Universidade de
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Margarida Sância da Cruz Fardilha, Professora Auxiliar com Agregação do Departamento de Ciências Médicas, Universidade de Aveiro e do Professor Doutor Pedro de Sousa Gomes, Professor Associado com Agregação da Faculdade de Medicina Dentária da Universidade do Porto

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palavras-chave

Células estaminais da polpa dentária, differenciação osteogénica/odontogénica, péptido disruptivo, proteina fosfatase 1, regeneração

resumo

A cárie dentária é uma doença crónica de elevada prevalência, que representa um desafio global de saúde publica, com uma prevalência de 49-83%, estando associada a uma elevada morbilidade e elevados custos de saúde. As estratégias terapêuticas atualmente utilizadas nas lesões de cárie mais avançadas, que atingem a polpa dentária, são procedimentos tecnicamente exigentes e invasivos, que envolvem a pulpectomia e o tratamento endodôntico radical, visando um resultado reparador que não permite a regeneração dos tecidos danificados. A polpa dentária possui, no entanto, um potencial reparativo e regenerativo após o dano, em que distintas vias de sinalização podem ser ativadas para promover processo biológicos como a adesão celular, proliferação, migração, angiogénese e diferenciação que células precursoras, levando à regeneração dos tecidos danificados. Apesar do reconhecimento destes processos, a manipulação das vias biológicas para promoção da regeneração pulpar ainda é essencialmente desconhecida.

A proteína fosfatase 1 (PP1) é uma fosfatase de serina/treonina que possui um papel fundamental na regulação celular através da remoção de grupos fosfato de resíduos de serina/treonina. Um estudo recente sugere que a ativação da PP1 em odontoblastos pode induzir a diferenciação osteogénica/odontogénica, modulando, assim, a reparação e a regeneração da polpa dentária. Neste sentido, o principal objetivo deste trabalho assenta na utilização de um péptido disruptivo- MSS1, com atividade de disrupção das interações da PP1, tornando-a ativa para modular as vias de sinalização associadas à PP1, e potencialmente promover a regeneração da polpa dentária. Os resultados demonstram que, *in vitro*, o MSS1 induz a proliferação celular em fases iniciais de culturas de células estromais da polpa dentária e, em fases intermédias-tardias, aumenta a diferenciação osteogénica/odontogénica, sugerindo o seu potencial para modular a reparação e regeneração da polpa dentária.

Em jeito de conclusão, este trabalho demonstrou o potencial uso do MSS1 para modular a sinalização da PP1 nas células estromais da polpa dentária, eventualmente suportando a regeneração dos tecidos, como uma estratégia terapêutica inovadora e não-invasiva para tratar lesões polpares.

keywords

Dental pulp stromal cells, osteogenic/odontogenic differentiation, disrupting peptide, protein phosphatase 1, regeneration.

abstract

Dental caries is a highly prevalent chronic disease in humans and represents a global public health challenge, with a prevalence of 49-83%, being associated with a high morbidity and extensive healthcare costs. Current state-of-art therapeutic strategies, used in more advanced caries that reach the dental pulp, are technically demanding and invasive procedures that involve pulpectomy and root canal treatment, aiming for a reparative outcome that does not allow the regeneration of the lost tissues. The dental pulp has a reparative and, as well, a regenerative potential, in which, after damage, signalling cascades may be activated to promote cell adhesion, proliferation, migration, angiogenesis and differentiation of precursor cells, leading to the regeneration of the lost dental tissues. Despite the acknowledgement, the putative manipulation of biological pathways to prime pulp regeneration is still largely unknown.

Protein phosphatase 1 (PP1) is a serine/threonine phosphatase that plays a fundamental role in cell regulation through the removal of phosphate groups from serine/threonine residues. A recent study suggests that PP1 activation in odontoblasts may induce osteogenic/odontogenic differentiation, modulating therefore dental pulp repair and regeneration. In this sense, the mail goal of this work is to use a disrupting peptide - MSS1 that has been reported to disrupt PP1 interactions, making PP1 active to modulate PP1-related signalling, and potentially promote dental pulp regeneration. The results showed that, in vitro, MSS1 induced cell proliferation in early stages of dental pulp stromal cells' cultures, and in mid-late stages, enhanced osteogenic/odontogenic differentiation, suggesting its potential to modulate dental pulp repair and regeneration.

In conclusion, this work demonstrated the potential use of MSS1 to modulate PP1 signalling in dental pulp cells, eventually assisting on tissue regeneration, as a potential and innovative, non-invasive therapeutic strategy to treat pulp-related lesions.

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List of Abbreviations, symbols and acronyms

ALP	Alkaline phosphatase activity
ALPL	Alkaline phosphatase
AP-1	Activator protein 1
bFGF	Basic fibroblast growth factor
BMP-2	Bone morphogenic protein 2
BMP-4	Bone morphogenic protein 4
BMPs	Bone morphogenic proteins
Col1α1	Collagen type I alpha I chain
Co-SMAD	Common mediator SMAD
CPPs	Cell-penetrating peptides
DMP-1	Dentin matrix protein 1
DPP	Dentin phosphoprotein
DPSCs	Dental pulp stromal cells
DSPP	Dentin sialo phosphoprotein
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
Fzd	Frizzled receptor
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GSK-3β	Glycogen synthase kinase-3β
hDPSCs	Human dental pulp stem cells
HP	Homing peptide
HP-CPP	HP conjugated with cell-penetrating peptides
IBSP	Integrin binding sialoprotein
IkB	Inhibitory kappa-B
IKK	Inhibitory kappa-B kinase
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-6	Interleukin-6
IL-8	Interleukin-8
I-SMAD	Inhibitory SMAD

JNKs	c-Jun N-terminal kinases
LPS	Lipopolysaccharides
LRP	Low-density lipoprotein-related receptor protein
MAP2Ks	Dual specificity mitogen-activated protein kinases
MAPKs	Mitogen-activated protein kinases
MEPE	Matrix extracellular phosphoglycoprotein
mTOR	Mammalian target of rapamycin
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
p38 MAPK	p38 mitogen-activated protein kinase
PAMPs	Pathogen-associated molecular patterns
PI3K/AKT	Phosphoinositide 3-kinase/protein kinase B
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PP1	Protein phosphatase 1
PP1c	PP1 catalytic subunit
PP2A	Protein phosphatase 2A
PP3	Calcineurin
PP4	Protein phosphatase 4
PP5	Protein phosphatase 5
PP6	Protein phosphatase 6
PP7	Protein phosphatase 7
PPIs	Protein-protein interactions
PPM	Metallo-dependent protein phosphatase
PPP	Phosphoprotein phosphatases
PRRs	The pathogen recognition receptors
PTMs	Post-translation modifications
PTP	Phosphotyrosine phosphatases
RIPPOs	Regulatory proteins of protein phosphatase 1
ROS	Reactive oxygen species
R-SMAD	Receptor-regulated SMAD
SLiMs	Short linear motifs
SMAD	Suppressor of mothers against decapentaplegic
SMAD-1	Suppressor of mothers against decapentaplegic 1

SMAD-2	Suppressor of mothers against decapentaplegic 2
SMAD-3	Suppressor of mothers against decapentaplegic 3
SMAD-4	Suppressor of mothers against decapentaplegic 4
SMAD-5	Suppressor of mothers against decapentaplegic 5
SMAD-8	Suppressor of mothers against decapentaplegic 8
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

I. Introduction

1. Teeth

1.1. General overview

Teeth are considered one of hardest and most resistant structures of the human body. Together, the tooth arrangement constitutes the dentition (Christensen et al., 2014). The principal functions of teeth are grasping, crushing food, speech articulation and they also influence facial appearance (Hovorakova et al., 2018). In humans, the dentition is classified as heterodont, which refers to the fact that there are four classes of teeth – the incisors, canines, premolars and molars - and as diphyodont, which means that there are two generations of functional teeth - the deciduous and the permanent teeth (Hovorakova et al., 2018).

