

2019

# Dental stem cells in tooth regeneration

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**DENTAL STEM CELLS IN TOOTH REGENERATION**

by

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B.S., Adelphi University, 2017

Submitted in partial fulfillment of the  
requirements for the degree of  
Master of Science

2019



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# **DENTAL STEM CELLS IN TOOTH REGENERATION**

**SULVA AHMED**

## **ABSTRACT**

The objective of this literature review was to highlight and summarize the key studies conducted in the field of tooth regeneration. The large amount of tooth loss and tooth decay indicate a need for tissue replacement therapies. Recent advancements made in the past fifteen years demonstrate the beginning of a new and continually developing field. Through the innovation of different isolation techniques and bioengineering methods, there have been significant breakthrough that have led to limited clinical trials. Researchers have been able to characterize DPSC's as somatic stem cells that have regenerative properties. Furthermore, a novel culture-pellet system was created to isolate these cells and grow them in quantities that would facilitate further research. Recently, research shifted into the clinical trial phase. Studies showed evidence of periodontal tissue repair, prevention of tooth loss, and even a successful regenerative endodontic procedure. These studies marked significant progress although there is need for more research as the field still is missing important components that hinder it from becoming fully applicable to the clinical setting at this point. There is large amount of potential that makes the field of tooth regeneration very promising in the near future.

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## LIST OF ABBREVIATIONS

BMP.....	Bone morphogenic protein
BMSC.....	Bone marrow stem cell
BrdU.....	5-bromo-2'-deoxyuridine
CD105.....	Cluster of differentiation 105
CD73.....	Cluster of differentiation 73
DMP.....	Dental matrix protein
DMP1.....	Dental matrix protein 1
DPSC.....	Dental pulp stem cell
DSP.....	Dentin Sialoprotein
Gdf11.....	Growth differentiation factor 11
G-SCF.....	Granulocyte-colony stimulating factory
H&E.....	Hematoxylin and eosin stain
MTA.....	Mineral trioxide aggregate
PDLSC.....	Periodontal ligament stem cells
RT-PCR.....	Reverse transcription polymerase chain reaction
SCAP.....	Stem cells of apical papilla
SCAP.....	Stem cells of the apical papilla
SHED.....	Stem cells from human exfoliated deciduous teeth

## INTRODUCTION

Oral health is often neglected, and poor oral hygiene has become a seemingly unnoticed worldwide epidemic. According to the National Institute of Dental and Craniofacial Research, 45.8% of adults between 20-65 had dental caries in their permanent teeth (Overman, 2008). The connection between oral and systemic health has long been known and linked to diseases such as diabetes and therefore dentists and other healthcare professionals are encouraging people to prioritize their oral health (Hollister and Weintraub, 1993). Dentistry is a rapidly evolving field that has seen many advancements and ongoing research has continued to develop new and innovate techniques to help people maintain oral health and function of their teeth (Yeung and Leung, 2018). The basics of tooth structure and development of dental disease are essential to understanding how a tooth can be regenerated. The need for advancements in dentistry in combination with current stem cell therapy research has led to the development of the field: regenerative dentistry (Duailibi et al., 2006).

### *Tooth Structure and Development*

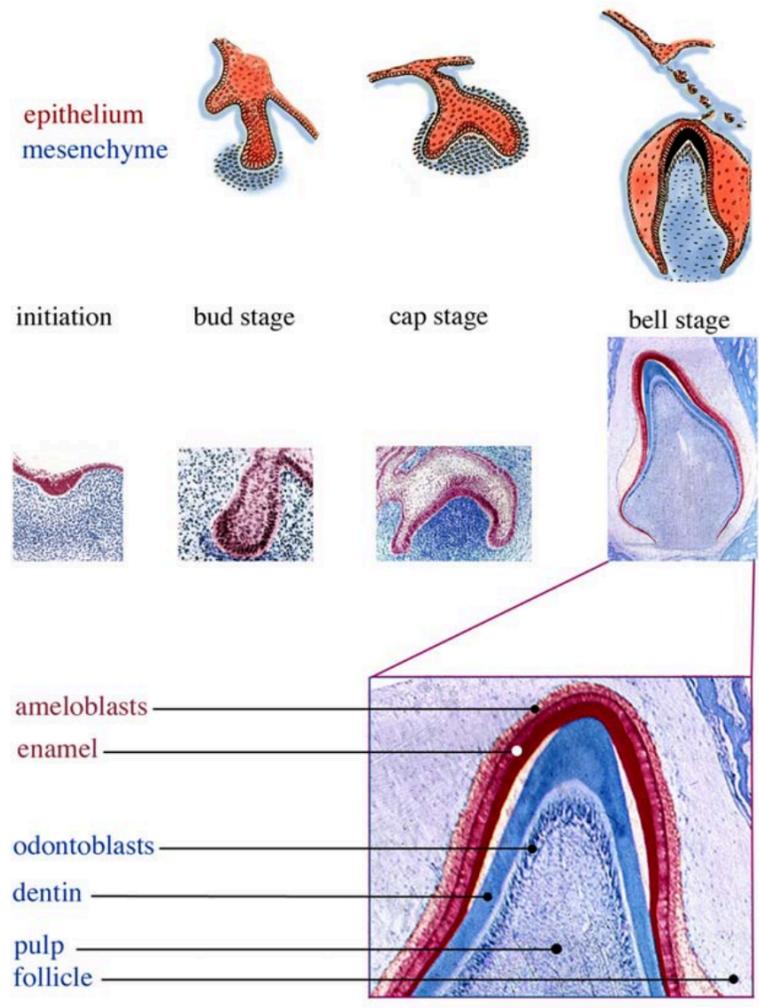
A tooth has three main structures a crown, neck, and root (Brudevold et al., 1960). The exposed crown at the top of the tooth is made of enamel which is composed of three organic substances known as calcium phosphate, calcium hydroxide, and calcium carbonate. Just below the crown is the neck which is surrounded by the gingiva and it joins the crown to the root of the tooth. The root of the tooth fits into the alveolar process of either the maxilla or the mandible.

The enamel is a hard, non-living tissue so it can no longer remodel or repair itself although it is resistant to decay and it is secreted by ameloblasts (Balic and Thesleff, 2015). Beneath the enamel is a structure called dentin. Dentin is made by odontoblasts or osteoblasts. It is made of mostly inorganic substances and houses dentinal tubules which are filled with tissue fluid. This structure encloses the pulp cavity which is lined by the odontoblasts. The pulp consists of pulp canals which house the blood vessels and where the nerves enter, and it is surrounded by connective tissue. The tooth itself is attached to the bone by cementum or the periodontal membrane which is made of hyaluronic acid (Li et al., 2017).

Tooth formation is a continuous process known as odontogenesis (Figure 1). This process involves the expression of more than 300 genes by signal molecules that regulate the sequential and reciprocal interactions between the epithelium and mesenchymal tissues through various stages of development (Bei, 2009). Teeth develop twice during childhood. Early on, there is a lining of ectoderm in the mouth which develops a C-shaped thickening, this is known as the dental lamina and it eventually moves into the underlying mesoderm. Tooth buds migrate down into the underlying mesoderm and detach from the surface and create teeth entirely within the maxilla. As the tooth bud moves into the underlying mesoderm it remains connected to the surface by the dental lamina and as it moves further in it forms a bell shape. It is then called the enamel organ which will produce the enamel (Bluteau 2007). The bell-shaped structure produces two laminae, the outer enamel epithelium and the inner enamel epithelium.

These layers are not yet mineralized enamel but will facilitate producing it. In between these layers, is the stellate reticulum which keeps these layers separate. As

development proceeds, the bud of permanent tooth will grow off the dental lamina which rescinds, degenerates and leaves the enamel organ completely free from the outside epithelium. At this point, the inner surface of the bell-shaped structure produces cells called ameloblasts. These cells will lay down the enamel which is the mineralized structures that covers the teeth (Caruso et al., 2016). Underneath those cells are another set of cells that line the papilla of the tooth called odontoblasts. These produces another mineralized structure known as dentin which will form the majority of the tooth. Odontoblasts surround the dental pulp at the center of each tooth. The dental pulp contains vessels and nerves for the tooth. The alveolar bone starts developing around the enamel organ, as the ameloblasts are forming more enamel and odontoblasts keeping producing more dentin. This continuous process forces the tooth to get closer to the surface, whereas the permanent tooth and its enamel organ keep migrating further in so that when it erupts it, the deciduous tooth will be displaced (Caruso et al., 2016).



