El-Hacène Zeghichi

RECENT STRATEGIES FOR TOOTH REGENERATION – A NARRATIVE REVIEW

Universidade Fernando Pessoa
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Atesto a originalidade do trabalho,

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(ElHacene Zeghichi)

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ABSTRACT

Complete dental regeneration through tissue engineering could be the treatment of the future, as an alternative to the current prosthetic ways of replacing a missing tooth, thus avoiding the disadvantages of the latter. For such an emergence of tissue engineering, a perfect knowledge of the embryological mechanisms of dental development is required, as well as the stem cells involved.

This narrative review will describe the current state of knowledge regarding tooth regeneration, focusing on the scientific advances made in this field and the tools used. However, there are limitations that will be described and analyzed in order to understand the challenges that must be overcome in this field before achieving the full functional outcome and large-scale clinical application of tissue-engineered dental regeneration in humans.

Keywords: Tooth regeneration, Regenerative dentistry, Tissue engineering, Tooth development, Dental embryology

RESUMO

A regeneração dentária completa através da engenharia de tecidos poderia ser o tratamento do futuro, como alternativa aos atuais meios protéticos de substituição de um dente em falta, evitando assim as desvantagens deste último. Para uma tal emergência de engenharia de tecidos, é necessário um conhecimento perfeito dos mecanismos embriológicos do desenvolvimento dentário, bem como das células estaminais envolvidas.

Esta narrativa descreverá o estado atual dos conhecimentos relativos à regeneração dentária completa, focando-se nos avanços científicos feitos neste campo e nas ferramentas utilizadas. No entanto, existem limitações que serão descritas e discutidas a fim de compreender os desafios que devem ser ultrapassados neste campo antes de ser possível alcançar o resultado funcional completo e a aplicação clínica em larga escala da regeneração dentária tecidual em humanos.

Palavras-chave: Regeneração dentária, Medicina dentária regenerativa, Engenharia de tecidos, Desenvolvimento dentário, Embriologia dentária

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List of Abbreviations

Barx: BarH-like Homeobox genes

BMP: Bone Morphogenetic Proteins

DFSC: Dental follicle stem cells

DPSC: Dental pulp stem cells

Dlx: Distal Less Homeobox genes

EDE: External Dental Epithelium

FGF: fibroblast growth factor

GMSC: Gingival mesenchymal stem cells

HERS: Hertwig's epithelial root sheath

HMG: High Mobility Group of proteins

IDE: Internal Dental Epithelium

IGF: Insulin-like Growth Factor

Lef-1: Lymphoid Enhancer Binding Factor 1 gene

Msx: Muscle Segment Homeobox genes

Pax: Paired Box genes

PDLSC: Periodontal ligament stem cells

PEK: Primary Enamel Knot

Pitx: Pituitary Homeobox genes

SCAP: Stem cells from the apical papilla

SHED: Stem cells from human exfoliated deciduous teeth

SHH: Sonic Hedgehog gene

TGFß: Transforming Growth Factor ß

TGPC: Tooth germ progenitor cells

INDEX

I. INTRODUCTION

Dental surgery is classically focused on the treatment of oral pathologies. However, it would be erratic to reduce this occupation only along this axis. In fact, the loss of a tooth in an individual is an irremediable stage. Currently, there are three different types of rehabilitation in case of tooth loss, such as conventional dentures, prosthodontics implants or self-transplantation. Each of these techniques has advantages and disadvantages. Tissue engineering is a set of techniques using the principles and methods of engineering and medicine to produce living tissue from cultured human cells with the objective of restoring, maintaining, or improving their functions (Letourneur and Bordenave, 2017). Over time, the level of knowledge on embryology and the stages of dental development, has increased, which is essential to be able to rebuild a living system and replace a destroyed or deteriorated structure in humans. The birth of tissue engineering has opened a new path in tooth organ replacement therapy, thanks to this level of in-depth knowledge of the various stages of dental development and the mastery of stem cell differentiation (Thisse and Zon, 2002). It is therefore a great challenge in tissue engineering to recreate an anatomically whole tooth, functional and complete. This challenge has already been taken in animal models, but no perfect results were obtained. In these studies, it was possible to create teeth, however their histological and morphological characteristics were not always like those of natural teeth. This suggests an optimistic forecast for the field of tooth regeneration despite the difficulties (Yang *et al.,* 2017). It is attractive to believe that, thanks to scientific advances, man will soon be able to replace missing teeth with new ones, histologically and morphologically equivalent to natural teeth and produced from individual own stem cells. Therefore, it seems obvious the question: where are we at the present time regarding the advances on the potential replacement of a missing tooth through complete tooth regeneration? In this narrative study, generalities concerning tooth development will be discussed, explaining all the embryological and post-birth stages of odontogenesis, focusing on the main biological elements interacting and allowing odontogenesis. Then, the use of stem cells and tissue engineering in dentistry will also be discussed, as well as its issues in regenerative dentistry.

a. 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 used keywords, in multiple combinations, were “tooth regeneration”; “odontogenesis”; “regenerative dentistry”; “tissue engineering” and “dental embryology”. Papers published in the last 29 years (from 1992 to 2021), in English, Portuguese and French were included. The search included narrative and systematic review and clinical cases articles. Publications

that did not obey the study goals were excluded. The search allowed the selection of 99 articles relevant for the writing of this thesis.

II. DEVELOPMENT

a. Dental development

i. Odontogenesis: Tooth development

Odontogenesis is a simple process involved in the development of the craniomaxillofacial complex, that leads to the formation and development of the dental organ (Brook *et al.,* 2014). Odontogenesis starts during the establishment of the dental rough, which begins with the interaction between the oral epithelium of ectodermal origin and the underlying ectomesenchyme, resulting from condensation of mesenchymal cells and neural crest derived cells. This process will lead to the development of dental organs (Piette and Goldberg, 2001).