Anatomically, teeth can be divided into two sections: the crown and the root. The crown is a functional part that is visible above the gum level, in the oral cavity; while the root is the unseen portion that extends into the alveolar bone through the periodontal ligament and holds the tooth in its place. The crown and root shape vary among different teeth and among different species (Türp & Alt, 1998).

All teeth have the same general structural organization, being composed of the same four tissues – enamel, dentine, cementum and pulp (Figure 1). Enamel is the outer layer that covers the crown of the tooth, it is majorly inorganic (96%) and is the hardest tissue in the body given the high mineral (hydroxyapatite – calcium phosphate) content. It does not have vascular or nerve tissue within, which means that it cannot be renewed or replaced, despite the ability to remineralize initial lesions through a chemical process (Hollins, 2012).

The dentin is synthesized by odontoblasts and forms the main bulk of the crown and the root extending almost the entire length of the tooth. It is covered by enamel in the crown portion and covered by cementum in the root section. The dentin gives support to these structures and provides a barrier of protection to the dental pulp. It is a mineralized tissue that has a high inorganic component (70%), being however inferior to that of enamel, thus sustaining inferior mechanical properties. Dentin is a tubular structure that contains tissue fluid, odontoblastic processes and nerve fibrils from the pulp tissue, allowing the detection of pain sensation. As the dentin is located directly above the pulp chamber, if enamel erodes the dentin becomes exposed and causes pain, a pulpar response may occur with inflammatory activation (Hollins, 2012).

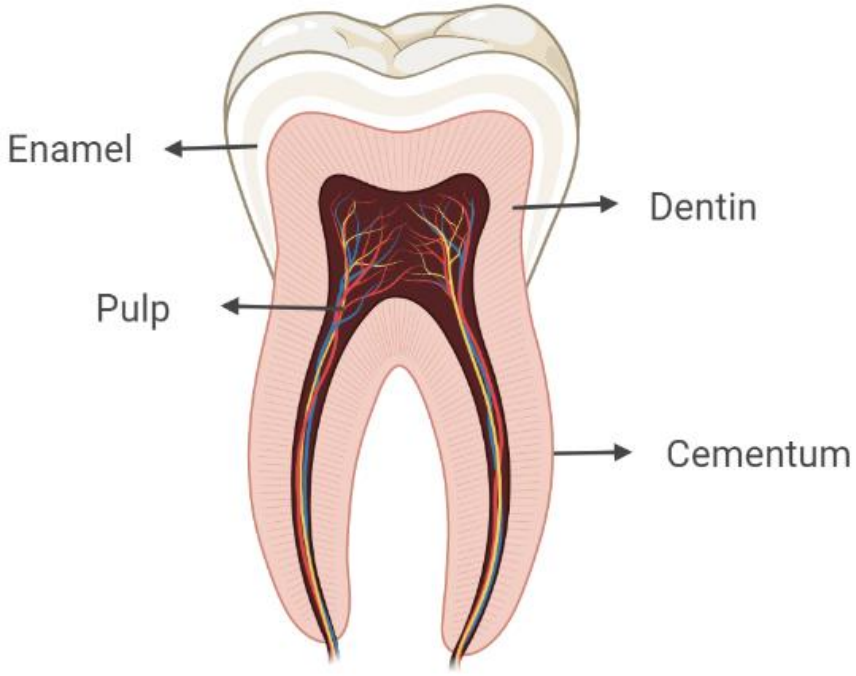

Figure 1: ***General structure of teeth***. Definitive human tooth is composed by four tissue types: enamel, dentin, cementum and dental pulp. Figure was created with BioRender.com (Adapted from: Gregory, 2009).

Cementum is similar to enamel and dentin – being mineralized, avascular and non-innervated – despite the reduced mineral content, as compared to other dental tissues, which substantiates the inferior mechanical properties. It provides a layer of coverage to the root and serves as a medium for the attachment of the periodontal ligament fibers that hold the tooth into the surrounding bone.

Lastly, the pulp is located in the most internal part of the teeth – the pulp chamber and pulp canals - and extends almost the whole length of the tooth and consists in the inner neurovascular tissue extremely rich in cells, tiny blood vessels and nerves (Hollins, 2012; Low et al., 2008). Enamel, dentin, and cementum constitute the hard tissues of the tooth and dental pulp is the soft tissue, with a gelatinous consistency (Hollins, 2012). In more detail, dental pulp is a highly vascularized and innervated mass of connective tissue. It contains different types of cells such as fibroblasts, odontoblasts, histiocytes, macrophages, mast cells and plasma cells. The extracellular matrix is composed of collagenous fibers and ground substance (Green D, 1955; Yu & Abbott, 2007). Dental pulp can be divided in two main regions, the peripheral and the central. The peripheral region is the area adjacent to the calcified dentin and is composed of different

structural layers. Of these, it is important to highlight a layer of columnar odontoblasts cells that produce reparative dentin in response to exogenous stimuli. Bellow this cell layer is the cell-free zone, that is rich in capillaries and nerve networks. To close the peripheral area, there is a layer rich in fibroblast and mesenchymal stem cells. The central region is outlined by the cell-rich layer. The dental pulp has several crucial functions, including the formation and nutrition of dentin, as well as, the innervation and immunological response within the tooth. The dentin formation and nutrition, as mentioned before, is carried out by the odontoblasts and blood vessels present in the pulp. Dentin innervation promotes the perception of pain caused by trauma, temperature, and pressure variations and, as with other triggers, activates the immune/inflammatory response. The defensive role is accomplished through the activation of odontoblasts for dentin formation or priming of precursor cell populations (mesenchymal stem cells) that differentiate and replace the odontoblasts (Ghannam et al., 2021; Masthan et al., 2013). The presence of various cellular constituents in the dental pulp, as odontoblasts, fibroblasts, cells of the immunological response, and stem cells, as well as a thorough vascular network, entails this tissue with regenerative capabilities. Dental pulp stem cells can form tissues such as dentin, pulp and periodontal ligament fibers. Thus, these cells have demonstrated a great potential in regenerative therapy (Ledesma-Martínez et al., 2016).

1.2. Teeth development

In humans, the development of teeth starts within the first weeks, in the early stages of embryological development, upon the establishment of the three germ layers - ectoderm, mesoderm and endoderm. Odontogenesis, or tooth formation, is similar to neurulation, since teeth arise from invaginations, developing from the oral ectoderm and the underlying neural crest derived mesenchyme (Sheldahl, 2020).

Early, the oral cavity is formed by ectoderm-derived epithelium and under this the mesenchyme derives from the interaction of mesoderm and neural crest cells. A continuous band of thickened epithelium, horse-shoe-like, originates around the mouth given the change in orientation of the mitotic spindle and cleavage plane of selected cells. Primary epithelial band is then established and ingrowns into the mesenchyme, originating the dental lamina. During this process localized areas proliferate and grow thicker, forming placodes (Sheldahl, 2020). On the anterior region of the dental lamina, active cell proliferation originates the establishment of distinct epithelial outgrowths into the mesenchyme, at locations corresponding to the place of the future teeth. Mesenchymal-derived cells accumulate around these regions. Upon this initiation stage, the

development of the tooth may be regarded into 3 stages: the bud, cap, and bell (Yamazak & Hayashi, 2004).

Within the bud stage, proliferation of ectodermal regions is attained, originating a morphological alteration of the structure, that appears around week 6. Neuro-mesenchymal cells also proliferate (Sheldahl, 2020).