**Figure 1. Schematic drawing and histological sections representing the different stages of odontogenesis.** Initiation Stage: when the dental lamina connects the developing tooth bud to the epithelial layer of the mouth Bud Stage: beginning of the 8<sup>th</sup> week of prenatal development for the primary dentition. Cap Stage: proliferation occurs to the point that it forms a cap around the dental papilla. Bell Stage: crown morphology is determined and the first dentin layer is formed/calcified. Figure taken from Bluteau et., al 2008

### ***Dental Caries Formation***

The prevalence of dental caries and periodontitis is found in a significant portion of the global population. These two factors are the leading cause of tooth loss. In the US alone, about 120 million people are missing at least one tooth (Frencken et al., 2017). Caries is the result of a variety of factors including diet, oral hygiene and most importantly the bacteria present in the oral cavity (Alvarez, 1995). Normally there is a balance between pathogenic factors that cause demineralization and protective factors that cause remineralization. The surface of enamel can attract various bacteria and various salivary glycoproteins producing a biofilm known as the pellicle layer. These bacteria come into contact with sucrose and use them for energy and to attach more firmly to the surface and form dental plaque (Alvarez, 1995). Certain harmful bacteria such as *Streptococcus mutans* or *Lactobacilli* can settle on the pellicle layer of teeth and attach to teeth using adhesions. Individual bacteria multiply and form larger microcolonies which create a biofilm. If the pH of the environment surrounding the tooth is above 5.5 the enamel will not demineralize. One factor that tips the progression towards caries is less bicarbonate-rich saliva. Sugar is a key component of demineralization since some bacteria ferment sucrose and that produces lactic acid as a byproduct. Acidic food and drinks can also cause the pH to lower and damage the teeth in two ways (Bluteau et al., 2007). If the pH of the saliva decreases, bacteria that thrive in a low pH start to dominate and as dental plaque grows thicker the oxygen level near the enamel decreases and that favors an environment for facultative anaerobic bacteria which can metabolize sugars. Lactic acid will soon build up and decrease the pH on the enamel. Calcium and phosphate start to dissolve into the fluid-filled channels and that starts the

process of demineralization (Frencken et al., 2017). Every time sugar enters the mouth, bacteria produce more acids. This destruction into the subsurface continues until the surface gets so thin that it collapses. Bacteria can then penetrate all the way to the dentin which then becomes infected and restorative procedures need to be implemented (Frencken et al., 2017).

### ***Current Restorative Techniques***

Previously the treatment for a caries infected tooth would have just been simple extraction but as new restorative techniques have developed, dentists have moved to procedures known today as fillings, root canals, though in some cases extractions are still necessary (Yeung and Leung, 2018). Fillings are used when a portion of the tooth has decayed and needs to be filled. Depending on the type of filling material, one may last longer than another but at one point most fillings have to be replaced due to everyday wear from eating and grinding. Fillings that are at the point of failure can leave entry points for bacteria to enter and work their way into the tooth causing more decay and eventually leading to another restorative procedure (Yeung and Leung, 2018). When the decay reaches the pulp, and endodontic procedure known as a root canal is needed. Since root canals are a more extensive procedure many complications can arise due to missed canals, fractures in the tooth itself and a variety of other factors eventually leading to either a retreat of the infected tooth or again an extraction of the tooth (Otsu 2014). The last restorative technique involves an extraction of the tooth first and replacement with synthetic structure known as an implant. The success of this procedures can be affected by a variety of factors. In some cases, improper dental hygiene after this procedure can

lead to infection or even bone loss. Since this method does not reproduce a natural root structure, jaw bone resorption occurs frequently (Otsu 2014). Although these procedures have been highly successful in treating caries and replacing teeth after extractions, they can produce variable outcomes and researchers are now shifting their focus to a whole different approach altogether.

### ***Basis of Stem Cell Therapy***

Stem cells are a highly studied subject in regenerative medicine and offer potential in every area of the healthcare industry (Fadini, Agostini, Avogaro, 2010). There has been a significant interest in changing the way doctors treat patients moving towards a more personalized approach based on an individual's specific needs. Researching stem cells was the first step in developing this new wave of thinking. These cells are known as "progenitor or precursor" cells and they have two key characteristics that set them apart from all other cells. They are able to differentiate into multiple lineages and can self-renew. They do not have any specific structure of functions. They do have the potential to become any other kind of cells in the body. The body uses stem cells to replace worn-out cells when they die. Scientists have been continuously researching to find ways in which to use stem cells to create new tissue and replace organs that are damaged by injury or disease. These cells have unparalleled and infinite applications and they helped develop the field know today as regenerative medicine (Fadini et., al 2010).

## ***Regenerative Medicine***

There has been a shift in the focus of regenerative medicine from improving restoration procedures to researching the steps needed to take regenerative dentistry into the clinical setting. Initial research only started about 15 years ago, while some of the most groundbreaking and promising findings have only been found just in the past few years (Otsu 2014).

The basis of a majority studies involves dental pulp stem cells (DPSCs) which have shown to have properties that can help with tooth damage since teeth themselves have limited repair capabilities. Dental pulp stem cells have two essential biological properties that make them an ideal subject in regenerative medicine. First, they are able to produce functional dental tissue and also have the ability to differentiate into various mesenchymal cell types. Since these cells are highly accessible and also offer high proliferation rates, they are the basis of regenerative medicine along with various other cell types. Stem cells from human exfoliated deciduous teeth (SHED) along with stem cells from the apical papilla (SCAP), and periodontal ligament stem cells (PDLSCs) are all types of dental stem cells that share the same unique that properties that make them ideal for experimentation. These cells facilitate research to help gain a better of understanding of how to generate a viable tooth. In dentistry, there is a clinical necessity to find an alternative from restorative procedures towards regenerative techniques that could produce healthy and viable teeth (Otsu 2014).

### *Specific Aims and Objectives*

There have been significant efforts to bring stem cell therapy into clinical practice specifically in medicine, but there has not been the same push for dentistry since it is a fairly new concept in comparison. Methods to maintain oral health and function have seen great advancements and therapies using stem cells may offer a promising approach to finding alternative treatments for patients.

This literature review aims to:

- Discuss if dental stem cells are a potential source for regeneration
- Identify the isolation techniques of dental pulp stem cells
- Discuss the potential therapeutic applications for dental stem cells
- Discuss gaps in the current research that still needs to be conducted to advance the field
- Discuss the future prospects of dental stem cells in medicine

This review will provide additional knowledge to the growing field of regenerative medicine supporting the further advancement of stem cell usage in both medicine and dentistry.

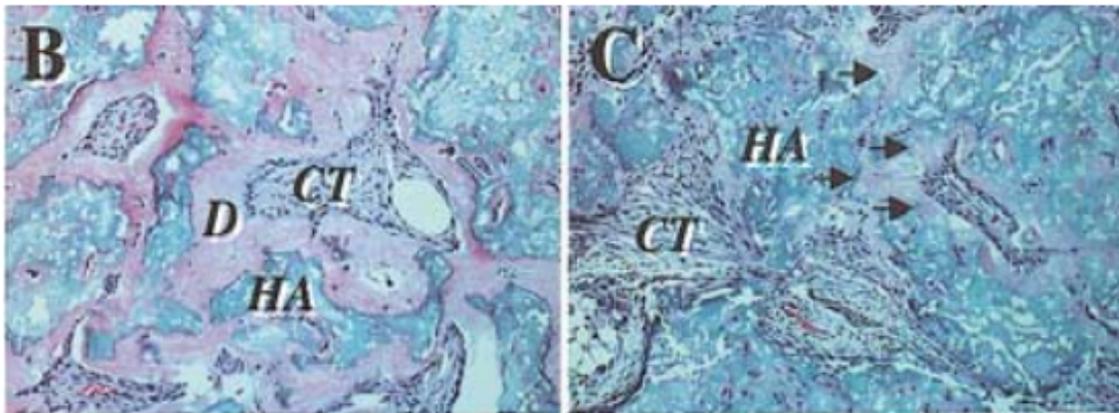
## LITERATURE REVIEW

### *Preliminary Findings - Dental Pulp Stem Cells*

The pilot study that provided the stepping stone for tooth regeneration was conducted in 2000 by researchers at the National Institute of Health. The study focused on comparing the generative capabilities of two different types of cells; DPSCs and human bone marrow stem cells (BMSCs) (Gronthos, Mankani, Brahini, Robey, Shi, 2000). Previous research showed that odontoblasts are associated with dental repair and their origin was known to be pulp tissue, but no associated precursor cell has been discovered yet (Gronthos et al., 2000). Extensive research had already been conducted regarding stem cells and their origin was known to be bone marrow. By analogy, researchers speculated that dental pulp could be the source of dental stem cells. In prior experiments a colony-forming efficiency assay was used to isolate BMSCs and the same technique was used in this experiment to isolate DPSC's. It confirmed that DPSCs were clonogenic and in comparison, to BMSCs they were shown to multiply faster *in vitro*. BMSCs were previously known to be able to differentiate into multiple lineages including adipocytes and osteoblasts. The main difference noted between these types of cells was that BMSC's had the ability to form a high density of calcified molecules and adipocytes while DPSC's formed a low quantity of granules and no adipocyte formation concluding that they are not capable of differentiating into a different lineage. This showed that the DPSCs failed to generate a functional tissue with the right architecture. To further test the regenerative capability of DPSC's, cells were placed in immunocompromised mice eventually forming a tissue that resembled pulp embedded