During the 4th week of intrauterine life, there is the formation of a cavity in the embryo, the stomodeum, being the origin of the pharyngeal arches, including the first pharyngeal arch. Thus, five buds are obtained (2 maxillary, 2 mandibular and 1 naso-frontal), formed by surrounding the stomodeum that corresponds to the primitive mouth. Therefore, in this arch, a continuous odontogenic epithelium develops, resulting from a fusion process of the different epithelia of the buds (Papon *et al.*, 2017). During the 6th week of intrauterine life, a molecular dialogue begins with epithelio-mesenchymal interactions. The mesenchyme underlying the odontogenic epithelium is transformed into ectomesenchyme by cells derived from neural ridges around future tooth buds. The cells of the odontogenic epithelium will thicken in the underlying mesenchyme to form a primitive lamina (Tucker and Sharpe, 2004). The primitive blade will undergo segmentation and regionalization allowing the formation of dental placodes that will induce the formation of 10 spherical tooth buds that penetrate the ectomesenchyme of both jaws. The epithelial invagination will have the future dental crown (Som and Miletich, 2018).

This bud stage is characterized by encirclement of the buds by ectomesenchymal cells and a progression of epithelial invagination (Tucker and Sharpe, 2004). At the end of this stage, the emergence of a complex of cells emitting signaling molecules and transcription factors, called Primary Enamel Knot (PEK) is observed, which will locally determine the cellular activity of the tissue territory (Matalova *et al.*, 2005). The epithelial and ectomesenchymal cells of each tooth rough will divide intensely leading to an increase in the volume of the buds, that are transformed into dental caps, characterized by an epithelial mass alveolus, which will partially envelop the future dental pulp (Ohazama *et al.*, 2004).

The cap stage is marked by an intense growth of epithelial cells, which results in the formation of a concavity in its internal aspect, justifying its designation as “cap” stage. This concavity is called enamel, and due to the accumulation of a big number of ectomesenchymal cells inside, the dental papilla is formed. Around the enamel organ and the dental papilla, a concentration of mesenchyme cells is present

that forms the tooth follicle. Epithelial cells located in the concavity adjacent to the ectomesenchyme make up the internal dental epithelium (IDE) of the enamel organ, and cells in the convex area make up the external dental epithelium (EDE). The cells in the central area acquire a branched appearance, forming a network called stellate reticulum. The dental papilla will soon produce dentin and pulp, the enamel organ will produce enamel, and the dental follicle will produce the periodontium, which are the supporting structures of the tooth. At the end of the cap stage, in the epithelial part, the disappearance of the PEK is observed due to apoptosis of its non-proliferative cells. At the ectomesenchymal level, a vascularization which seems more organized, and a beginning of innervation can be observed (Goldberg and Gaucher, 2011).

The dental cap stage is followed by the dental bell stage. The lower surface of the cap expands, deepens, and forms a kind of a bell. This stage corresponds to histological and morphological differentiations as well as determination of the pattern of the dental crown or morpho-differentiation. In fact, the secreting cells of the crown hard tissues (ameloblasts and odontoblasts) obtain their distinctive phenotype during this stage (Smith and Lesot, 2001). At the epithelial level, the appearance of Secondary Enamel Nodes can be observed only on pluricuspid teeth, which will guide the differentiation of the epithelium and the morphogenesis of each cusp tip during this dental bell stage. After this stage, these nodes will disappear by apoptosis (Goldberg and Gaucher, 2011).In the central area of the bell, the IDE suffers damage, and develops a more elongated shape through cell differentiation, giving rise to the future ameloblast cells. In contrast, the ectomesenchymal cells that face the IDE cells, separated by a basement membrane, will differentiate into odontoblasts cells. IDE and EDE meet to form a double epithelial layer, the reflection zone of the enamel that constitutes the Hertwig's epithelial root sheath (HERS), formed by fusion and invagination of layers of the connective tissue. The HERS will induce the differentiation of pre-odontoblasts into odontoblasts producing root dentin. Other cells will persist after the formation of the first dentinal layers as Malassez epithelial rests (Goldberg and Gaucher, 2011). The enamel organ takes the shape of a bell, and most of its cells form the stellate reticulum. The condensed ectomesenchyme located at the periphery of the enamel organ and the dental papilla forms the dental follicle. It will give rise to the cementum, the alveolar ligament, and the alveolar bone (Nanci *et al.*, 2008).

In the next stage, amelogenesis, the enamel is secreted by ameloblasts derived from the IDE, that lose their secretory activity at the end of the amelogenesis and disappear after the eruption of the tooth (Bartlett *et al.*, 2006). The pre-ameloblasts, precursors of ameloblasts, will only differentiate in the presence of functional odontoblasts (Peters *et al.*, 1998). To secrete enamel, pre-ameloblasts differentiate into ameloblasts accompanied by intracellular changes involving exit from the cell cycle, with concomitant degradation of the basal lamina. Finally, ameloblasts go through the maturation phase and then the protection phase (Goldberg and Gaucher, 2011). The enamel has an ectodermal origin, and its formation occurs only during crown formation, from the 14th week of in-utero development. The cell

responsible for amelogenesis is destroyed when the tooth erupts in the oral cavity, so, if damaged, the enamel cannot regenerate (Goldberg and Gaucher, 2011). The amelogenesis begins with the synthesis and secretion of enamel matrix molecules, followed by their mineralization in the maturation phase. Then, the shrinkage of this matrix, followed by increased mineral deposition is observed, forming the enamel structure. In fact, the enamel is not an organic tissue but a structure because of its acellular state and without innervation. It is the most mineralized structure in the body (Piette and Goldberg, 2001).