By week 10, ectoderm continues to proliferate and collide with the dental papilla (cluster of neuro-mesenchymal populations) that forces the ectoderm tooth bud to grow around it, initiating the cap stage (Sheldahl, 2020). The epithelial cells close to the dental papilla are designated as the enamel organ – given rise to enamel-forming cell populations; and the mesenchyme-derived populations of the dental papilla originate dentin and pulp – and those outside the involving bell are designated as dental sac (or follicle) – which form the periodontal ligament, cementum and alveolar bone. The enamel organ, dental papilla and dental sac collectively form the tooth germ.

Around week 11-12 of development, ectoderm and mesenchyme continue proliferating. The epithelial cap becomes larger, initiating the bell stage. At this stage, the enamel organ is formed by cells from the inner enamel epithelium (which will differentiate into ameloblasts) and the outer enamel epithelium (involved in tooth eruption and formation of the junctional epithelium). Within this area, stratum intermedium and stellate reticulum, of ectodermal origin, assist inner enamel epithelium on differentiation and enamel formation. At this time, dental papilla can be subdivided into a central and an outer region. Central cells will give rise to pulp cellular populations while outer cells will differentiate into odontoblasts (Sheldahl, 2020; Yamazak & Hayashi, 2004).

Upon the bell stage, the late bell stage (or crown stage) initiates, being characterized by a productive activity of ameloblasts and odontoblasts. Odontoblasts lay down layers of dentin matrix between themselves and basement membrane, thus the cells gradually move away from the basement matrix. This process stimulates the amelogenesis and ameloblast lay down of enamel matrix forming the crowns of teeth. At this stage, the dental pulp also starts to be invaded by blood vessels. The dental epithelium will move into the underlying mesenchyme, forming the epithelial root sheath, that in years, will allow root formation. Mesenchyme on outside of tooth differentiates into cementoblasts, which produces cementum. With the further narrowing of the root the crown gets pushed into the cavity and, jumping forward in time, will originate the eruption of the tooth (Pansky, 1982; Sheldahl, 2020; Yamazak & Hayashi, 2004).

1.3. Dental damage and repair

Enamel and dentin may become damaged by different factors, for example the presence of microorganisms that can cause inflammation in the dental pulp and the root apex. The entrance of microorganisms can be caused by dental caries, crack of dentinal tubules of teeth, chemical or mechanical irritation during procedures, trauma or orthodontic movement of the teeth (Sanders & Houck, 2020). Thus, dental damage can be caused by trauma, physic and biological changes that may initiate inflammatory cascades leading to the degeneration or necrosis of dental pulp (Park et al., 2015).

In this section we will address modifications with biological origin- microorganisms. Symbiotic microbial communities cover the crowns of erupted teeth. These communities are primarily constituted of Gram-positive bacteria, that normally are harmless to the tooth, and form biofilms on the mineralized enamel that create an impermeable barrier to pathogenic microorganisms, protecting the underlying dentin and consequently the dental pulp. In a sugar-rich environment, *Streptococcus, Lactobacillus, Neisseria, Actinomyces* and *Veillonella* species release organic acids as lactic, propionic, and acetic acid. These organic acids decrease the overall pH of the oral cavity and initiate the process of dental caries (Gross et al., 2012; Hahn & Liewehr, 2007; Park et al., 2015). When the pH reaches 5.5, enamel starts to demineralize, and an early carious lesion develops. At this point, remineralization of the enamel can occur through a modification of the microenvironment, for instance by the application of topical fluoride, but if cavitation occurs and the infection reaches dentin, only restoration can restore the tooth structure (Hollins, 2012).

As the carious lesion develops through the dentine structure, tissue damage may achieve a threshold in which it is not able to support the remaining enamel, leading to its fracture, opening the cavity to a more widespread tissue destruction. Upon dentinal lesion, pain sensation will be recognized within the pulp, being defined as a reversible pulpitis (Hollins, 2012). If the caries approaches the pulp chamber, the odontoblasts will synthesize a secondary dentine layer to avoid breach by microorganisms. If failed, the pulp chamber will be contaminated and microbial contamination will lead to pulp cell death, initially only localized within the region of infection. When this happens, a stage of irreversible pulpitis will be achieved. If untreated, it can result in dental infection or abscess with widespread pulpal necrosis (Hollins, 2012). Untreated caries is considered one the most prevalent health conditions across the globe, with a prevalence of 66% worldwide and has high morbidity and extensive healthcare costs- $544.41 billion in 2015 (Kassebaum et al., 2015; Kazeminia et al., 2020; Righolt et al., 2018). The current therapeutic strategies for advanced caries are technically demanding and invasive procedures that aim the

restoration of the lost dental tissues, without focusing on a regenerative outcome that restores the anatomical, aesthetical, and functional settings of the dental structures (El karim et al., 2021).

Damage repair can be defined as the restoration of tissue architecture and function after an injury, and it comprises two separate processes: tissue regeneration and replacement (Krafts, 2010). In the first one, new tissue growth that restores the damaged tissue to their pre-disease state occurs, while within the second consists in the deposition of connective tissue in severely damaged tissue that cannot be regenerated. The repair process that takes place depends on the type and severity of the damage. The regeneration is favored in tissues with more proliferative capacities and that are rich in stem cells. Indeed, dental pulp contains resident population of adult stem cells and migratory progenitor cell population that contribute to reparative processes (El karim et al., 2021; Peterson et al., 2015).

In this frame, initial lesion of the dentin structure may be repaired upon the activation of tertiary dentinogenesis, the process of hard tissue repair and regeneration on the dentin-pulp complex. It consists in the focal secretion of dentin matrix in the site of injury in the mature dentin-pulp complex (Farges et al., 2015). Despite the formation of new tissue, with histological similarity, true regeneration does not occur and though a reparative response is attained. This response can be considered a biological defense response and function as a barrier to further injury, or it can be a reparative activation, set in order to restore tissue function and architecture. There are two types of tertiary dentin formation: reactionary and reparative dentinogenesis (Figure 2). The first occurs after a mild stimulus, where primary odontoblast secrete tertiary dentine matrix. On the other hand, the second occurs when the tissue damage results in the death of the primary odontoblasts, leading to the recruitment and differentiation of stem cells from the pulp into odontoblasts-like cells (A. J. Smith et al., 2012).

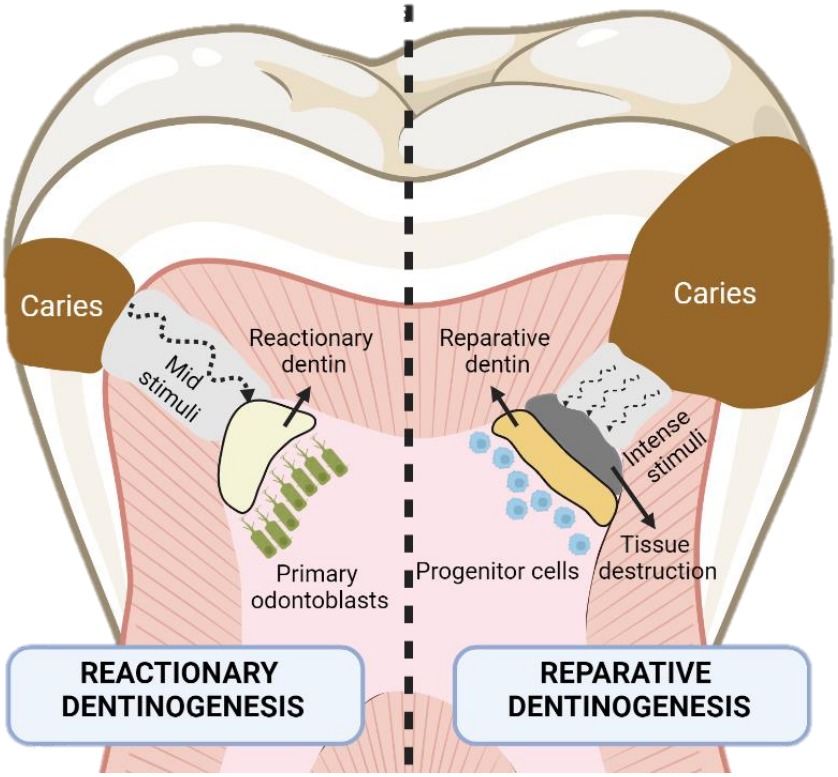

Figure 2: Tertiary dentine formation. *Reactionary dentinogenesis occurs when mid stimuli induce primary odontoblasts to secret reactionary dentin and reparative dentinogenesis occurs when intense stimuli promote tissue destruction and primary odontoblasts death followed by stem/progenitor cell recruitment that differentiate into odontoblast-like cells and secret reparative dentin. Figure was created with BioRender.com (Source: da Rosa et al., 2018)*