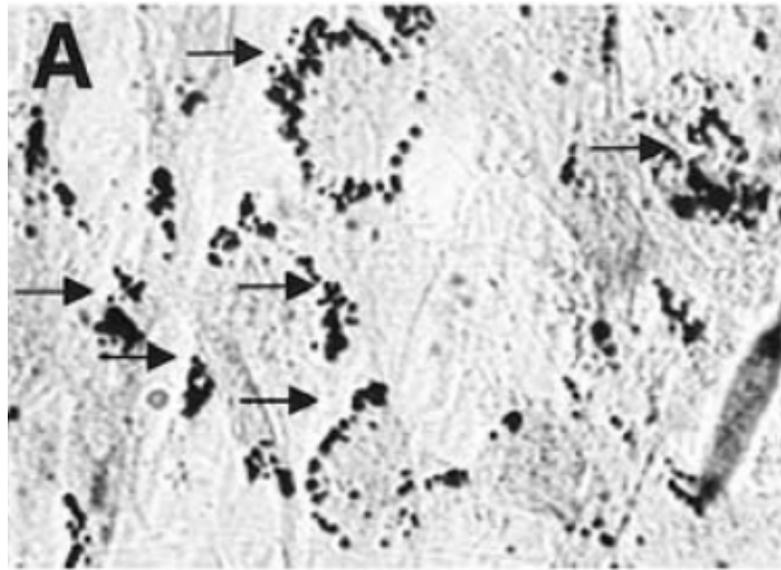
with a similar product of odontoblasts, known as dentin. This study was the first to exemplify the potential of DPSC's by comparing them to the regenerative capabilities of human bone marrow stem cells. The isolated cells satisfied two main criteria when characterizing postnatal somatic stem cells (Gronthos et al., 2000).

Further research by Gronthos et al. (2002) focused on investigating the key properties needed for dental pulp stem cells to be characterized as a potential source for regeneration. To determine if DPSC's were multipotential and had the capability to proliferate into an undifferentiated state, stromal cells were re-isolated from previous DPSC's transplants and the majority were shown to have produced ectopic dentin. The other remaining cells formed none to only minute quantities of dentin (Figure 2) (Gronthos et al., 2002).



**Figure 2. Cloning Efficiency of 12 single-colony derived DPSC strains.** B. 2/3 of the single-colony-derived DPSC strains generated abundant ectopic dentin C. Remaining 1/3 single-colony-derived DPSC strains that formed moderate to low amount of dentin. Figure amended from Gronthos et al., 2000).

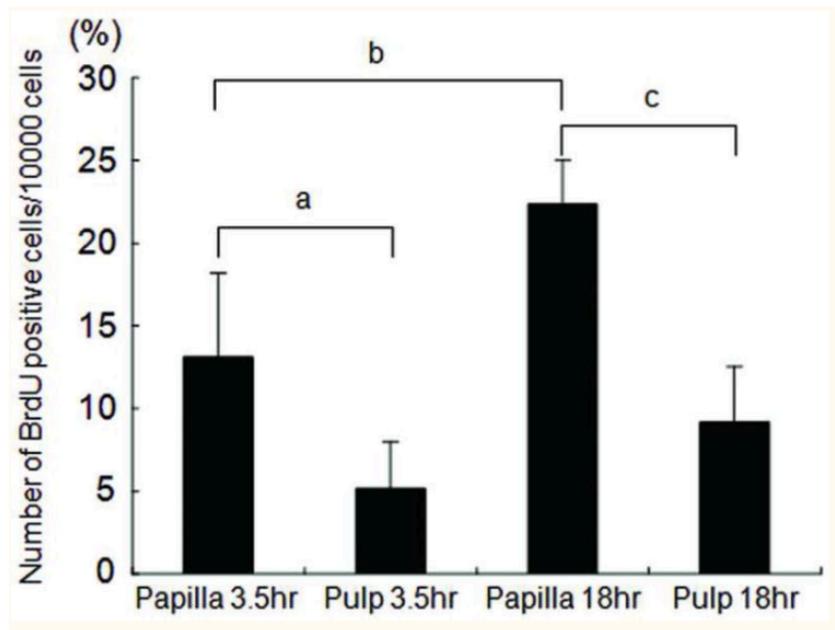
Furthermore, another key characteristic of stem cells is known as multi-lineage differentiation (Gronthos et al., 2002). Previously, it was shown that these cells were not able to differentiate into adipocytes but in this study after culturing the cells in a more potent adipocyte inducing medium, the cells were able to differentiate into adipocytes. This was shown after a 5-week culture that produced oil red O-positive lipid clusters in combination with an up-regulation of two adipocyte specific markers PPAR $\gamma$ 2 and lipoprotein lipase that confirmed by performing a reverse transcription polymerase chain reaction (RT-PCR). This confirmed the ability of DPSC's to differentiate into a specific lineage when induced with certain mediums (Figure 3) (Gronthos et al., 2002).



**Figure 3. Adipocyte differentiation of human DPSC's** In a adipogenic medium DPSCs formed Oil red O-positive lipid clusters (arrows) and showed a significant up-regulation of lipoprotein lipase (LPL) and PPAR $\gamma$ 2 in the experimental group as compared with the control group by RT-PCR. Figure amended from Gronthos et al., 2002.

Researchers subsequently discovered a novel type of dental stem cells that were isolated from the apical papilla named SCAP (Sonoyama et al., 2008). These cells from the papilla had been previously found to be a source of odontoblasts and eventually formed pulp tissue. Researchers found that the apical papilla could be removed from the pulp quite easily indicating a loose connection. When researchers looked at this region histologically, they found an abundance of cells. The researchers wanted to compare these cells to the stem cells found in the pulp. The main key difference noted was the rate of cell proliferation (Sonoyama et al. 2008). Cell proliferation is sometimes measured by using thymidine analog BrdU (5-bromo-2'-deoxyuridine) following its integration into DNA that has been newly synthesized and afterward its detection with an anti-BrdU antibody.

After placing the apical papilla in cultures, cells that were within the tissue showed more BrdU incorporation in comparison to those in the pulp (Figure 4).



**Figure 4. BrdU positive cells in the pulp and apical papilla** Results of the one-way factorial ANOVA followed by Fisher's exact test that shows that the proliferation rate was higher in the tissue of the apical papilla in comparison to the dental pulp of the same tooth at 3.5 hrs and 18hrs based on the number of BrdU positive cells. Error bars: SD; a,  $p=0.0091$ ; b,  $p<0.0001$ ; c,  $p=0.0002$ .