In dentinogenesis dentin is secreted by odontoblasts through the process of dentinogenesis, that includes two essential steps: the synthesis and secretion of pre-dentin and the mineralization of the pre-dentin to form dentin. Dentinogenesis is a phenomenon that takes place gradually throughout the life of the tooth, being faster during tooth formation, until it becomes very slow in the final stage, until the end of life (Linde and Goldberg, 1993). Functional differentiation of odontoblasts takes place, beginning at the cusp tip and proceeding apically. Like ameloblasts, odontoblasts differentiate through different stages accompanied by intracellular changes. First, the pro-odontoblasts, precursors of odontoblasts, divide at the end of the cell cycle. These cells undergo a final division with two goals: first to keep a stock of daughter cells which can differentiate into odontoblasts in case of future aggressions, and second to have a cell that will become an odontoblast and will allow the secretion of dentin matrix and dentin (Yildrim, 2012). Among the organic constituents of the dentin matrix, collagens, large quantities of glycoproteins involved in mineralization and small amounts of proteoglycans and matrix metalloproteinases, lipids, some growth factors, among others (serum proteins, phospholipids and enamel proteins) (Peters *et al.*, 1998).

ii. Molecular interactions and dental development in odontogenesis

As mentioned earlier, dental development is shaped by a series of interactions between the odontogenic epithelium and the ectomesenchyme. The general mechanisms of dental development such as migration, adhesion, differentiation or cell apoptosis, are the consequence of molecular events controlled by numerous interactions between genes. These molecular events are managed by families of signaling molecules that often act repeatedly in the spatio-temporal context (Cobourne and Sharpe, 2010). Thus, to allow the growth of a tooth from scratch, one must be fully aware of this pattern constituting the process of epithelial-mesenchymal interactions. This process is regulated by more than 300 genes and 100 growth and differentiation factors, which intervene in a given space and time (Jernvall and Thesleff, 2000). The study of the nature of these genes and the mechanisms that control their expression will give interesting models in initiation, morphogenesis and cell differentiation. Several genes and transcription factors are involved in odontogenesis. An example are homeotic genes (homeogenes) are genes that define the location and specify the characteristics of each territory of the human body by governing the synthesis of transcription factors that regulate the activity of structural genes. Thus, these genes control

the synthesis of those transcription factors, defining the fate of a cell in the organs. Transcription factors are proteins that act on transcription regulation, by having a homeodomain that allows them to attach to DNA and modulate the expression of certain genes (Goldberg and Gaucher, 2001). The synthesis of growth factors is also determined by homeotic genes, whose role is to act on cells at specific times during development (Kieffer-Combeau *et al.,* 2001).

The classification of homeoproteins is based on an analogy scale within the homeodomain present, and it is possible to distinguish the Hox genes responsible for assigning positional identities to nascent embryonic tissues, i.e. they manage the placement of the different body segments (Deschamps, 2007). However, they are not expressed in the head. They only control along the rostro-caudal axis of the axial skeleton and the rhomboencephalon (Stock *et al.,* 1997). The divergent or Parahox genes expressed by neural crest cells in the head play an important role during craniofacial formation (Maxson *et al.,* 2003). Other involved genes are the ones coding for specific transcription factors. *Msx* (Muscle Segment Homeobox) family genes has 3 members: *Msx-1* to *3*. *Msx-1* and *2* are expressed in the first branchial arch and dental sprouts. These genes contribute to dental histomorphogenesis and cytodifferentiation (Ohazama *et al.,* 2004). A study was able to demonstrate that *Msx-1* and *2* genes regulate epithelial-mesenchymal signaling in certain stages of dental development. In fact, the absence of *Msx-1* and *2* genes in mice leads to oral malformation such as anodontia or hypodontia (Puthiyaveetil *et al.,* 2016). The *Msx-3* gene is not expressed in either neural crest cells or orofacial structures (Sharpe, 1995).

Dlx (Distal Less Homeobox) gene family has 6 members: *Dlx-1* to *6*. *Dlx-1* and *2* are expressed in both the maxillary and mandibular arches during orofacial development (Zhao *et al.,* 2000). *Dlx-3, 5* and *6* are expressed only in the mandible, and their roles have not yet been clearly defined (McCollum and Sharpe, 2001).

Pitx (Pituitary Homeobox) gene family has 3 members: *Pitx-1* to *3*. *Pitx-1* and *2* act on the regionalization and formation of the first branchial arch (Mitsiadis and Smith, 2006) and their absence influences dental development. One study showed that the absence of these genes leads to an alteration in molar shape or a complete absence of molars from the dentition. On the other hand, *Pitx-3* has no role in dental development (Tucker and Sharpe, 2004).

Pax (Paired Box) gene family (*Pax-1* to *9*) function as transcription factors present in the dental ectomesenchyme from the earliest stages of odontogenesis (Zhao *et al.,* 2013). The expression of the *Pax-9* gene is a pivotal axis in the positioning of future dental sprouts. To corroborate this, a study showed that agenesis is a direct consequence of the absence or mutation on the *Pax-9* gene (Hlousková *et al.,* 2015).

Barx (BarH-like Homeobox) gene family (*Barx-1* and *2*) are expressed in the maxilla and mandibula (Doshi *et al.,* 2016). However, *Barx-1* gene is only expressed posteriorly in the first branchial arch, specifically in the ectomesenchyme of the molars (Mitsiadis and Smith, 2006).

The *Lef-1* (Lymphoid Enhancer Binding Factor 1) gene belongs to the HMG (High Mobility Group) protein family. This gene is expressed in tooth germs and hair follicles, being essential for dental morphogenesis, influencing the dental papilla development (Sasaki *et al.*, 2005).