When the bacterial infection, bacterial by-products or trauma affects the pulp-dentin complex, odontoblasts recognize pathogenic signals and initiate the localized healing response. After severe injury, a significant depletion of resident odontoblasts in dentil-pulp interface may occur and the pathogen recognition receptors (PRRs) of local and recruited cell populations trigger a protective inflammatory response. This response is characterized by the release of potent signalling molecules, including inflammatory markers, cytokines and antibacterial agents, further recruiting immune and reparative cells to the infected locus (Durand et al., 2006). El karim et al. (2021) divided the reparative process of inflamed dental pulp in three consecutive phases: inflammatory, proliferative and remodeling phase (Figure 3) (El karim et al., 2021).

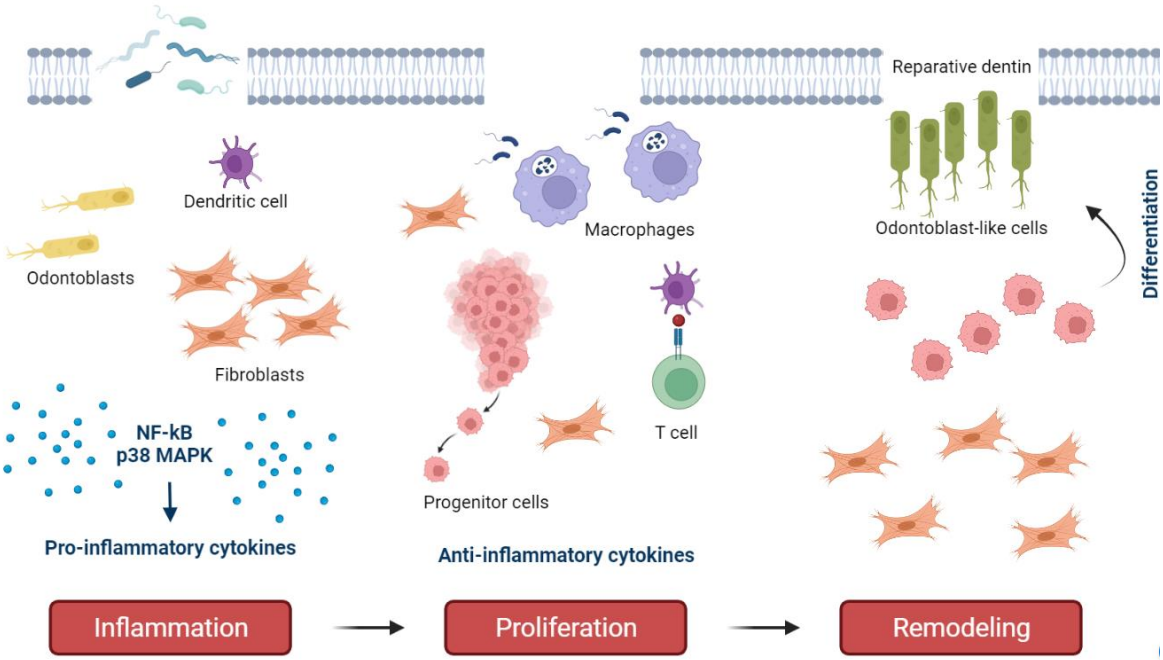

Figure 3: Schematic illustration of the three phases of the reparative process of inflamed dental pulp. *Following carious infection and lesion progression, the inflammatory phase becomes characterized by the recruitment of immune cells and production of inflammatory cytokines. Many of the cytokines which drove the inflammatory response along with newly expressed molecules promote proliferative responses as the disease context changes toward a healing response. Proliferation and migration of stem/progenitor cells continues to complete the healing process by the differentiation of new pulp tissue and odontoblast-like cells that produce reparative dentine during the remodeling phase.* Figure was created with BioRender.com *(Source: El karim et al., 2021).*

The inflammatory phase is characterized by the dynamic release of inflammatory mediators that combat the invading bacteria and support the repair process. It is initiated when PRRs recognize pathogen-associated molecular patterns (PAMPs). This leads to the activation of nuclear factor kappa-light-chain-enhancer (NF-KB) and p38 mitogen-activated protein kinase (p38 MAPK) signalling cascades, that constitute a hallmark of the inflammatory process, and results in the release of pro-inflammatory cytokines that exacerbate some signalling pathways such as the NF-KB, activator protein 1 (AP-1) and signal transducer and activator of transcription (STAT), to amplify cytokine response (Chin Lo Hahn & Liewehr, 2007; Rechenberg et al., 2016). Although the exact role of these cytokines is largely unknown in the pulpal environment, there are evidence that TNF-α promotes dental pulp stromal cells (DPSCs) functions, as migration, proliferation, and

odontogenic differentiation (Qin et al., 2015; Silva et al., 2009; Ueda et al., 2014; X. Yang et al., 2012).

The pulpal nerves also respond to injury through the release of neuropeptides that regulate pulpal blood flow, influence the production of angiogenic growth factors that facilitate healing and may further exert an antimicrobial activity (El karim et al., 2009, 2021).

Pulp cells also play an important role in this inflammatory phase: odontoblasts are the primarily local response cells and they are responsible for dentin formation and due to their location, they display sensory and immunological properties (Farges et al., 2009); fibroblasts are the most abundant cells in pulp, and they produce numerous cytokines and growth factors that facilitate healing, have antimicrobial activity and the ability to recruit stem cells (Chmilewsky et al., 2014; Rufas et al., 2016); stem cells are important in tissue regeneration and inflammation control, through immunomodulatory activity (Z. Li et al., 2014).

The proliferative phase is characterized by the activation, proliferation, migration and differentiation of cells that are recruited when pulp inflammation occurs. Dendritic cells present the antigens to T cells, activating the adaptative immune response (Abbas, 2003; Randolph et al., 1999). Natural killer T cells may further target autologous cells infiltrated by pathogens, supporting the immune response (Kawashima et al., 2006). Other immune cells involved are the macrophages, that phagocyte the invading pathogens. First, they produce pro-inflammatory cytokines and then to control inflammation, anti-inflammatory cytokines as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) (Matsukawa et al., 2005; Wynn & Vannella, 2016).

The remodeling phase involves tissue formation and maturation and is only possible when the infection and associated inflammation are controlled. The DPSCs and the other cells recruited in the proliferative phase differentiate into odontoblastic-like cells and replace the original odontoblasts that were destroyed during the tissue infection. These cells synthesize reparative tertiary dentin that is less organized and lacks the continuous tubular structure of the original dentin, which reduces the barrier efficacy and permeability that protects the pulp from further damage (Izumi et al., 2001).