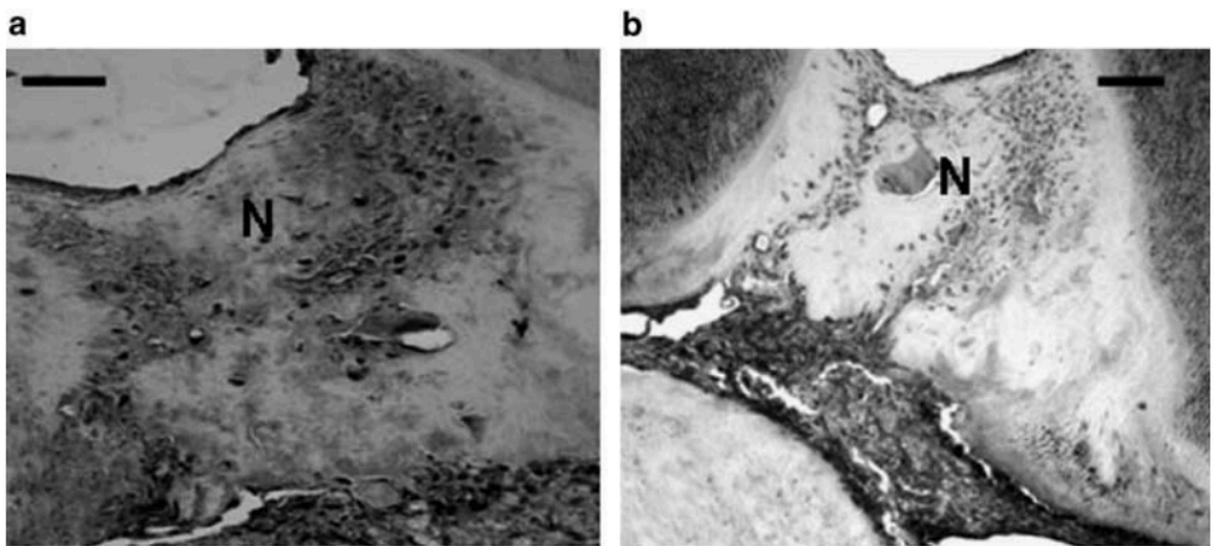
### ***Growth Factors/ Proteins and Activation***

Once DPSC's were found to possess the qualities needed to form dentin and also differentiate into multiple lineages, the research shifted to finding a technique that could be implemented in pulp to induce growth and form 3-D tissue *in vitro*. It has been shown in previous studies that an expression of a certain protein called bone morphogenic protein (BMP) is involved in the odontoblast formation process (Nakashima 1994). It was suggested that its role was regulatory and permitted the conversion of pulp cells to odontoblasts when exposed to a stimulus such as trauma. In 2004, a study conducted by Iohara and his team at Kyushu University, used a culture pellet system using porcine-

derived pulp from third molars to determine the effect of the protein BMP (Iohara et al., 2004). Researchers used a combination of hydroxyapatite/tricalcium phosphate and the pulp cells derived from porcine which was then implanted into immunocompromised mice. The monolayer culture was monitored and displayed a shape change of the cells. The porcine pulp cells grown in the monolayer culture underwent a characteristic process of dedifferentiation and redifferentiation. This indicated differentiation of pulp cells into odontoblasts was more complicated. In the next phase of the experiment, the culture pellet system was treated with BMP2 to see if there was production of dentin. The results displayed a higher number of dentin formation in the BMP2 pellet system in comparison to the control. This study by Iohara et al., (2004) was significant for two reasons as it was able to isolate stem cells from the pulp effectively with a new technique and was also manipulating growth with the protein BMP2. This demonstrates the possibility of cell therapy with BMP2 which could potentially lead to regenerative endodontic therapy (Iohara et al., 2004).

Another study investigated the role of dentin matrix protein I in the cytoformation of odontoblasts. Certain signal molecules are required for cells to differentiate and this experiment investigated the role of dentin matrix protein I (DMP1) (Almushayt, Narayanan, Zaki, George 2006). This noncollagenous protein has been found to be involved in mineralization. The undifferentiated cells in the pulp have the potential to cytodifferentiate into cells that resemble odontoblasts. To study the role of this matrix protein, a rat model was used. Researchers drilled holes in the molar of these rats to create cavities and expose the pulp. Three groups were tested; one with a recombinant DMP1 matrix, one with a calcium hydroxide matrix and then one with a collagen matrix

which served as the control. They were further subdivided into two groups. One group was incubated for two weeks while the other was incubated for four weeks. The cells formed a structure similar to odontoblasts and this was confirmed by measuring the level of specific markers such as dentin sialoprotein (DSP) and the formation of a collagenous matrix along with calcified nodules (Figure 5). The results showed that this protein was capable of acting as a signal molecule for cells that had not been differentiated (Almushayt et al., 2006).

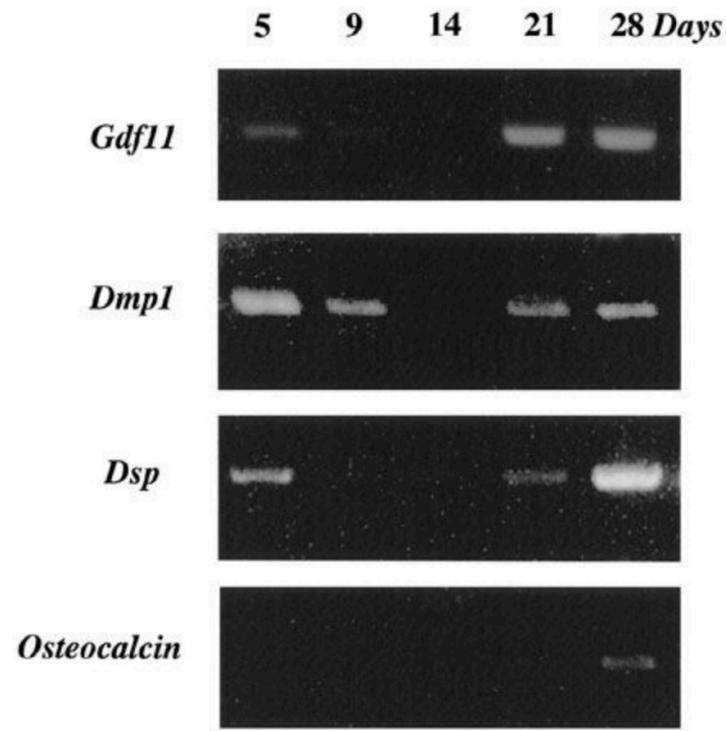


**Figure 5. Immunostaining with DSP and DMP1 antibody** (a) Samples from groups 1B showed that the cells at the site of new tissue formation expressed DMP1. (b) Tissue sections from group 1B stained positive for DSP which is a marker for terminally differentiated odontoblast cells.

In 2002, a study conducted by Nakashima and fellow colleagues investigated a certain growth factor and its role in stem cell differentiation. Growth differentiation factor 11 (Gdf11) was studied to see if it enhanced the regeneration and healing capabilities of pulp stem cells into odontoblasts (Nakashima, Miznuma, Murakami, Akamine 2002).

The experiment was done in dental papilla mesenchyme of mice in organ culture. Gdf11 was transfected to by electroporation and the results showed that this growth factor enhanced the expression of dentin sialoprotein (DSP). DSP is matrix protein that has a crucial role in the generation of dentin and differentiation of odontoblasts (Yuan, Yang, Wu, Chen Z, Chen S, 2010). The researchers used gene transfer to induce odontoblasts differentiation by *in vitro*. To measure the differentiation, three markers including dentin matrix protein 1 and dentin sialoprotein, and osteocalcin were measured all of which are known to increase expression during normal odontogenesis. *Gdf11* initially increased then did not reappear until days 21-28. *Dmp1* and *Dsp* showed a similar pattern and displayed a significant increase in days 21-28. *Osteocalcin* was only found on day 28 (Nakashima et al., 2002).

**Figure 1**



**Figure 6. RT-PCR analyses for Gdf11** (product size: 0.5 kb), *Dmp1* (0.5 kb), *Dsp* (0.4 kb), *Osteocalcin* (0.3 kb) and  $\beta$ -actin (0.5 kb) in the primary dental pulp cell culture.

In 2006, researchers at the University of Illinois discovered a novel dentin matrix protein consisting of 579 amino acids that were specific to odontoblasts and was subsequently given the name DMP4. The key distinction between this protein and other dentin matrix protein was a calcium binding domain and another unknown function that was not present in any other protein in that family. When odontoblasts are synthesized from precursor cells now known to be DPSC's, many proteins are secreted. Some that have collagenous properties and some that do not. The non-collagenous proteins were previously found to be involved in mineralization and these were grouped

into dentin matrix family. Researchers obtained a cDNA library to better understand the genes associated with dentin formation and odontoblasts. These cells were isolated from immortalized T4-4 and mouse preosteoblastic cells (MC3T3-E1 and grown in a medium. Once the poly (A) RNA was isolated from each type of cell, a technique called suppressive subtractive hybridization was implemented. A part of the cDNA of DMP4 was isolated and cloned to identify the primary sequence. A database search then revealed a corresponding gene in humans with 69% homology (Hao, Narayan, Muni, Ramachandran 2007).