Besides the described gene products, growth factors and signaling molecules are also required in odontogenesis. Growth factors are polyvalent signaling molecules that promote different molecular biological processes such as cell division or differentiation (Byer *et al.*, 2003). They are versatile agents that can have stimulating effects in different cell types. However, different responses from different cells or tissues can be observed by the action of the same growth factor, showing variable specificities. Many signaling pathways and molecules that influence the determination of morphological characteristics of teeth such as crown size, type of cusp pattern and tooth length, as well as the repair of pulp and dentin, have been identified. These signaling components generally regulate epithelial-mesenchymal cell interactions (Thesleff, 2003).

TGFß (Transforming Growth Factor ß) molecules represent a superfamily of growth factors that are active in tooth development, particularly during dentin formation and pulp protection, through the expression of the genes coding for TGFß-I and TGFß-II receptors in odontoblasts and pulp cells (Sloan *et al.*, 1999). The genes encoding these three proteins are intensely expressed during the amelodontoblastic differentiation (Thesleff and Mikkola, 2002).

TGFß-I activates or inhibits the proliferation and differentiation of epithelial and mesenchymal cell lines. Moreover, in association with BMP2 (Bone Morphogenetic Protein 2), it stimulates another growth factor that acts on the secretion of the dentinal matrix by dental papillae cells. This growth factor acts as an activity modulator of the cytological and functional differentiation of odontoblasts (Bègue-Kirn *et al.*, 1992).

TGFß-II inhibits cell proliferation and differentiation of the enamel organ as well as the dental papilla, influencing incisors and molars morphology and size (Piette and Goldberg, 2001). Although the exact role of TGFß-III in tooth development is not known, one study showed that inhibition of this growth factor alone can lead to failure of palate fusion (Brunet *et al.*, 1995).

BMPs are signaling proteins belonging to the TGFß superfamily, that influence organogenesis and bone and cartilage development. BMPs are considered osteoinductive factors, having the power to regulate the bone and dentin formation (Åberg *et al.*, 1997). These are important molecules in cell signaling for odontoblast differentiation and stimulation of reactive dentin formation (Nakashima, 2005). These molecules also participate in the inductive interactions between the epithelium and the dental ectomesenchyme (Bethan *et al.*, 1997), by stimulating the expression of the transcript factors Msx-1, Msx-2 and Dlx-2 (Jernvall and Thesleff, 2000).

BMP-2, *4*, *6*, *7* and *Gdf11* genes are expressed, and the coded proteins have a coordinated action during odontoblasts differentiation, while *BMP-4* and *5* are expressed during the differentiation of ameloblasts

(Zhang *et al.,* 2005). *BMP-2* and *7* genes have similar expression patterns, being expressed in the dental epithelium and the enamel node (Thesleff, 2003).

However, BMP-2 alone is required for the activation of odontoblast differentiation (Casagrande *et al.*, 2010). BMP-4 has a central role in epitheliomesenchymal exchange at different stages of odontogenesis. It is an inducer of the forming enamel node and stimulates the synthesis of some transcription factors such as Msx-1 and Msx-2 (Tucker and Sharpe, 2004).

fibroblast growth factors (FGFs) belong to a large family of 24 growth factors, which influence embryonic development by affecting cellular functions such as proliferation, differentiation, division or migration and adhesion. Many FGFs are expressed during dental development and stimulate epithelial and mesenchymal cell divisions at different stages (Thesleff and Mikkola, 2002). The most important FGFs are FGF-4 which acts at the enamel node (Jernvall and Thesleff, 2000), and FGF-8 and 9 which act as an epithelial signal, structuring the mesenchyme of the first arch and influencing tooth initiation. FGF-8 also stimulates the expression of certain transcription factors such as Msx-1 or Pax-9 (Tucker and Sharpe, 2004).

Besides growth factors, during dental development, several other signaling molecules that act/affect different molecular mechanisms, can be found. An example is retinoic acid, required for the initiation of odontogenesis by stimulating the proliferation of epithelial cells of the dental lamina, and generating the proliferation of the maxilla and mandible cells, leading to a higher number of these cells in the incisal sector than in the molar sector (Piette and Goldberg, 2001).

The *SHH* (Sonic Hedgehog) gene is also an example of a gene coding for a signaling protein. Its expression is observed in the dental lamina, at the spots of future teeth formation. It is involved in the development of dental sprouts, influencing the enamel node, and participating in dental morphogenesis (Thesleff and Mikkola, 2002).

There is also *Notch* gene involved in the Notch signaling pathway. This pathway determines the development of stem cells playing a central role in the diversification of cellular phenotypes during development. It is also present in the odontogenic epithelium, during its thickening, and in all the epithelial cells that will become ameloblasts (Thesleff and Mikkola, 2002).

Teeth have different shapes and consequently divergent functions depending on their location on the dental arch: incisors have a cutting and shearing role while canines tear and premolars/molars are responsible for crushing food. The development of dentition has suffered changes during the evolution of vertebrates, and the modification of the dentition allowed the adaptation of the organism to new feeding strategies (Ohazama *et al.,* 2010). Teeth form a segmented organization, showing quantitative morphological differences between them in terms of size and shape. A biological mechanism will determine the location and identity of each tooth, following a dental pattern or scheme. Different theories have been proposed to explain this (Mitsiadis and Smith, 2006).