For the wound healing/repair process, vascularization is essential to provide nutrition and oxygen. Vascularization is supported by signalling of angiogenic molecules, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and TGF-β, that are released from inflammatory cell populations and the damaged pulp cells. Thus, after the inflammatory phase, proliferation and differentiation phases take place, and together collaborate to enrich a natural

healing process for pulp-dentin tissue (Mathieu et al., 2005; Roberts-Clark & Smith, 2000; Velnar et al., 2009).

2. Molecular pathways involved in dental pulp repair

Tertiary dentinogenesis consists of a complex group of molecular pathways intended to induce odontoblast secretory activity. In general, signalling molecules can bind and many times induce phosphorylation of different receptors, activating many signalling transduction pathways, which results in a panoply of outcomes. To have a better understanding on how the dental repair occurs, different molecular pathways that regulate this process will be briefly analyzed. Recent studies have described the set of signalling pathways involved in dentinogenesis, including the NF-kB, the mitogen-activated protein kinases (MAPKs), the suppressor of mothers against decapentaplegic (SMAD) pathway, the Wnt/β-catenin and the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways (Figure 4).

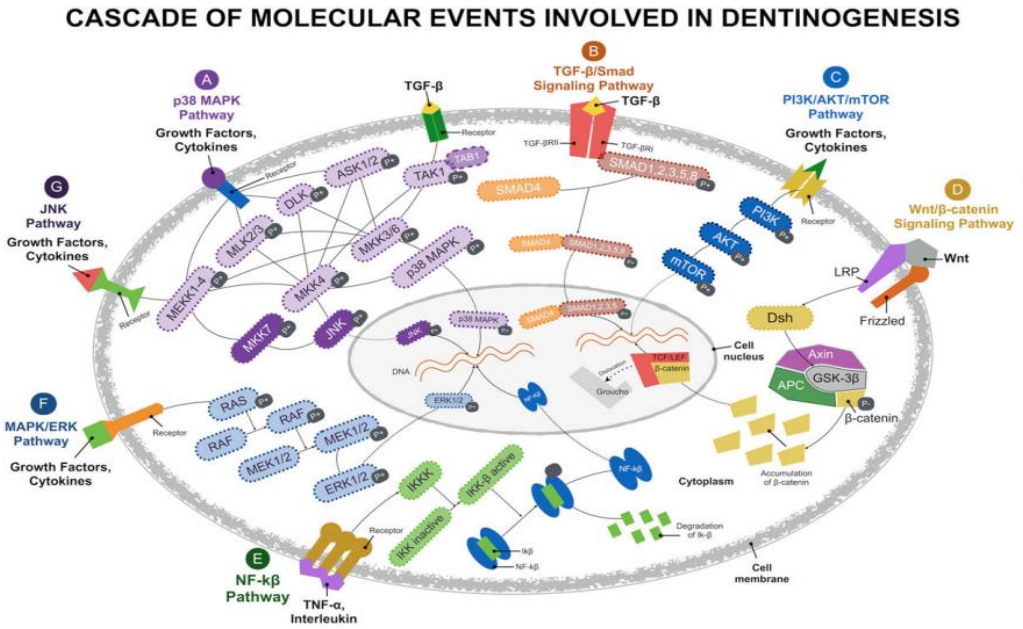

Figure 4: Schematic representation of the different signalling pathways involved in tertiary dentinogenesis. *(A) p38 MAPK Pathway; (B) TGF-β-Smad signalling pathway; (C) PI3K/AKT/mTOR pathway; (D) Wnt/b-catenin signalling pathway; (E) NF-kb pathway; (F) MAPK/ERK pathway; (G) JNK pathway. (Source: da Rosa et al., 2018).*

2.1. Nuclear factor kappa-light-chain-enhancer of activated B cells pathway NF-kB pathway

Dental caries is characterized by the invasion of dental pulp by bacteria that cause infection and inflammation. Some studies have identified lipopolysaccharides (LPS) as a major component present in the membrane of the invaders, that stimulate the production of pro-inflammatory

cytokines through the activation of transcription factor NF-kB (Darveau et al., 2002; Nara et al., 2019; Rupf et al., 2000; Wang et al., 1997).

NF-kB is a redox-sensitive transcription factor that regulates a battery of inflammatory genes, that has been found to play an important role in the development of numerous pathological conditions (Valen et al., 2001). Under physiological conditions, the NF-kB is present in the cytoplasm, where it binds with inhibitory kappa-B (IkB), preventing its translocation into the nucleus and stimulation of gene transcription (Q. Zhang et al., 2017). If damage to the pulp occurs, NF-kB pathway has been shown to be activated (Wang et al., 2009; Yao et al., 2008). Therefore, in the presence of cellular stresses caused by the lesion as reactive oxygen species (ROS), cytokines and LPS, the IkB is phosphorylated by IkB kinase (IKK), releasing NF-kB that translocates into the nucleus and regulate genes involved in both innate and adaptative immunity, including chemokines and cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-8 (IL-8), that contribute to a pro-inflammatory environment. NF-kB also induces the expression of adhesion molecules, that lead to the recruitment of leukocytes to inflammation sites (Figure 5) (Tripathi & Aggarwal, 2006). TNF-α and IL-1 have the ability to activate this pathway (Bonizzi & Karin, 2004).

The NF-kB pathway has a very important role in regulating immune responses to dental pulp damage, being activated in the inflammatory phase to combat bacteria, addressed previously (Bonizzi & Karin, 2004). Some studies suggest that prolonged inflammatory signalling impedes odontogenic differentiation and consequently dental pulp regeneration (Sloan & Smith, 2007; Wisithphrom & Windsor, 2006; XC Yang et al., 2011). Thus, the activation of this pathway must be controlled, since its exacerbated stimulation may prolong the dental pulp healing process (Nirwana et al., 2017).

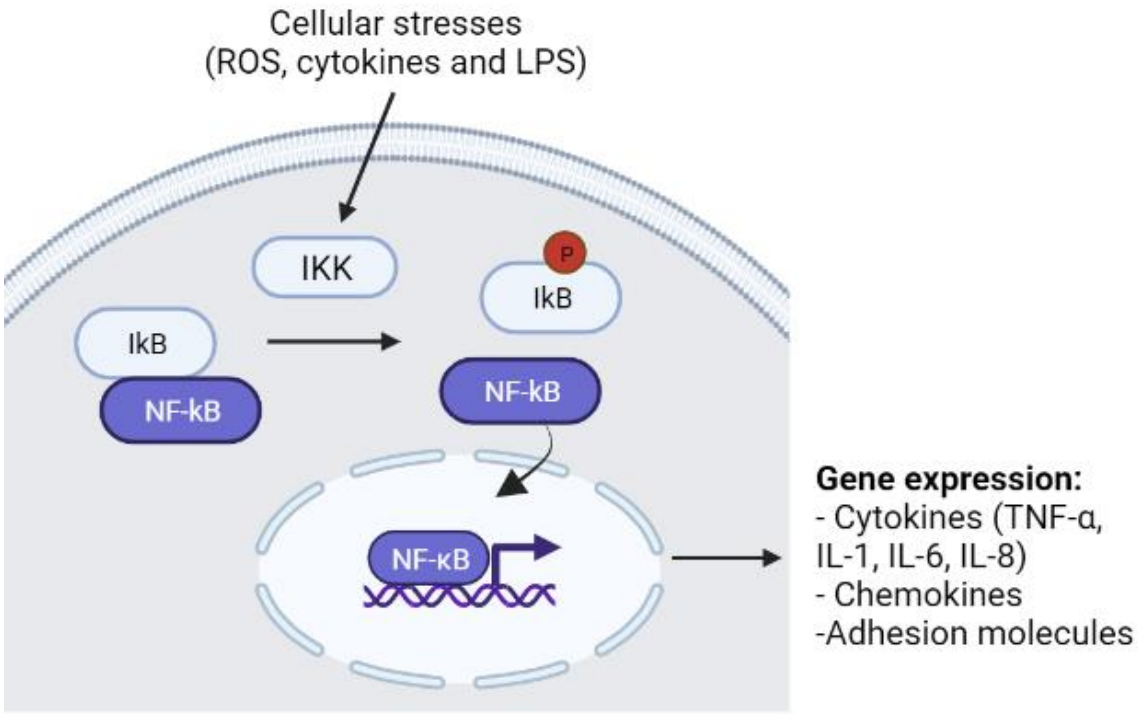

Figure 5: Schematic representation of activation of the transcription nuclear factor kappa-B (NFκB) molecular pathway. *In the cytoplasm the NF-kB bound with the inhibitory kappa-B (IkB). Stimuli stimulates IkB phosphorylation resulting in the release of NF-kB that translocates into the nucleus and promotes gene transcription of chemokines, cytokines and adhesion molecules. Figure was created with BioRender.com (Adapted from: Valen et al., 2001).*

