp. 154-160.

Bechtle S., *et al.* (2010). The fracture behaviour of dental enamel. *Biomaterials*, 31(2), pp. 375-384.

Bhattacharjee R., *et al*. (2017). CRISPR/Cas9 genome editing system in the diagnosis and treatment of cancer. *Journal of RNAi and Gene Silencing*, 13, pp. 585-591.

Bonnot J. & Pillon F. (2013). Une bonne hygiène bucco-dentaire pour prévenir les caries. *Actualités Pharmaceutiques*, 52, pp. 39-44.

Brokowski C. & Adli M. (2019). CRISPR ethics: Moral considerations for application of a powerful tool. *Journal of Molecular Biology*, 431(1), pp. 88-101.

Casagrande L., *et al*. (2011). Dental pulp stem cells in regenerative dentistry. *Odontology*, 99(1), pp. 1-7.

Chai A., *et al.* (2020). Genome-wide CRISPR screens of oral squamous cell carcinoma reveals fitness genes in the Hippo pathway. *eLife*, 9(1), pp. 1-34.

Chala S., *et al*. (2011). Apexification of immature teeth with calcium hydroxide or mineral trioxide aggregate: systematic review and meta-analysis. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, 112(4), pp. 36-42.

Charpentier E. & Marraffini L. A. (2014). Harnessing CRISPR-Cas9 immunity for genetic engineering. *Current Opinion in Microbiology*, 19(1), pp. 114-119.

Chavez-Granados P. A., *et al*. (2022). CRISPR/Cas gene-editing technology and its advances in dentistry. *Biochimie*, 194(1), pp. 96-107.

Chevallay B. & Herbage D. (2000). Collagen-based biomaterials as 3D scaffold for cell cultures: application for tissue engineering and gene therapy. *Medical & Biological Engineering & Computing*, 38(2), pp. 211-218.

Chew W. L., *et al*. (2016). A multifunctional AAV-CRISPR-Cas9 and its host response. *Nature Methods*, 13(10), pp. 868-874.

Chiarello E., *et al*. (2013). Autograft, allograft and bone substitutes in reconstructive orthopedic surgery. *Aging Clinical and Experimental Research*, 25(1), pp. 101-103.

Colque-Navarro P., *et al.* (2010). Levels of Antibody against 11 *Staphylococcus aureus* Antigens in a Healthy Population. *Clinical and Vaccine Immunology*, 17(7), pp. 1117-1123.

Couly G., *et al*. (2015). Embryologie et chirurgie embryologique des six fentes orales. *EMC Chirurgie Orale et Maxillo-Faciale*, 10(2), pp. 1-22.

Crudele J. M. & Chamberlain J. S. (2018). Cas9 immunity creates challenges for CRISPR gene editing therapies. *Nature Communications*, 9(1), pp. 3497.

Dash S., *et al*. (2020). Regenerative endodontics treatment in maxillary central incisor: a 6-month case report. *Indian Journal of Forensic Medicine & Toxicology*, 14(4), pp. 8376-8380.

Dimitrova-Nakov S., *et al*. (2014). Pulp stem cells: implication in reparative dentin formation. *Journal of Endodontics*, 40(4), pp. 13-18.

Doudna J. A. & Charpentier E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science (New York, N.Y.)*, 346(6213), pp. 1258096.

Freyman T., *et al*. (2001). Cellular materials as porous scaffolds for tissue engineering. *Progress in Materials Science*, 46(3-4), pp. 273-282.

Galler K. M. & D'Souza R. N. (2011). Tissue engineering approaches for regenerative dentistry. *Regenerative Medicine*, 6(1), pp. 111-124.

Goldberg M. & Gaucher C. (2011). Embryologie de la dent. *EMC Médecine Buccale*, 28-085-U-50, pp. 1-22.

Garhnayak M., *et al.* (2023). CRISPR in dentistry: a boon or bane!. *Microbes and Infectious Diseases*, 10-21608, pp. 1-5

Gonatopoulos-Pournatzis T., *et al*. (2020). Genetic interaction mapping and exon-resolution functional genomics with a hybrid Cas9-Cas12a platform. *Nature Biotechnology*, 38(5), pp. 638-648.

Gong T., *et al.* (2018). Genome editing in Streptococcus mutans through self-targeting CRISPR arrays. *Molecular Oral Microbiology*, 33(6), pp. 440-449.

Groll J., *et al*. (2016). Biofabrication: reappraising the definition of an evolving field. *Biofabrication*, 8(1), pp. 1-5.

Hansson S. & Halldin A. (2012). Alveolar ridge resorption after tooth extraction: A consequence of a fundamental principle of bone physiology. *Journal of Dental Biomechanics*, 3, pp. 1-8.