The field theory was formulated by Butler in 1939, and states that the various types of teeth develop from identical bases. In a single set, the type and shape of teeth are controlled by a gradient field of concentration of signaling molecules (BMP and FGF) along an anteroposterior axis divided into three regions: incisor, caniform and molariform (premolars are included with molars). The BMP gradient concerns the formation of anterior teeth (incisors) and the FGF gradient concerns posterior teeth (Mitsiadis and Smith, 2006). Another theory proposed by Osborn in 1978, states that tooth types correspond to the progeny of three different clones of distinctly derived mesenchymal cells. Each initial tooth outline would therefore be fundamentally different, and the differences in tooth pattern would be due to changes in the cell lineages overtime (Mitsiadis and Smith, 2006). There is another code that differs from the field and clonal theories. This code corresponds to a combinatorial expression of homeobox genes that specifically control the dental pattern and the development of maxillofacial elements. It is a "homeobox code" that sets up a regional diversity within the regions of tooth formation in the branchial arch. It is the expression of this "specific-region" code in the mesenchyme of the branchial arch that determines the identity of each tooth (Mitsiadis and Smith, 2006). It can therefore be seen that there are certain genes from this homeobox family that are expressed in a spatial-temporal manner. For example, just before the initiation of odontogenesis, Msx-1 has specific expression domains in the anterior region of the first arch which corresponds to the future incisor regions (Tucker and Sharpe, 2004). One study attempted to modify the molecular signaling at the epithelial level by suppressing the expression of BMP in the incisal territory and observed a transformation of the dental morphology: instead of incisors, these authors obtained molars (Tucker *et al.,* 1998). Therefore, it is possible to change the dental identity through the modification of the expression of homeobox genes in the mesenchyme (Mitsiadis and Smith, 2006). By combining the clonal theory and the dental homeocode, a decrease or absence of teeth is observed. A new model has been proposed to try to explain this phenomenon. First, the appearance of point mutations in genes expressed in mesenchymal dental clones can affect cell proliferation and their ability to produce normal number of cells needed to form a specific number of tooth types. In addition, the signaling molecule produced by the cells affected by the point mutation will have reduced functionality due to its lower production. All these factors contribute to a reduction in the size or complete loss of a tooth. Thus, the number of teeth of a given type decreases in individuals with such mutations (Mitsiadis and Smith, 2006).

b. Stem cells and tissue engineering

i. Stem cells in Odontology

The term "stem cell" appeared as early as 1883 and refers to undifferentiated cells present in almost all multicellular organisms (Stappenbeck and Miyoshi, 2009), that can divide and differentiate into specialized cell types. They have self-renewal capacity producing other stem cells *in vivo* or *in vitro*,

being seen as an internal repair system. They can be used for the healing and renewal of the cell pool by regeneration or recreation of destroyed tissues (cell therapy) (Huang *et al.*, 2013). The sources of stem cells can be the embryo or fetus (ante-natal), the umbilical cord (peri-natal) or post-natal (after birth) (Huang *et al.*, 2013). Four types of stem cells can be identified according to their differentiation potential: totipotent stem cells that give rise to any type of differentiated cell, i.e., they have the highest differentiation potential. These cells are only present at the beginning of embryogenesis and one single totipotent cell can give rise to an entire organism (Condic, 2014); pluripotent stem cells can produce almost all cell types of an organism except the embryonic appendages, and are found at the blastocyst stage (Smith, 2006); multipotent stem cells are already engaged in differentiation pathways. These cells give rise to different types of differentiated cells. For example, hematopoietic stem cells will give rise to all cells of the blood lineage (such as red blood cells), but cannot naturally differentiate into nerve cells (Condic, 2014); unipotent stem cells can only produce one differentiated cell type. These cells still have the capacity of self-renewal shared by all stem cells (Smith, 2006).

Epithelial stem cells are present and implicated in embryogenesis since the first stages. These cells are formed from the epithelium of the primitive mouth, and will differentiate thereafter, thanks to epithelial-mesenchymal interactions, to form the various components of enamel. One study, focused on dental epithelial stem cells that participate in the renewal of the mouse incisor, observed that the rodent incisor presents a continuous growth, with permanent production of enamel (Michon, 2013). The reflection zone contains a niche of these cells that work on the renewal of all dental epithelia, including ameloblasts that secrete enamel (Michon, 2013).

Dental pulp stem cells (DPSC) have retained the ability to form functional odontoblasts that can produce dentin. This capacity can be observed with the formation of reparative dentin in cases of caries or mild trauma (Shi *et al.*, 2005). Therefore, studies have assumed that there are mesenchymal stem cells in the dental pulp with the ability to differentiate and form new dentin to repair injuries. The first adult human dental pulp stem cells isolated were named dental pulp stem cells (DPSC) (Gronthos *et al.*, 2000), being phenotypically similar to bone marrow stem cells with properties specific of adult stem cells such as self-renewal, multi-differentiation or regeneration of the pulp-dentinal complex, representing an important and accessible post-natal cellular stock. They are physiologically in a state of rest, however, when solicited due to an aggression or a trauma, they are activated and allow the formation of specialized cells, the odontoblast-like cells that will lead to pulp healing by replacing the destroyed odontoblasts (Casagrande *et al.*, 2011).