2.2. Mitogen-activated protein kinases pathway

Under physiological conditions of mature teeth, odontoblasts synthesise TGF-β that is trapped into the dentin. Upon dental damage TGF-β is released and stimulates odontoblast-like cells differentiation and pulp tissue repair, through the activation of some molecular pathways including the MAPK pathway (Tziafas et al., 2000; Yongchaitrakul & Pavasant, 2007). This pathway can also be activated through a variety of cellular stresses including exposure to ROS, cytokines and LPS generated during bacterial challenge invasion (Goldberg, 2014). MAPKs consist of serine/threonine specific protein kinases that are expressed in mammalian cells and have an essential role in different cellular processes, including proliferation, differentiation and apoptosis. MAPKs can be divided into 3 subfamilies: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNKs) and p38 MAPK (Johnson & Lapadat, 2002). In a general matter, Figure 6 illustrates the signalling cascade that result from MAPK pathway activation: dental pulp damage

activates receptors that led to dual specificity mitogen-activated protein kinases (MAP2Ks) phosphorylation that consequently phosphorylates MAPK on threonine and tyrosine residue in the activation loop, activating the signalling (Dérijard et al., 1995). There are six different MAP2Ks, that have selective substrate specificities, activating different MAPK pathways. These pathways modulate some cellular activities as proliferation, differentiation, apoptosis and survival (Davis, 2000).

TNF-α that is release upon inflammatory response, stimulates differentiation of dental pulp cells towards an odontoblastic phenotype via p38 MAPK, increasing the expression of dentin phosphoprotein (DPP) and dentin sialoprotein (DSPP) and inducing therefore tertiary dentinogenesis (Silva et al., 2009).

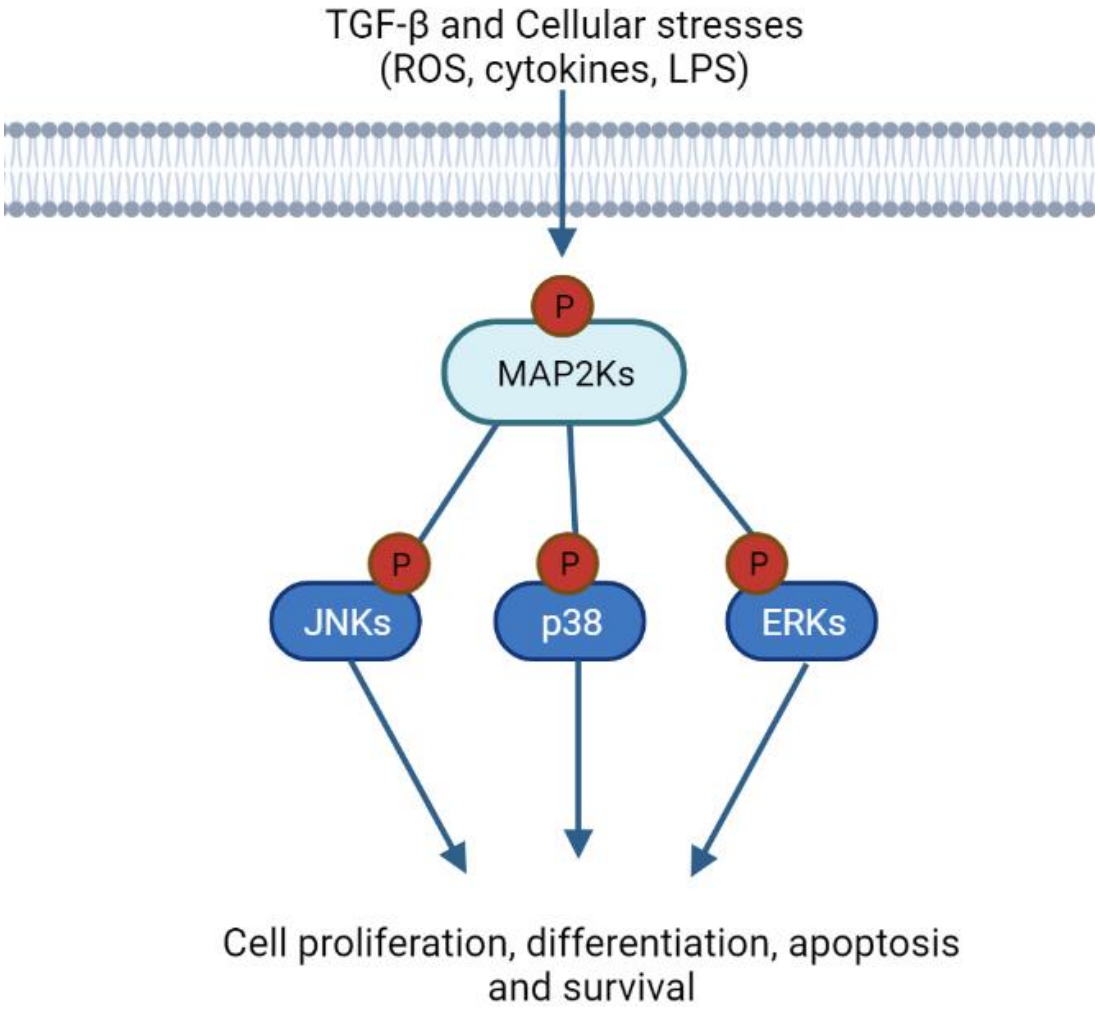

Figure 6: Schematic representation of the MAPKs signalling pathway. *Stimuli promotes the phosphorylation of MAP2Ks that are responsible for the activation of JNKs, p38 MAPKs and ERKs. The activation of MAPKs pathway contributes to cell, proliferation, differentiation, apoptosis and survival. Figure was created with BioRender.com (Adapted from: J. Wang & Xia, 2012).*

2.3. Suppressor of mothers against decapentaplegic pathway

Dental pulp cells express SMAD proteins that consist of intracellular signalling proteins that are active in response to members of the TGF-β family, including bone morphogenic proteins (BMPs) (Souchelnytskyi et al., 2002). This family has been implicated in odontoblast differentiation and dentin mineralization and in tooth development and repair after injury (Korrodi-Gregório et al., 2014; Massagué, 1998). It is possible to distinguish three types of SMADs: receptor-regulated (R-SMAD), common mediator (Co-SMAD) and inhibitory (I-SMAD) (Shi & Massagué, 2003). There are two types of serine/threonine receptors, type I and II, where TGF-β binds and activates the SMAD signalling pathway (He et al., 2004). When the receptors are stimulated, R-SMADs are phosphorylated, followed by the formation of heterodimeric complexes with SMAD-4, that translocate into the nucleus and regulate the transcription of target genes. Depending on the molecule that stimulates the receptors, different SMADs are phosphorylated (Jadlowiec et al., 2006; Korrodi-Gregório et al., 2014). If BMPs phosphorylate the receptor, SMAD-1, SMAD-5 and SMAD-8 will be used as substrates. However, if TGF-β phosphorylates the receptor, the substrates will be SMAD-2 and SMAD-3 (Da Rosa et al., 2018). Bone morphogenic protein 2 (BMP-2) plays a key role in odontoblast dental pulp cells differentiation and TGF-β in the stimulation of odontoblast differentiation and mineralization (Ahmed et al., 2020; Niwa et al., 2018; Tóth et al., 2020). When the BMP-2 and bone morphogenic protein 4 (BMP-4) expression is enhanced, the dentin matrix protein 1 (DMP-1) and DSPP gene expression increases, thus promoting odontogenic differentiation (He et al., 2008). Haniastudi et al., described the influence of TGF-β in reactionary dentinogenesis and reparative dentinogenesis. In the reactionary, it stimulates the upregulation of odontoblasts secretory activity and in the reparative it induces the proliferation, migration and differentiation of odontoblast (Haniastuti et al., 1996).