Heler R., *et al*. (2015). Cas9 specifies functional viral targets during CRISPR-Cas adaptation. *Nature*, 519(7542), pp. 199-202.

Hemmat S., *et al*. (2010). An introduction to stem cell biology. *Facial Plastic Surgery: FPS*, 26(5), pp. 343-349.

Honda M. J., *et al*. (2010). Dental follicle stem cells and tissue engineering. *Journal of Oral Science*, 52(4), pp. 541-552.

Horst O. V., *et al*. (2012). Stem cell and biomaterials research in dental tissue engineering and regeneration. *Dental Clinics of North America*, 56(3), pp. 495-520.

Howard H. C., *et al*. (2018). One small edit for humans, one giant edit for humankind? Points and questions to consider for a responsible way forward for gene editing in humans. *European Journal of Human Genetics*, 26(1), pp. 1-11.

Hryhorowicz M., *et al*. (2017). CRISPR/Cas9 Immune system as a tool for genome engineering. *Archivum Immunologiae et Therapiae Experimentalis*, 65(3), pp. 233-240.

Ishino Y., *et al*. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *Journal of Bacteriology*, 169(12), pp. 5429-5433.

Ivanovski S., *et al*. (2006). Stem cells in the periodontal ligament. *Oral Disease*, 12, pp. 358-363.

Janebodin K. & Reyes M. (2012). Neural crest-derived dental pulp stem cells function as ectomesenchyme to support salivary gland tissue formation. *Dentistry*, 13, pp. 2161-2169.

Jangam D. K. & Talreja K. M. (2020). Application of CRISPR technology in dentistry: a review. *European Journal of Biomedical and Pharmaceutical Sciences*, 7(6), pp. 314-320.

Jansen R., *et al*. (2002). Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular Microbiology*, 43(6), pp. 1565-1575.

Johansson-Åkhe I., *et al*. (2019). Predicting protein-peptide interaction sites using distant protein complexes as structural templates. *Scientific Reports*, 9(1), pp. 4267.

Johnson P. (2011). Atomic gardening: Day of the irradiated peanuts. *RadSafe Web Archive*, http://health.phys.iit.edu/archives/2011-June/032767.html .

Kačarević Ž. P., *et al*. (2020). An introduction to bone tissue engineering. *The International Journal of Artificial Organs*, 43(2), pp. 69-86.

Kaigler D., *et al*. (2013). Stem cell therapy for craniofacial bone regeneration: a randomized, controlled feasibility trial. *Cell Transplantation*, 22(5), pp. 767-777.

Kanellopoulos-Langevin C., *et al*. (2003). Tolerance of the fetus by the maternal immune system: role of inflammatory mediators at the feto-maternal interface. *Reproductive Biology and Endocrinology*, 1, pp. 121.

Kang J., *et al*. (2019). Stem cells from the apical papilla: a promising source for stem cell-based therapy. *BioMed Research International*, 6104738, pp. 1-8.

Kang S., *et al*. (2020). Prediction-based highly sensitive CRISPR off-target validation using target-specific DNA enrichment. *Nature Communications*, 11(1), pp. 3596.

Kawashima N. (2012). Characterization of dental pulp stem cells: a new horizon for tissue regeneration? *Archives of Oral Biology*, 57(11), pp. 1439-1458.

Keller G. (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes & Development*, 19(10), pp. 1129-1155.

Kirkham J., *et al*. (2017). Enamel research: priorities and future directions. *Frontiers in Physiology*, 8, pp. 513.

Klein O. D., *et al*. (2017). Meeting report: a hard look at the state of enamel research. *International Journal of Oral Science*, 9(11), pp. 1-7.

Kim S. G., *et al*. (2018). Regenerative endodontics: a comprehensive review. *International Endodontic Journal*, 51(12), pp. 1367-1388.

Lademann F., et al. (2020). The Bone Morphogenetic Protein Pathway: The Osteoclastic Perspective. Frontiers in Cell and Developmental Biology, 8.

Langer R. & Vacanti P. J. (1993). Tissue Engineering. *Science*, 260, pp. 920-926.

Lentsch E., *et al*. (2019). CRISPR/Cas9-mediated knock-out of $Kras^{G12D}$ mutated pancreatic cancer cell lines. *International Journal of Molecular Sciences*, 20(22), pp. 5706.

Li H., *et al*. (2013). 25-hydroxyvitamin D3 ameliorates periodontitis by modulating the expression of inflammation-associated factors in diabetic mice. *Steroids*, 78(2), pp. 115-120.