Stem cells from human exfoliated deciduous teeth (SHED) can induce bone formation and generate dentin. Miura *et al.* (2003) observed the *in vitro* differentiation of non-dental mesenchymal cell derivatives. Moreover, a study of Sakai *et al.* (2010) showed that SHEDs can differentiate into functional odontoblasts and induce bone matrix formation and generate tubular dentin, after *in vivo* subcutaneous

transplantation in immunodeficient mice. SHEDs are multipotent stem cells, with the same ectomesenchymal origin as DPSCs, from the cranial neural crest. SHEDs have higher proliferation rates compared to DPSCs and other bone-marrow derived mesenchymal stem cells (Nakamura *et al.,* 2009). However, they differ in the expression of genes involved in embryogenesis. Specific genes are expressed in SHEDs, being involved in extracellular matrix formation pathways, and several growth factors such as fibroblast growth factor (FGF) and transforming growth factor TGF-ß. TGF-ß is particularly important since it is released after dentinal lesions and can mobilize pulp stem cells to differentiate into odontoblasts (Sloan *et al.,* 1999). SHEDs are highly proliferative, exhibit high plasticity and retain their stem cell characteristics after prolonged culture. Every individual naturally loses 20 deciduous teeth allowing easy access to SHEDs. In fact, more and more banks of these cells have been created to allow their use when the child becomes adult. However, SHEDs do not retain their properties beyond two years of cryopreservation (Wood *et al.,* 2009). This suggests a potential use as an allogenic source of mesenchymal stem cells but limited as autologous cells to children who have not lost their temporary teeth.

The periodontal ligament is a specialized connective tissue located between the alveolar bone and the cementum. Its role is to maintain and support the tooth, absorbing shocks during mastication and being a sensory receptor for the positioning of the jaw. The periodontal ligament completes its formation during the eruption of the tooth (Zhu and Liang, 2015). However, studies have shown that its continuous regeneration involves mesenchymal progenitor cells from the dental follicle. In fact, the periodontal ligament contains different types of cells, which can differentiate into cementoblasts and osteoblasts (Gay *et al.*, 2007). Isolated periodontal ligament stem cells (PDLSC) have a morphology similar to fibroblasts and present a clonogenic nature. They have a high proliferation rate compared to DPSCs and express stem cell markers. PDLSCs can differentiate *in vivo* and *in vitro,* as mesenchymal cell lines, into cementoblasts, adipocytes, osteoblasts and fibroblasts (Gay *et al.*, 2007). Therefore, the periodontal ligament contains progenitor cells that can be activated for self-renewal and regeneration of tissues such as cementum and alveolar bone. Studies showed the differentiation capacity of PDLSCs in multiple lineages, undergoing osteogenic, adipogenic and chondrogenic differentiation when cultured in an appropriate medium (Seo *et al.*, 2004).

Stem cells have been found in the papillary tissue in the apex of tooth root formation, which are mesenchymal stem cells. The apical papillary tissue is only present before tooth eruption, during root development. These stem cells from the apical papilla (SCAP) can differentiate into odontoblasts, adipocytes, osteoblasts and chondrocytes (Sonoyama *et al.,* 2006). SCAPs have a higher *in vitro* proliferation rate than DPSCs, differing in cellular and vascular composition. Sonoyama *et al.* (2006), by co-transplanting SCAP and PDLSC cells into tooth sockets, observed the formation of dentin and periodontal ligament. This suggests that SCAPs, as well as PDLSCs, can be used to create a biological

root where a crown could be placed. The dental follicle is a loose ectomesenchymal connective tissue that surrounds the enamel organ and the dental papilla of the developing tooth germ (Nanci *et al.*, 2008). It has long been considered a multipotent tissue, since it contains progenitor cells for cementoblasts, periodontal ligament cells and osteoblasts, leading to its ability to generate cementum, periodontal ligament and bone (Handa *et al.*, 2002). Precursors have been isolated from dental follicles derived from third molar germ: the dental follicle stem cells (DFSC). These cells can differentiate into osteoblasts, cementoblasts, chondrocytes and adipocytes depending on the culture medium (Lin *et al.*, 2008). In the same way as SCAPs, DFSCs are cells of a developing tissue and therefore may exhibit bigger plasticity than other dental stem cells. However, like SCAPs, more research is needed on the properties and potential uses of these cells (Estrela *et al.*, 2011).

ii. Tissue engineering

The total or partial loss of an organ in an individual is a major clinical problem. There are many difficulties on its replacement surgically, due to many factors, such as the shortage of organ donors or the increased risk of infection. This is why, in 1993, Langer and Vacanti introduced the concept of tissue engineering, that would make possible to regenerate a tissue by growing specific cells on a biodegradable material (Langer and Vacanti, 1993). An example of an application of modern tissue engineering is the formation of artificially cultured skin, consisting of fibroblast cells seeded on patterns composed of collagen, which is used clinically in the treatment of diabetic burns and ulcers (Chua *et al.*, 2016).

The goal of tissue engineering is to replace, maintain or improve the function of human tissues with biological substitutes developed from cells or matrices. Tissue engineering is also referred as regenerative medicine, having broad interesting therapeutic potential (Chen *et al.*, 2011). Reconstruct a tooth by regenerative medicine is a real challenge. Some approaches use a 3-dimensional matrix model called scaffolds to guide the growth and morphogenesis of the future dental organ (Dutta and Dutta, 2009). Other laboratories use biomimetic techniques to reproduce the growth stages of the dental organ (Pandya and Diekwisch, 2019). Different concepts and research strategies in tissue engineering can be used, having in common the use of polymeric materials (Baum and Mooney, 2000). In the Conductive approach, biomaterials with a passive role are used to allow the growth of regenerative capacity of an existing tissue as in periodontology, for example, where membranes are used to guide regeneration. The polymer is used as a barrier with a specific filtration rate, avoiding the entrance of cells that can disturb the regeneration process (Kaigler and Mooney, 2001). The Inductive approach uses competent cells recruited and activated in the vicinity of the defective site by specific biological signals such as bioactive molecules. The inductive approach uses biodegradable polymers as carriers of cells where genes and coding proteins can be expressed and act on the target cells to promote tissue formation (Kaigler and Mooney, 2001). The Cell transplantation approach uses competent cells biopsied from the animal,