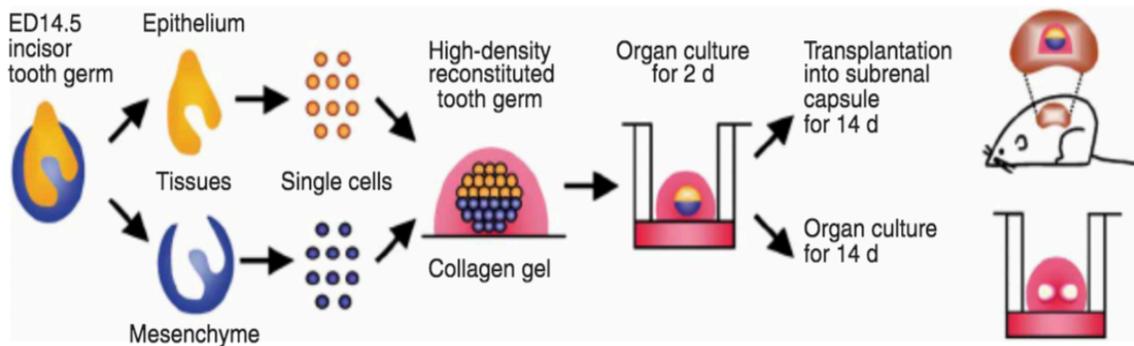
The next study focused on the activation of the precursor stem cells found in pulp in response to injury (Téclès et al., 2005). The focus was to determine the migration pattern after a noxious stimulus such as a cavity. To determine this, researchers used human third molars that had been subjected to cavity formation. The pulp was exposed BrdU labeling which identifies cell proliferation. The results showed that after a progression of four weeks, the cells migrated from a larger area to a more localized region near the cavity and showed little to no cells in teeth that had little to no cavity formation. The BrdU immunolabeling exhibited a gradient which was localized just around the vicinity of the cavity after two weeks but localized to the cavity at the four week time point. The authors concluded that injury or cavity formation was a stimulus for migration (Téclès et al., 2005).

### ***Isolation Techniques***

A study in 2006 by Yu et al. focused on the obstacle that there are a limited number of odontoblasts readily available in the pulp (Yu et al., 2006). The goal was to find a cell culture system that allowed DPSC's to differentiate into odontoblasts. The researchers tested the effect of a medium that came from developing tooth germ cells both *in vivo* and *in vitro* as well. Rat incisors were used to obtain DPSCs and isolated enzymatically. Once the cells were cultured in the medium, the researchers performed a qualitative comparison to odontoblasts. Many similar qualities were found between the two, including morphological shape, protein levels, and the formation of mineralized nodules. The structure contained dentin tubules creating a dentin-pulp complex in contrast to cells that were cultured in the control medium that produced tissue that resembled bone instead. The researchers were able to create an environment that favored the differentiation into odontoblasts in an effective manner (Yu et al., 2006).

One of the most important studies in this field was conducted in 2007. This study explored a bioengineering technique termed the organ germ method (Nakao et. al 2007). The purpose of this study was to use tooth and whisker follicle as models to improve bioengineering methods using mesenchymal cells. First, single cells from epithelial and mesenchymal tissues that were derived from an incisor tooth germ that was at the cap stage, were isolated. The cells were then injected with a collagen gel drop to form the right cell compartmentalization (Figure 7). Once they observed a formation of tooth germ they transplanted it into the subrenal capsules of mice for a ten day period where the histology was observed. What was discovered was that this germ could produce the

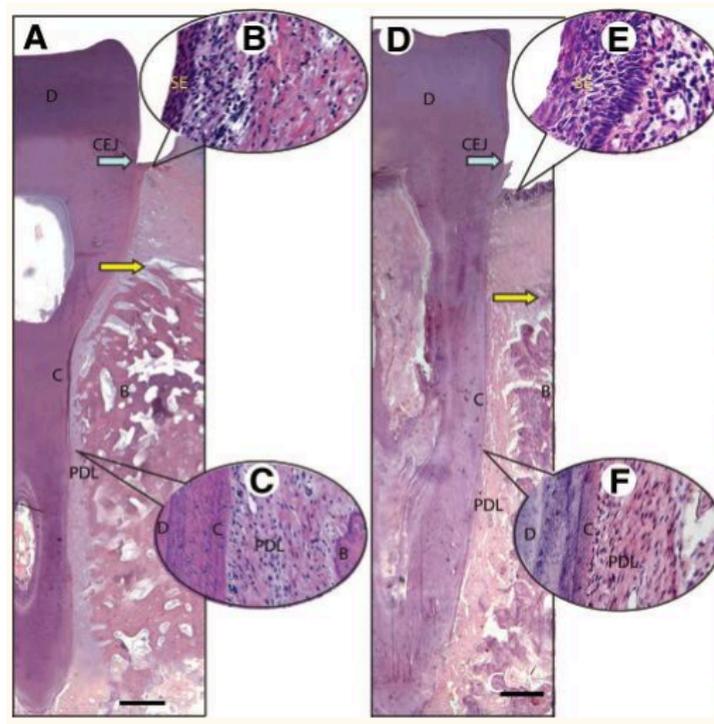
basic structural elements of tooth such as dentin, odontoblasts, enamel, dental pulp, and blood vessels. This was found in a 100% of all 50 transplants that were done. The next phase of the study was to determine if the tooth germ could be transplanted successfully and reform in a tooth cavity after removing the mandibular incisor in an 8- week old mouse. It was shown that the primordia could generate a single tooth after a 14-day incubation, both *in vitro* and in the subrenal capsule. The transplantation of explants with the epithelial cells and GFP-transgenic mouse-derived mesenchymal also displayed the same result by producing a bioengineered tooth. The tooth had all the correct structural components such as the periodontal ligament, blood vessels, and nerve fibers (Nakao et al., 2007).



**Figure 7. Schematic of the tooth germ method.** Tissue from the mesenchyme and the epithelium was isolated from incisor tooth germ of ED14.5 mice and reduced to single cells. The tooth germ was then reconstructed using these cells that showed cell compartmentalization at a high cell density. The explants were either transplanted beneath a subrenal capsule or were continuously cultured. Figure amended from Nakao et al., 2007.

### *Clinical Applications*

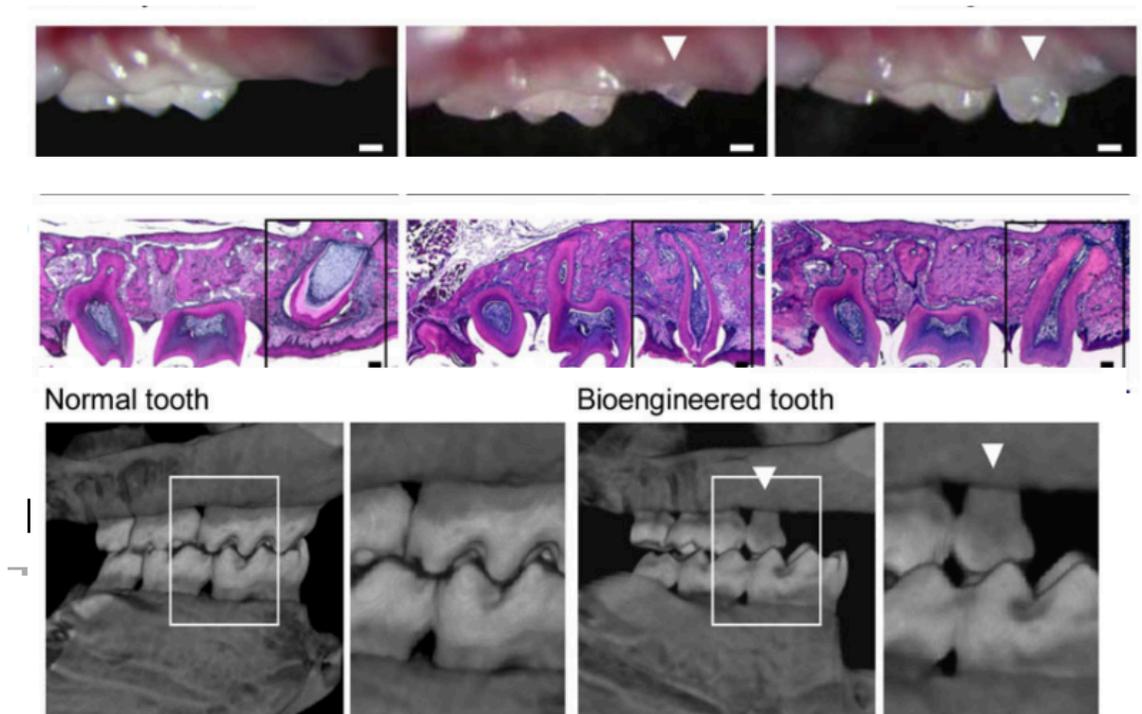
Researchers looked at using a different type of stem cells called periodontal ligament stem cells called (PDLCS), not for tooth regeneration but to treat periodontitis (Liu et al., 2008). Liu and his colleagues (2008) used a porcine model of periodontitis and extracted PDLCS from teeth from pigs and then cultured them to produce a substantial amount of cells. They were then transferred to an area of infected gums that were created surgically and displayed regeneration potential of periodontal tissues. In order to view the amount of periodontal tissue generation, and a hematoxylin and eosin stain (H&E) stain was used. There was significantly less inflamed tissue and production of bone and cementum were also seen ( Figure 8) (Liu et al., 2008).