2.4. Wnt/β-catenin pathway

Wnt/β-catenin pathway, also known as the canonical Wnt pathway has also been involved in tertiary dentinogenesis (Rosa et al., 2018). When Wnt ligand binds to the Frizzled receptor (Fzd) and to the low-density lipoprotein-related receptor protein (LRP), they form the complex Wnt-Fzd-LRP. This complex recruits the Axin complex, that is responsible for the β-catenin phosphorylation. Thus, when the Wnt binds to the receptor it inhibits the phosphorylation of β-catenin (Gordon & Nusse, 2006). The cytoplasmatic β-catenin accumulates and translocates into the nucleus, activating Wnt target genes, as is the case of transcription factor *Runx2*, that seems to be involved in odontogenic differentiation (Han et al., 2014). The signalling of this pathway in dental pulp is not enough to promote odontogenic differentiation and dentin regeneration, and

both positive and negative effects of Wnt/β-catenin signalling have been reported in dentinogenesis. A study demonstrated that the overexpression of a Wnt target gene decreased the expression of odontoblast differentiation-related genes (Z. Zhang et al., 2014), but overall, a trend for increased cell survival, proliferation and enhanced odontogenic differentiation has been demonstrated in the literature (Chen et al., 2016; Z. Zhang et al., 2016).

2.5. Phosphoinositide 3-kinase/protein kinase B pathway

The PI3K/AKT pathway consists of a very important pathway in the human body, that has been associated with cell proliferation, differentiation, metabolism, and apoptosis. It is composed by PI3K, AKT and downstream target proteins (F. Zhang et al., 2020). PI3Ks are a group of plasma membrane-associated lipid kinases, that are composed by a regulatory subunit, normally the p85 regulatory subunit, and a catalytic subunit, the p110 catalytic subunit. The PI3K signalling can be activated by different extracellular stimuli, including growth factors, cytokines, and hormones. When the receptors are activated, the catalytic subunits phosphorylate the phosphatidylinositol 4,5-bisphosphate (PIP2) in phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that binds with lipid-biding domains of downstream targets to the cell membrane. Signalling proteins as AKT and PDK1 bind with the lipid products of PI3K and activate cell growth and survival pathways (Sun & Meng, 2020; Yang et al., 2019). AKT is a serine/threonine kinase that, when is activated, can phosphorylate target protein effectors, as mammalian target of rapamycin (mTOR), glycogen synthase kinase-3β (GSK-3β) and NF-kB (Zhang et al., 2020). The RAS-MEK-ERK signalling pathway is highly interconnected with PI3K signalling. Thus, this pathway can also activate cell growth and survival pathways through AKT (Figure 7) (Buckles et al., 2017; F. Zhang et al., 2020). TNF-α, a cytokine early released in the repair of pulp damage, has been reported to activate this pathway. Once PI3K/AKT signalling is activated, mTOR, GSK-3β or NF-kB will be phosphorylated and will promote odontogenic differentiation and dental pulp repair (Kim et al., 2011; Qin et al., 2015). There are several studies that demonstrate the ability of PI3K/AKT pathway to regulate differentiation and self-renewal of various cells. Lee et al. showed that this signalling pathway promotes osteogenic differentiation of bone marrow mesenchymal stem cells (Lee et al., 2013) and human embryonic stem cells (Lee et al., 2010). A recent study, suggest that this pathway may have an important role in proliferation, migration, and odontogenic differentiation of human dental pulp stromal cells (hDPSCs) (Zhang et al., 2020).

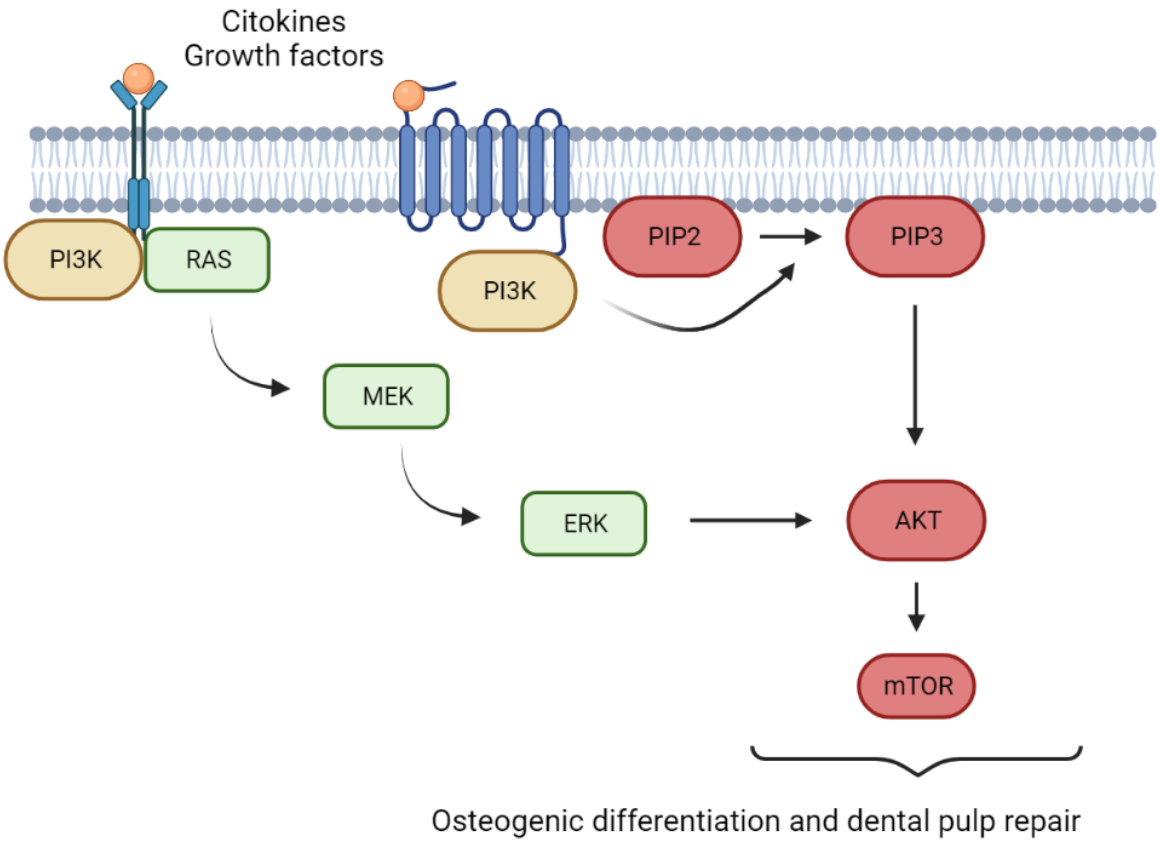

Figure 7: Schematic representation of PI3K/AKT pathway. *PI3k activation promotes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) in phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that binds with AKT and activate cell growth and survival pathways resulting in odontogenic differentiation and dental pulp repair. The RAS-MEK-ERK signalling pathway is highly interconnected with PI3K signalling. Figure was created with BioRender.com (Adapted from: Sun & Meng, 2020).*