cultured to multiply and maintain their function in the laboratory, and then seeded onto a specific support for subsequent transplantation into a host. After transplantation, the support degrades or is remodeled by the transplanted cells, resulting in a natural tissue (Kaigler and Mooney, 2001). Tissue engineering is conceived as a combination of three factors, namely cells, biomaterials and bioactive growth factors. The main strategy is to regenerate an organ or tissue remaining as faithful as possible its physiological development mechanisms (Garcia and Murray, 2010). The cells used in tissue engineering can be multiple and from different origins. Mesenchymal and epithelial cells have ideal biological properties for tissue engineering. To be able to use a source of stem cells in practice, several characteristics must be fulfilled such as the possibility of cryopreserving the cells (Costa *et al.*, 2012), an angiogenic activity to allow the growth by mitosis or by other cellular mechanism (Howard *et al.*, 2008), the possibility of auto or allogeneic transfer and a known relation between these stem cells and the infectious agents (Koh and Atala, 2004). The main sources of cells used in regenerative medicine are autologous (from the host itself) and allogeneic (from a donor) cells. Autologous cells are well tolerated by the body, raise fewer ethical issues and can be multiplied *in vitro*. However, they have a limited supply (Jorgensen *et al.*, 2004). Allogeneic cells are available in greater numbers than autologous cells. However, they frequently activate immune reactions (Yoshiko, 2006). There are even cases where xenogeneic cells from a different species are used, which are easier to obtain, but have risks of viral transmission from one species to another (Schmidt *et al.*, 2007). Bioactive molecules can have various origins being involved in tooth regeneration. They include genes, growth factors, transcription factors, proteins or signal molecules. Growth factors play a major role in the recruitment of cells and in the stimulation of the synthesis of matrix proteins, such as TGF-ß, BMPs (Galler and D'Souza, 2011) or IGF (Insulin-like Growth Factor) which would induce dentin bridge formation (Lovschall *et al.*, 2001). According to Almushayt *et al.* (2005), dentin matrix phosphoprotein-1 induces mineralization, cytodifferenciation of pulp stem cells into odontoblast-like cells, and the production of calcium deposits after direct pulp capping in rats. Moreover, dexamethasone decreases cell proliferation and induces odontoblast markers in human pulp cell cultures (Alliot-Licht *et al.*, 2005). To choose bioactive molecules, it is necessary to know beforehand the administration route and their properties, since some of them, such as growth factors, are rapidly eliminated by the organism. Thus, it is necessary to study the implementation of a system that will allow the sustainable integration of these factors in a controlled and local way (Scheller *et al.*, 2009).

iii. Supporting biomaterials or scaffolds

The regeneration mechanism is regulated by an extracellular matrix. Used scaffold acts as such, artificially, and in the same way as natural extracellular matrix, plays a role as a barrier, a reserve of nutrients, oxygen and a signaling molecule (Guilak *et al.*, 2009). In the case of dental regeneration by tissue engineering, templates made of natural or synthetic biomaterials are used. Scaffolds that work as

a support, provide an environment for the adhesion, migration, cellular differentiation of transplanted cells and the delivery of bioactive molecules required to form the new tissue (Dutta and Dutta, 2009). Thus, the scaffold is a temporary biocompatible structure that will degrade overtime in a controlled manner, being eventually replaced by an extracellular matrix and the newly formed tissue of interest (Kaigler and Mooney, 2001). There are two major types of scaffolds in tissue engineering: biological polymer scaffolds such as collagen, elastin, glycosaminoglycans, fibrin, silk, chitosan or hyaluronic acid offer a good solid structure, biocompatibility and bioactivity, however, are not very suitable for modification (Galler and D'Souza, 2011); synthetic polymer scaffolds (polyactic acid, polyglycolic acid, polyactic-co-glycolic acid or inorganic calcium and phosphate materials such as hydroxyapatite) allow great control of mechanical and chemical properties during manufacture. However, they lack bioactivity and inherent biological recognition (Place *et al.*, 2009). Hydrogels, another type of scaffold made of water and fibrin gel associated with polyethylene glycol or glycosaminoglycans, can be used. Their viscoelastic properties similar to tissue, their capacity to transport nutrients and metabolic products as well as their capacity to be injected makes their use particularly interesting (Galler and D'Souza, 2011).

c. Research strategies in Odontology

To create a complete tooth, a synchronized generation of the crown, root and periodontal ligament should occur. The natural process of tooth germ formation, with epithelial-mesenchymal interactions, would have to be mimicked to have a functional tooth. Any mesenchymal or epithelial cell cannot independently regenerate appropriate dental structures. The exact interactions are needed to mimic the natural tooth formation (Battistella *et al.*, 2010). Two major strategies are currently used to reconstruct a tooth: reconstitution of the embryonic development of natural teeth by *in vivo* stem cell implantation (Ikeda *et al.*, 2009); *in vivo* transplantation of stem cells, prepared *in vitro* on scaffolds, into the host (Oshima *et al.*, 2011). The *in vivo* cell implantation, to reconstitute embryonic dental development, is based on the fact that aggregates of dissociated dental germ cells have the potential to form a germ and proliferate further into a tooth after *in vivo* transplantation (Yamamoto *et al.*, 2003). Ohazama *et al.* (2004) was able to form a tooth with adult bone marrow-derived mesenchymal cells combined with embryonic dental epithelium at the inductive stage. In 2007, a three-dimensional dental germ culture method was unveiled. This dental germ was generated *in vitro* in a culture medium and implanted *in vivo* into a dental cavity, showing vascular and nervous activity (Nakao *et al.*, 2007). Normal teeth were produced in another study, using epithelial and mesenchymal tissues of the tooth germs that were separated, cells dissociated and then recombined. This tooth developed after transplantation of these aggregates, with a correct structure and architecture (Ikeda *et al.*, 2009).A whole series of other studies aimed to recreate complete teeth, by strategies such as using only human gingival epithelial cells and embryonic mesenchyme from mouse tooth (Volponi *et al.*, 2013) or reusing the method of Nakao *et al.* (2007) by *in vitro* culture of dissociated