Lino A. C., *et al*. (2018). Delivering CRISPR: a review of the challenges and approaches. *Drug Delivery*, 25(1), pp. 1234-1257.

Liu G., *et al*. (2020). Advances in pluripotent stem cells: history, mechanisms, technologies, and applications. *Stem Cell Reviews and Reports*, 16(1), pp. 3-32.

Ma D., *et al*. (2013). Locally injection of cell sheet fragments enhances new bone formation in mandibular distraction osteogenesis: a rabbit model. *Journal of Orthopedic Research: official publication of the Orthopedic Research Society*, 31(7), pp. 1082-1088.

Ma Y., *et al.* (2014). Genome modification by CRISPR/Cas9. *The FEBS Journal*, 281(23), pp. 5186-5193.

Makarova K. S. & Koonin E. V. (2015). Annotation and Classification of CRISPR-Cas Systems. *Methods in Molecular Biology (Clifton, N.J.)*, 1311, pp. 47-75.

Martínez-Lage M., *et al*. (2018). CRISPR/Cas9 for Cancer Therapy: Hopes and Challenges. *Biomedicines*, 6(4), pp. 105.

Milgrom P., *et al.* (2020). Addition of Povidone-Iodine to Fluoride Varnish for Dental Caries: a randomized clinical trial. *JDR Clinical and translational research*, 6(2), pp. 195-204.

Mitsiadis T. A. & Graf D. (2009). Cell fate determination during tooth development and regeneration. *Birth Defects Research. Part C, Embryo today: reviews*, 87(3), pp. 199-211.

Miura H., *et al*. (2018). Easi-CRISPR for creating knock-in and conditional knockout mouse models using long ssDNA donors. *Nature Protocols*, 13(1), pp. 195-215.

Miura M., *et al*. (2003). SHED: stem cells from human exfoliated deciduous teeth. *Proceedings of the National Academy of Sciences of the United States of America*, 100(10), pp. 5807-5812.

Moffatt P., *et al*. (2006). Cloning of rat amelotin and localization of the protein to the basal lamina of maturation stage ameloblasts and junctional epithelium. *The Biochemical Journal*, 399(1), pp. 37-46.

Mojica F. J. M., *et al.* (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution*, 60(2), pp. 174-182.

Monica F. J., *et al*. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution*, 60(2), pp. 174-182.

Moradian-Oldak J. (2012). Protein-mediated enamel mineralization. *Frontiers in Bioscience (Landmark edition)*, 17(6), pp. 1996-2023.

Mosterd C. & Moineau S. (2020). Characterization of a Type II-A CRISPR-Cas System in *Streptococcus mutans*. *mSphere*, *5*(3), pp. e00235-20.

Mout R., *et al.* (2017). In vivo delivery of CRISPR/Cas9 for therapeutic gene editing: progress and challenges. *Bioconjugate Chemistry*, 28(4), pp. 880-884.

Nakashima M. & Reddi, A. H. (2003). The application of bone morphogenetic proteins to dental tissue engineering. Nature Biotechnology, 21(9), pp. 1025-1032.

Nakashima M. (2005). Tissue engineering in endodontics. *Australian Endodontic Journal*, 31(3), pp. 111-113.

Nanduri L., *et al*. (2013). Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland. *Radiotherapy and Oncology: Journal of the European Society for Therapeutic Radiology and Oncology*, *108*(3), pp. 458-463.

Ogawa M. & Tsuji T. (2015). Reconstitution of a bioengineered salivary gland using a three-dimensional cell manipulation method. *Current Protocols in Cell Biology*, 66, pp. 19.17.1–19.17.13.

Ozturk A., *et al*. (2010). The antimicrobial peptide DEFB1 is associated with caries. *Journal of Dental Research*, 89(6), pp. 631-636.

Pandya M. & Diekwisch T. G. H. (2019). Enamel biomimetics-fiction or future of dentistry. *International Journal of Oral Science*, 11(1), pp. 8.

Pawluk A., *et al.* (2016). Naturally occurring off-switches for CRISPR-Cas9. *Cell*, 167(7), pp. 1829-1838.

Peng R., *et al*. (2016). Potential pitfalls of CRISPR/Cas9-mediated genome editing. *The FEBS Journal*, 283(7), pp. 1218-1231.

Place E. S., *et al*. (2009). Synthetic polymer scaffolds for tissue engineering. *Chemical Society Reviews*, 38(4), pp. 1139-1151.

Prateeptongkum E., *et al*. (2016). Characterization of progenitor cells and stem cells from the periodontal ligament tissue derived from a single person. *Journal of Periodontal Research*, 51(2), pp. 265-272.