**Figure 8. H&E staining of periodontal tissue.** A. Sulcular epithelium with less inflamed tissue. B. Periodontal ligament which shows normal structure development. C. Formation of cemento-apical junction. D. Regeneration of periodontal ligament E The sulcular epithelium in PDLSC-mediated group was thicker, and the epithelial pegs and dermal papillae were short and blunt, with fewer inflammatory cells F. New bone, cementum, and periodontal ligament were regenerated in the periodontal defect area. Figure amended from (Liu et al., 2008).

Further research focused on not only producing a bioengineered tooth with normal structure and components but Etsuko Ikeda as his colleagues (2009) designed an experiment to produce a fully-function tooth as well. These functions include mastication, compatibility with surrounding tissue and response to sensory stimulation. This study was actually used as a model for organ development since the process of odontogenesis has overlap with this area of study. The initial development process that occurs in the germ and the reciprocal interactions in the embryo between epithelial and

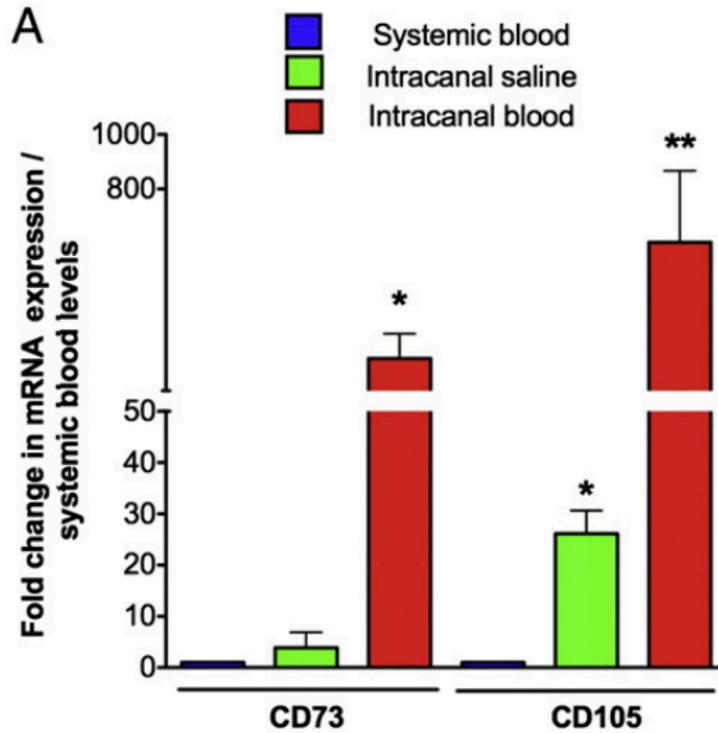
the mesenchyme has been the basis of tooth replacement, but it can also be used a model to further develop clinical strategies for organ development since there is much overlap between the two. Using the organ germ method, and placement of a bioengineered tooth germ into the alveolar bone in an adult mouse (Figure 9). The transplanted germ was culture for a week until it had developed to the bell stage and then placed in the 8-week old mice. It took an average of 50 days after transplantation to obtain correct occlusion with the plane. This tooth erupted with the correct structure but also with hardness needed for mastication and response to noxious stimuli (Ikeda, E., Morita, R., Nakao, K., Ishida, K., Nakamura, T., Takano-Yamamoto, T. & Tsuji, T. 2009).



**Figure 9. Bioengineered tooth (photograph and histology)** A. Oral photograph of eruption/ occlusion. B. Histology of eruption /occlusion showing a correct structure of odontoblasts, alveolar bone, dentin, ameloblasts, and blood vessels. C. Micro CT images of normal vs bioengineered teeth

Lovelance et al. focused on a potential application of stem cell research and how it could be used to attempt a regenerative endodontic procedure (Lovelance, Henry, Hargreaves, Diogenes, 2011). The goal of this study was to regenerate the vitality of the pulp and to induce root development. This was the first study to test how beneficial stem cells could be in a canal space. Lovelance and colleague designed an experiment to evaluate whether regenerative endodontic procedures can transport stem cells into the canal space of deciduous teeth in young patients.

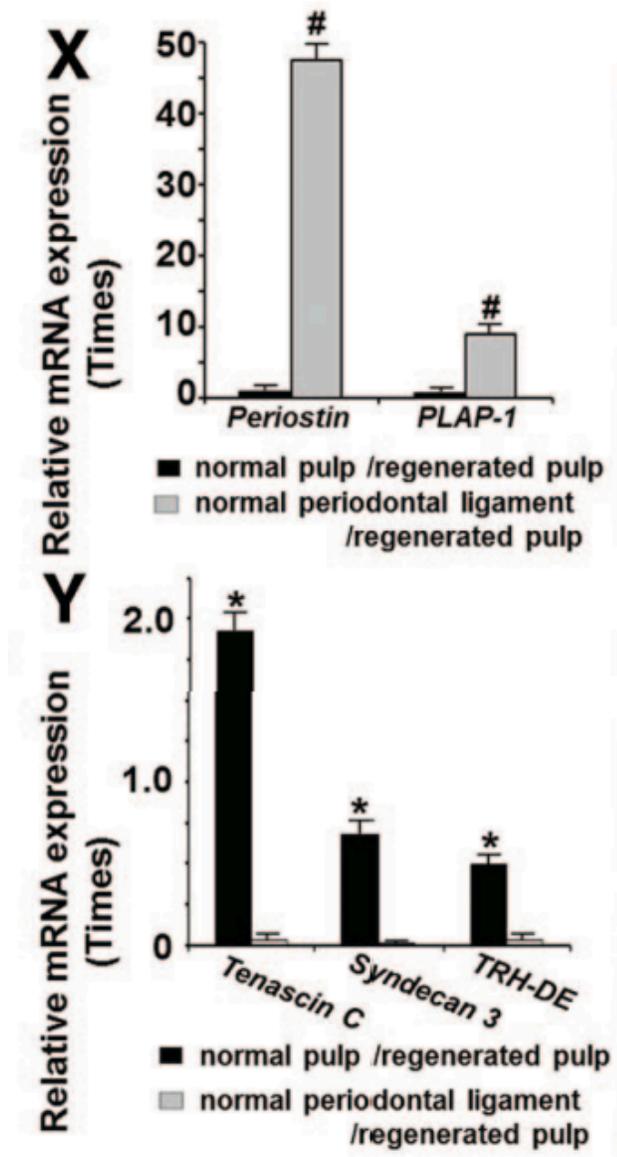
In the first appointment, an antibiotic paste was used with sodium hypochlorite irrigation (Lovelance et al., 2011). The next appointment was not until a month later where the canal space was irrigated with saline and bleeding was induced. Immunohistochemistry and reverse-transcription polymerase chain reaction was used to compare the transcript of genes in the root canal versus those found in samples from the systemic circulation. Stem cell markers are genes and their protein products are used to identify stem cells. The stem cell markers used in this experiment were CD73 and CD105 and they were found at a level of 600-fold in comparison to values of systemic blood samples (Figure 10) The researchers found that by inducing bleeding, it triggers the accumulation of undifferentiated stem cells in the canal space where they could potentially regenerate pulp tissue (Lovelance et al., 2011).