cells from a tooth germ and then recombination and *in vivo* implantation in a dog (Ono *et al.*, 2017). In the *In vitro* cell culture with biodegradable scaffolds and *in vivo* transplantation, biodegradable scaffolds can be used as a support, seeded with dissociated dental germ stem cells. The seeded cells contain epithelial and mesenchymal cells that will interact to produce the different tissues that make up the tooth. Work of Young *et al.* (2002) observed that once these cells were transplanted into a host mouse, recognizable dental structures with developed enamel, dentin and pulp tissue are obtained. Sumita *et al.* (2006) have evaluated and examined the different parameters that make up the construction of scaffolds such as materials or mechanical constraints, to improve the success rate of dental regeneration. The use of biodegradable scaffolds has advantages, such as the control of the shape or size of the regenerated tooth, however, fundamental problems concerning this dental regeneration have not yet been solved. In fact, the biggest challenge is, after *in vivo* transplantation, the presence of residual material from the scaffold that persists and may cause irregularities in the structure of the dental tissue (Honda *et al.*, 2003). Moreover, the generation of dental structures with scaffolds requires complex junctions between enamel, dentin and cementum by a very precise spatio-temporal positioning of epithelial-mesenchymal cells, as in the natural development of teeth (Nakao *et al.*, 2007). However, biodegradable scaffolds still have a high potential for dental tissue regeneration, with research and studies continuing to search for the ideal materials for such regeneration (O'Brien, 2011). Several studies focused on the search for an alternative source of epithelial cells have been carried out, including one showing the use of bone marrow-derived cells expressing the c-kit receptor, together with dental epithelial cells to generate ameloblast-like cells, but they must be cultured in re-association with dental mesenchyme (Hu *et al.*, 2006). Another study demonstrated that post-natal non-dental epithelium can become odontogenic when reassociated with embryonic dental mesenchyme (Nakagawa *et al.*, 2009). In fact, these studies demonstrate a technical limitation that is the need for a source of embryonic, epithelial or mesenchymal cells with odontogenic potential, in order to regenerate a tooth. Attempts to use epithelial-mesenchymal cells have their own technical limitations that complicate the generation of the tooth germ. Despite the fact that adult stem cells can respond to an inductive odontogenic signal and participate in tooth formation, only embryonic odontogenic cells derived from embryonic or postnatal tooth germ tissue (epithelial or mesenchymal) are endowed with inductive capacity (Yang *et al.*, 2017). Therefore, to overcome this technical limitation, it is necessary to clearly identify adult cell populations that retain their odontogenic potential and can be cultured in large numbers to be able to exploit them. At present, tissue engineering can reproduce the entire dental tissue (enamel, dentin and periodontium). However, limitations need to be addressed such as problems with tooth structure. As mentioned, the ideal stem cell sources for harvesting and exploitation remain to be determined. Currently, immature wisdom tooth germ from a young patient is considered a potential candidate for tissue-engineered tooth germ reconstruction (Avery, 2001). One study was able to reconstruct a total tooth replacement using these postnatal germ cells (Ono *et al.*, 2007).

If large scale culture of these epithelial/mesenchymal germ cells could be achieved, this bioengineered tooth technology would be able to treat a large number of missing teeth (Ono *et al.,* 2007). However, elderly patients no longer have a developing tooth germ that can be used. Hence, this limitation must be overcome. Other uncertainties concern the immunogenicity of stem cells and their potential for tumor transformation. And not only stem cells, but also the use of growth factors might promote a multiplication of cells with risk of uncontrolled growth, with a carcinogenic nature (Nakahara *et al.*, 2011). Finally, the other major handicap is financial (Knight and Evans, 2004).

III. DISCUSSION

With the current state of knowledge, the complete tooth regeneration is possible to reproduce. Numerous studies that attempted to recreate a tooth by the different methods previously discussed were used in this work. The quality of the results is difficult to gauge in the majority of this studies, since they employed different experimental methods leading to varying success rates. Most studies used in this work conducted their experiments in rodents. The transition from a small animal model to a human model is therefore not an easy task. A rodent, with a smaller size and therefore a larger defect control, has a higher remodeling rate in the event of a defect. In humans, the size of such a defect increases, so the ability to correct it decreases, due to a more difficult histophysiological change. This is a major challenge to overcome for dental regeneration (Scheller *et al*., 2009). Finally, most of the mentioned studies used embryonic cells of animal dental germ, which are pluripotent cells of great interest for use in regenerative medicine. However, if embryonic stem cells of human origin are considered, ethical problems would arise, hence the strict regulations concerning this subject. Therefore, another source of cells capable of generating dental sprouts should be considered to avoid an ethical conflict (Martinat *et al*., 2019).

IV. CONCLUSION

The complete dental regeneration by tissue engineering is a vast and very interesting subject, provided that the basics of dental development and the principles of tissue engineering are perfectly mastered. In the current state of knowledge, it was shown that the complete recreation of a tooth in animals, with a crown, root and alveolar bone, physiologically organized and possessing a functional vascularization and innervation, is possible. This suggests that there are very promising prospects for dental tissue engineering. However, there are clearly discussed limitations in the various attempts, which result from teeth often being of different morphology and size than natural teeth. Many obstacles will have to be overcome, ranging from ethical to technical, to achieve total and functional dental regeneration in humans, and its large-scale application. Once these obstacles are overcome, dental medicine will be able to consider a purely regenerative orientation and profoundly change the current therapeutic goals.

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