Qin B., et al. (2014). Epidemiology of primary Sjögren's syndrome: a systematic review and meta-analysis. Annals of the Rheumatic Diseases, 74 (11), pp. 1983-1989.

Ramalho-Santos M. & Willenbring H. (2007). On the origin of the term "stem cell". *Cell Stem Cell*, 1(1), pp. 35-38.

Rodríguez-Lozano F. J., *et al*. (2011). Mesenchymal stem cells derived from dental tissues. *International Endodontic Journal*, 44(9), pp. 800-806.

Sharpe P. (2020). Regenerative Dentistry. *Frontiers in Dental Medicine*, 1(3), pp. 1-2.

Shen B., *et al*. (2014). Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nature Methods*, 11(4), pp. 399-402.

Shinwari Z. K., *et al*. (2018). Ethical issues regarding CRISPR mediated genome editing. *Current Issues in Molecular Biology*, 26, pp. 103-110.

Slack J. M. (2000). Stem cells in epithelial tissues. *Science (New York, N.Y.)*, 287(5457), pp. 1431-1433.

Sobhani A., *et al*. (2017). Multipotent stem cell and current application. *Acta Medica Iranica*, 55(1), pp. 6-23.

Stadtmauer E. A., *et al*. (2020). CRISPR-engineered T cells in patients with refractory cancer. *Science (New York, N.Y.)*, 367(6481), pp. 7365.

Szczelkun M. D., *et al*. (2014). Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. *Proceedings of the National Academy of Sciences of the United States of America*, 111(27), pp. 9798-9803.

Szczes A., *et al*. (2017). Synthesis of hydroxyapatite for biomedical applications. *Advances in Colloid and Interface Science*, 249, pp. 321-330.

Talamini R., *et al*. (2000). Oral hygiene, dentition, sexual habits and risk of oral cancer. *British Journal of Cancer*, 83(9), pp. 1238-1242.

Tu Z., *et al*. (2017). Promoting Cas9 degradation reduces mosaic mutations in non-human primate embryos. *Scientific Reports*, *7*, pp. 42081.

Vacanti C. A. (2006). The history of tissue engineering. *Journal of Cellular and Molecular Medicine*, 10(3), pp. 569-576.

Wang S., et al. (2014). Odontogenic differentiation and dentin formation of dental pulp cells under nanobioactive glass induction. Acta Biomaterialia, 10(6), pp. 2792-2803.

Weissman I. L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. *Cell*, 100(1), pp. 157-168.

Wigler R., *et al.* (2013). Revascularization: a treatment for permanent teeth with necrotic pulp and incomplete root development. *Journal of Endodontics*, 39(3), pp. 319-326.

Xiao L. & Nasu M. (2014). From regenerative dentistry to regenerative medicine: progress, challenges, and potential applications of oral stem cells. *Stem Cells and Cloning: advances and applications*, 7, pp. 89-99.

Xiao Y., et al. (2003). Tissue Engineering for Bone Regeneration Using Differentiated Alveolar Bone Cells in Collagen Scaffolds. Tissue Engineering, 9(6), pp. 1167-1177.

Xie J., *et al.* (2019). IGF2BP1 promotes LPS-induced NFκB activation and pro-inflammatory cytokines production in human macrophages and monocytes. *Biochemical and Biophysical Research Communications*, 513(4), pp. 820-826.

Yang J. W., *et al.* (2015). Dental pulp tissue engineering with bFGF-incorporated silk fibroin scaffolds. *Journal of Biomaterials Applications*, 30(2), pp. 221-229.

Yang M., *et al.* (2019). Impact of CXCR4 and CXCR7 knockout by CRISPR/Cas9 on the function of triple-negative breast cancer cells. *OncoTargets and Therapy*, 12, pp. 3849-3858.

Yip B. H. (2020). Recent Advances in CRISPR/Cas9 Delivery Strategies. *Biomolecules*, 10(6), pp. 839.

Yu B., *et al.* (2022). Transforming growth factor-β1 promotes early odontoblastic differentiation of dental pulp stem cells via activating AKT, Erk1/2 and p38 MAPK pathways. *Journal of Dental Sciences*, volume 18, pp. 87-94.

Yu N., *et al*. (2018). Genome Editing: A New Horizon for Oral and Craniofacial Research. *Journal of dental research*, 98(1), pp. 36-45.

Zai Q., *et al*. (2019). Dental stem cell and dental tissue regeneration. *Frontiers of medicine*, 13(2), pp. 152-159.

Zischewski J., *et al*. (2017). Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases. *Biotechnology Advances*, 35(1), pp. 95-104.