**Figure 10. Upregulation of stem cell markers CD73 and CD 105 due to inducing bleeding in canal space** Systemic blood, saline irrigation, and intracanal blood samples were collected during second visit of regenerative procedures. Real-time RT-PCR was performed by using RNA isolated from each sample as template. Expression of CD73 and CD 105 was up-regulated after evoking bleeding Figure amended from Lovelance et al., 2011.

Iohara and her team in 2013 designed an experiment to see if dental stem cells from the pulp could be safely transplanted and if that would be effective in treating pulpitis (Iohara et al., 2013). Canine pulp stem cells were isolated using novel method utilizing granulocyte-colony stimulating factory (G-CSF), cell mobilization, and a colony forming assay. In order to model this experiment as a precursor to a clinical trial, multiple tests were conducted to determine if the cells were up to clinical standards. They were tested

to see if they had to appropriate expression of stem cell markers including *SOX2* and *Stat3*. In order to evaluate the safety before transplantation the karyotype, sterile nature and viability was assessed. After a pulpectomy in dogs, the pulp stem cells were transplanted, and the efficacy was measured. The results showed regeneration of the pulp tissue with the appropriate vasculature that filled the canal of the root, and dentin was formed in the coronal region. There was also a decrease in apoptotic and inflammatory cells. The pulp regeneration was confirmed by assessing the gene expression. The researchers compared the mRNA expression of specific markers in normal periodontal ligament and the regenerated tissue (Figure 11). The expression of *Tenascin*, *Syndecan 3*, and *TRH-DE* was significantly greater by 5 folds or more in the regenerated tissue. This experiment showed G-CSF in combination with a conditioned medium of pulp stem cells stimulated cell migration and demonstrated prerequisites of safety for future clinical trials (Iohara et al., 2013).



**Figure 11. Relative mRNA gene expression of normal pulp/regenerated pulp and normal periodontal ligament.** The mRNA expression levels of *periostin* and *asporin/periodontal ligament-associated protein 1 (PLAP-1)* were much higher in the normal periodontal ligament than in the regenerated tissue on day 28. The expression levels of *tenascin C*, *syndecan 3*, and *TRH-DE*, known to be highly expressed in pulp, were higher in the regenerated tissue than in the periodontal ligament. Figure amended from Iohara et al., 2013.

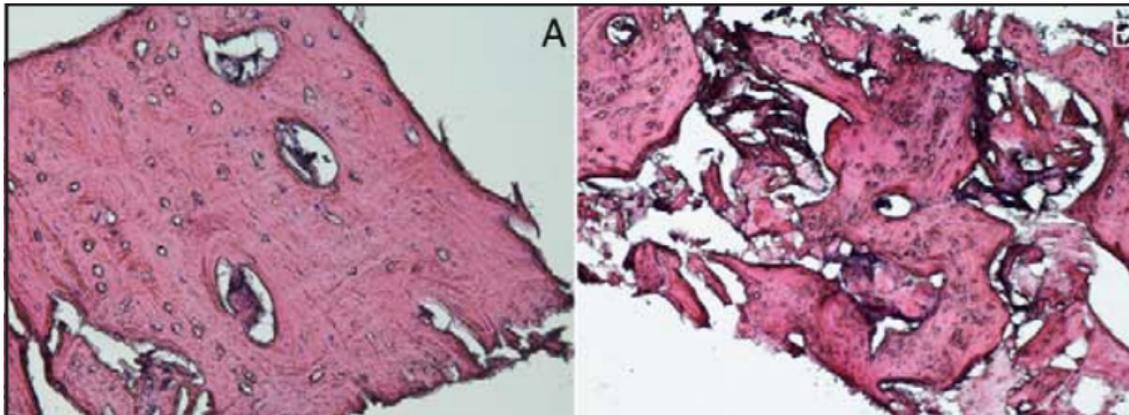
In order to evaluate the safety of the transplantation, a toxicology report was done and found no negative effects along with normal appearance, food consumption, and normal body weight of the dogs used for this experiment. Urine and serum sample were normal, and a histological evaluation revealed no abnormalities in any organs a month after transplantation.

### ***Pre-Clinical/Clinical Trials***

In 2018 a randomized clinical trial was conducted at the University of Turin which looked at 29 patients with chronic periodontitis. The goal of this study was to see if DPSC's that were implanted into intrabony defects would induce periodontal regeneration (Ferrarotti et al., 2018) . In each patient, one viable tooth was extracted to obtain the dental pulp in order to isolate the stem cells. Tests sites for 15 patients, included the stem cells in combination with collagen sponge while the 14 control patients only received the collagen sponge. Radiographic images were taken at the time of the procedure and then 6, 9, and 12 months afterward. The experimental group had a reduction in the probing depth from an average of 4.9mm to 3.4mm. There was also a gain of clinical attachment from 2.9mm to 4.5mm (Ferrarotti et al., 2018)

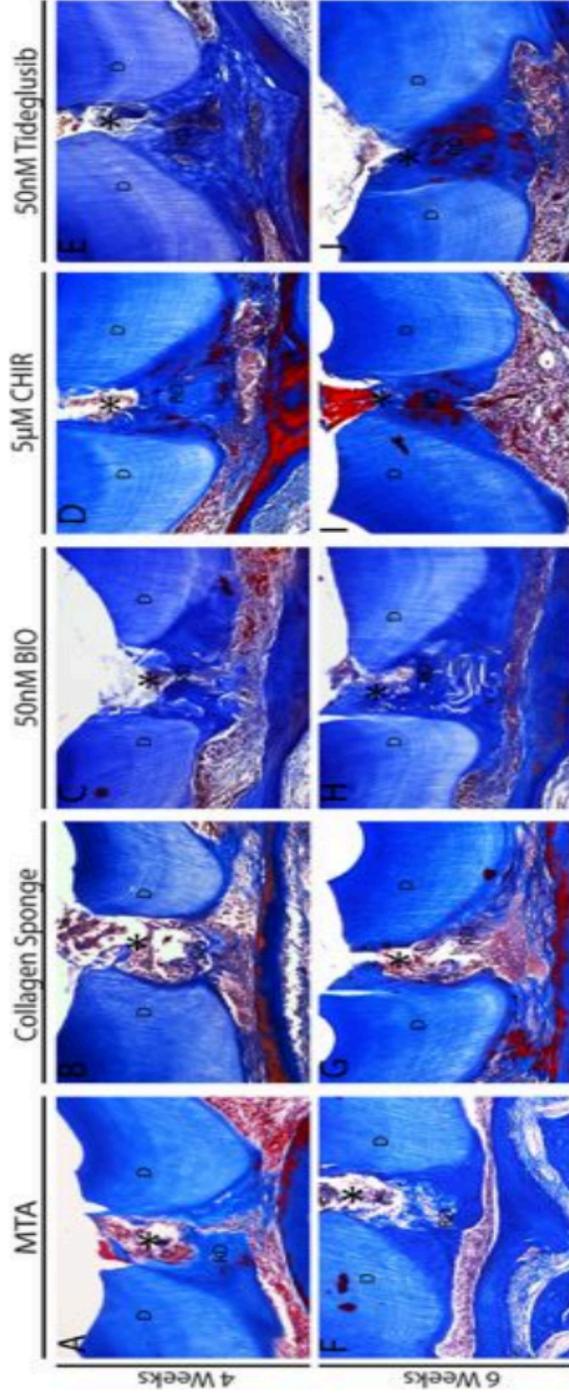
Another notable human trial was conducted by Riccardo d'Aquino and his team which focused on using dental pulp stem cells and a collagen sponge scaffold for bone tissue repair in patients that needed their third molars extracted (d'Aquino et al., 2009). All patients recruited had a defect due to impaction on the third molar that prevented bone repair after extraction and eventual loss of the second molar. The first step in this study was to extract third molars in order to isolate the stem cells and it was then seeded

on a collagen sponge scaffold. This material was then filled at the site post extraction of the third molar. To examine the results, radiographic imaging and clinical imaging were used. There was increased vertical repair and total restoration of periodontal tissue near the second molars. When the area was looked at histologically, there was a total regeneration of bone as the injured site, this was after a year of grafting (Figure 12). In comparison to the control site where bone resorption occurred, there was substantial bone formation at the injury site. The H&E staining revealed highly vascularized bone with complete Haversian canals completely surrounded by lamellar structure at the injury site (d'Aquino et al., 2009).



**Figure 12. Histological staining of extraction site** A. H&E evaluation of injury site 3 months post-operation B. H&E evaluation of test site (control) 3 months post operation.

The most recent development and highly noted study by researchers was done at Kings College in London in 2017 (Neves et al., 2017). Researchers used third molars of wild-type mouse to model a clinical approach that could develop into a cost-effective therapy dentist could use in their practice. An injured site was created on the two upper first molars and it was capped by one of five materials to seal the site. Researchers wanted to target the Wnt/ $\beta$ catenin signaling pathway which was known to be a universal early response to tissue damage. Using collagen sponges as a delivery system, a small molecule glycogen synthase kinase (GSK-3) antagonists was given in small doses to activate the pathway and stimulate reparative dentin formation. The delivery system gradually degraded over time and left behind dentin. The formation of dentin was induced by activation of the stem cells already present in the tooth pulp. The results showed increased mineralization with the highest associated with mineral trioxide aggregate (MTA) treatment (Figure 13) (Neves et.,al 2017).



**Figure 13. Micro computed tomographic scanning** -Dentin formation at 4 and 6 weeks with each of the 5 different capping materials. A. Dentin formation underneath material. B. little dentin formation in pulp. C, D, E, H, I, . Increased dentin formation in pulp at 4- and 6-weeks J. Tideglusib treatment shows complete repair with vital dental pulp after 6 weeks. Figure amended from Neves et al. 2017.

## DISCUSSION

Odontoblasts have some repairing and remodeling capabilities after development of the primary dentition (Gronthos et., al 2000). However, after eruption, both deciduous and permanent teeth are subject to many diseases and physical trauma, hence the development of the field of dentistry. Right now, dentists focus on repairing and replacing teeth with synthetic materials that are subject to many different of stresses that can cause failure. The effort to combine dentistry with regeneration hope to revolutionize dental care.

Stem cells have been studied extensively and it is their properties that demonstrate their potential in regenerative medicine. The speculation that there might be progenitor cells in the dental pulp that were similar to bone marrow stem cells was the first step in developing the field of regenerative dentistry. This first study that experimented on the basis of this speculation was able determine the existence of progenitor cell in the dental pulp (Gronthos et al., 2000). That study also highlighted key characteristics of those cells as being highly proliferative, clonogenic, and capable of regenerating tissue. These properties demonstrate their potential for dentinal repair. Further research demonstrated their capability of self- renewal *in vivo*. More importantly, the ability to create more stem cells when induced by certain signals suggest that dental stem cells can commit to odontoblasts lineage (Gronthos et al., 2002). These studies were significant because they demonstrated the possible regenerative priorities that could be further researched and exploited. Besides DPSC's another type of stem cell was discovered in the apical papilla termed SCAP. This was an important discovery because there not much literature on the apical papilla and it was compared to the proliferation in dental pulp cells, it was found to

have a higher rate indicating another source for potential regeneration of tissue damage and remodeling (Sonoyama et. al 2009). These cells met the criteria for stem cells due to their capability of self-renewal and eventual differentiation into specialized stem cells.

Once potential dental stem sources were discovered, there was ongoing research concerning certain activators, proteins and growth factors that were all involved in the process of tissue generation and odontogenesis. Particularly, bone morphogenic protein was of high interest due to its known role in odontoblast formation process (Iohara et al., 2004). The novel culture-pellet system that was invented during this experiment, led to results that demonstrated that a large amount of reparative dentin can form in bovine pulp that has been amputated when supplemented with bone morphogenic protein- 2. This study was important for two reasons as it developed a new technique to isolate human pulp stem cells and induce their growth efficiently and further enhance the process with BMP2. This was one of the small steps towards using this methodology in a clinical setting (Iohara et al., 2004). Another study looked at cytoformation of odontoblasts and the role of a certain protein called DMP1, this was significant as the results showed this protein could induce undifferentiated cells at repair site. The *in-vivo* potential of DMP1 to act as an inducer demonstrated the components necessary to produce dentin. This study showed that contact with DMP1 membrane could potentially enhance mobilization of cells that are exposed to trauma or disease as the increase blood flow drew cells into the area (Almushayt 2006). Gdf 11 is an important growth factor that was also looked at to see if it enhanced the regeneration and healing capabilities of pulp stem cells (Nakashima et. al 2002). This study was significant because the reparative dentin formed in the damaged pulp in canines, provided a model for gene therapy regarding endodontic

procedures. In 2006, a novel dental matrix protein was discovered subsequently named DMP4 since three other proteins with similar characteristics had previously been discovered prior (Hao et. al 2007). This study demonstrated that this protein had the potential to induce differentiation in precursor cells in the mesenchyme that could have potentially have odontoblasts function and it was also discovered to expressed at high levels in odontoblasts in comparison to other cells such as ameloblasts. This indicates that protein is also a key regulator in odontogenesis.

Other recent studies focused on the isolation techniques and method needed to bring stem cell therapy into clinical practice. A problem many researchers encountered was the number of odontoblasts that are readily available in the pulp. Researchers discovered an innovate culture medium that could help facilitate that problem. By using a medium from developing tooth germ cells to cultivate a cell culture system, Jinhua Yu and his colleagues were able to create an odontogenic environment that could help facilitate investigation in every study afterward that needed a substantial supply of odontoblast (Yu et al., 2006). One of the most notable and important advancements in this field is known as the organ germ method. This technique allowed a generation of a structurally correct tooth, both in vitro and in vivo. This study was a huge step forward in understanding regenerative therapies and the advancement led to progress that eventually utilized this method in clinical trials (Nakao et al., 2007).

Utilizing dental stem cells eventually branched off from solely attempting to regenerating viable teeth to curing dental diseases such as periodontitis. Periodontal ligament cells were used, and periodontitis was created in a porcine model and the results demonstrated a decreased in severity of the disease (Liu et al., 2008). Another study in

2017 looked at patients with chronic periodontitis and transplanted DPSC's instead and found reduction of probing depth which means the severity of periodontist was decreasing. Another one of the first human clinical trials attempted to use stem cell therapy strategies to conduct a regenerative endodontic procedure (Lovelance et al., 2011). The positive results did demonstrate that evoking bleeding could trigger stem cells to move into the canal space where they could potentially regenerate pulp tissue. This study was one of the first to use human patients and utilize the knowledge of dental stem cells and demonstrate how this could replace endodontic procedures all together. A preclinical trial in dogs looked at pulpitis which is inflammation of the dental pulp. The researchers used G-CSF with a conditioned medium of stem cells to regenerate pulp and dentin while also monitoring the safety of the procedure. The positive results demonstrated that this medium could be used clinically and met the basic prerequisites of safety measures. This was significant, since many of the studies done prior had not assessed the adverse effects some techniques could potentially have (Iohara et al., 2013). Another one of the few clinical trials looked at using DPSC's after wisdom teeth removal to prevent loss of the second molar in patients that had decreased bone repairing ability due to an impacted molar (d'Aquino et al., 2009). This study demonstrated a complete repair of the periodontal tissue which was significant because it was one of the first human trials to restore viability and prevent loss of teeth in patients without any adverse effects following up with patients up until a year after post-operation. The most recent study, that brought dental regeneration into the news and shed light on the subject to the general public for this first time focused on the Wnt/ $\beta$ catenin signaling pathway. The researchers innovate technique was to use the teeth's own repairing ability and stimulate

this pathway and mobilize, these stem cells in the dental pulp by using various drugs that had previously been tested to treat Alzheimer's and other neurological diseases. Rats were used as a model and the caries induced teeth eventually were restored to their original healthy state. This was the first study to demonstrate that potential of a pharmaceutical treatment that could be used routinely, easily and cost effectively in regular clinical practices (Neves et al. 2017).

## **CONCLUSION**

The branch of regenerative dentistry is fairly recent and preliminary findings only date back to early 2000. There has more than enough evidence to state that dental pulp stem cells have regenerative capabilities in animal models but developing the techniques that could be implemented in practice are still far from the clinical trial stage. Also, since there has been very few clinical trials done and most being outside the U.S, there are a lot of steps and information that needs to be assessed regarding safety, cost effectiveness, and practicality. The field does however, have a very promising future based on the number of discoveries made in the past 15 years. To develop and replace the current restorative techniques and change the field of dentistry all together, it is going to take more studies, research and innovative thinking before it can be implemented in clinical practices.

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**CURRICULUM VITAE**