3. Protein phosphatase 1 and pulp regeneration- what is known

In typical eukaryotic cells, most of the proteins are phosphorylated on serine, threonine, and tyrosine residues. This phosphorylation is catalyzed by protein kinases that transfer γ-phosphate from ATP to its protein substrates. On the other hand, protein phosphatases catalyze the transfer of the phosphate group to a water molecule. Even though these proteins catalyze opposite reactions, they work together in the modulation of protein functions (Cheng et al., 2011). The phosphorylation state of a protein results from the balance between the action of protein kinases and phosphatases. Interruption of this equilibrium causes aberrant phosphorylation, which is associated with a large range of human diseases, including cancer, Alzheimer's disease, neurodegeneration, among others (McConnell & Wadzinski, 2009).

Based on the substrate preference, inhibitor sensitivity and catalytic mechanism, protein phosphatases can be classified into three major classes: phosphoprotein phosphatases (PPP), metallo-dependent protein phosphatase (PPM) and phosphotyrosine phosphatases (PTP) (Korrodi-Gregório et al., 2014).

The PPP family consists of protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), protein phosphatase 2B also known as calcineurin (PP3), protein phosphatase 4 (PP4), protein phosphatase 5 (PP5), protein phosphatase 6 (PP6) and protein phosphatase 7 (PP7) (Nasa & Kettenbach, 2018). PP1 is a major serine/threonine phosphatase widely expressed and highly conserved in all eukaryotes (Chen et al., 2017). Initially, it was identified as the responsible protein for the conversion of phosphorylase a in phosphorylase b, however over the years, it has been shown to be a key regulator of vital cellular processes, including cell cycle progression, meiosis, protein synthesis, metabolism, cytoskeletal reorganization and, signal transduction (Ceulemans & Bollen, 2004; Cohen, 2002).

The functional diversity of PP1 is achieved by the existence of different isoforms of the catalytic subunit (PP1c) (Honkanen & Golden, 2012). There are three different genes (*PPP1CA, PPP1CB, PPP1CC*) that encode four nearly identical PP1c isoforms (PP1α, PP1β, PP1γ1, PP1γ2), that share about 90% of the sequence identity and diverge at their extremities, N- and C-terminal (Nasa & Kettenbach, 2018). The *PPP1CC* gene encodes PP1γ1 and PP1γ2, through alternative splice transcripts, which consists in the deletion or retention of the last intron of the gene, respectively. The PP1c is highly conserved among the isoforms and folds in a single elliptical domain with a central β-sandwich structure of two mixed β-sheets with a binuclear metal site in its active site (Peti et al., 2013). Oxygen atoms mediate the dephosphorylation reaction through the surrounding of the central phosphorus atom in the phosphate molecule (Egloff et al., 1997;

Goldberg et al., 1995). This domain has low intrinsic specificity, meaning that it does not select the substrates and most of the time it is coupled with interacting proteins, preventing uncontrolled dephosphorylation events (Peti et al., 2013; Verbinnen et al., 2017).

To achieve specificity to substrates, PP1 forms heterodimers that consists in the binding of the catalytic subunit to diverse regulatory proteins (RIPPOs) (Bollen et al., 2010). More than five hundred proteins have been identified as RIPPOs and they function as targeting subunits, substrates and/or inhibitors (Alanis-Lobato et al., 2017; Korrodi-Gregório et al., 2014). Therefore, a wide range of PP1 holoenzymes can result from the binding of the catalytic and the regulatory subunits. Depending on the RIPPO, the holoenzyme can be in different subcellular compartments and regulate different cellular processes (Felgueiras et al., 2020). Thus, RIPPOs are structurally unrelated proteins that define where and when PP1 is active and to which substrates (Ferreira et al., 2019). Contrarily to PP1, RIPPOs exist freely and have distinct functions beyond regulation of PP1c (Felgueiras et al., 2020).

In terms of structure, PP1 is constituted by three substrate-binding grooves, the acidic groove, the C-terminal groove, and the hydrophobic groove that form a Y-shape. In the intersection of the three-binding grooves lies the binuclear metal site - the PP1 active site. The RIPPOs that function as targeting subunits, achieve this ability by modulating the binding sites, adding substrate specific docking sites, or blocking substrate binding sites. Some of the inhibitors are phosphorylation-dependent, which means that the phosphorylated residue is pushed into the active site and other cover the active site inhibiting therefore PP1 (Bollen et al., 2010). Short linear motifs (SLiMs), also known as PP1 docking motifs, facilitate the binding of RIPPOs to PP1 (Ferreira et al., 2019). The most studied SLiMs, RVxF, is present in almost all known RIPPOs (90%) and it may be the primary interaction point for further interactions (Hendrickx et al., 2009). It is a short linear motif, which binds into the hydrophobic groove of the catalytic pocket of PP1 and in PPP family it is specific to PP1 (Chatterjee et al., 2012; Egloff et al., 1997; Hendrickx et al., 2009). Since PP1 isoforms diverge at their extremity at the N- and C-terminal, some RIPPOs interact with PP1 in an isoform-dependent manner, suggesting that they possess isoform-specific docking sites to the isoform that they bind (Bollen et al., 2010).

PP1 has been associated with the development of different organs, including lungs (Tazawa et al., 2017) and eyes (Butler, 1998) and, with the differentiation of different cell types, as neuronal (Sloan, 2015), myogenic (Yu & Abbott, 2007) and more recently odontoblasts (Kim et al., 2017). In 2017 Kim et al. explored the presence of PP1 in dental pulp cells and its role in odontoblastic differentiation and angiogenesis. The authors first confirmed the PP1 expression at the different

developmental stages of pulp tissue in mice, showing that it is mainly expressed in preodontoblasts, odontoblasts, endothelial cells, and dental pulp cells. These results suggested that PP1 may be a key regulator of odontoblast development. *In vitro* experiments, allowed to conclude that PP1 levels increased during differentiation and mineralization states, suggesting that it may play a key role in these processes. Since PP1 is an *in vitro* target for natural ceramides, they used C2 ceramide as an activator of PP1 and concluded that this molecule increased the expression of osteogenesis markers, as alkaline phosphatase activity (ALP) and DSPP and DMP-1. These results suggested that PP1 activation lead to odontogenic differentiation. Angiogenesis is an essential process in response to injuries and, since dental pulp is a well-vascularized connective tissue that has the potential to heal, the authors further evaluated the expression of angiogenic factors, vascular endothelial growth factor and angiopoietin 1, in the presence of C2 ceramide. The presence of C2 ceramide resulted in the up regulation of their expression, suggesting that PP1 activation stimulates angiogenesis *in vitro* (Kim et al., 2017).

Use of a biopeptide to disrupt PP1 complexes and potentially modulate pulp regeneration

The regulation of signal transduction is made by cells through different mechanisms, for example, post-translation modifications (PTMs) and protein-protein interactions (PPIs). PTMs consist in the covalent events that cleave or add chemical groups to proteins. The most common PTM is phosphorylation (Ramazi & Zahiri, 2021). PPIs consist in the physical contact between proteins through selective molecular docking (De las rivas & Fontanillo, 2012). Since PP1 has a large number of RIPPOs, in the past years, it emerged as a promising drug target (Arkin et al., 2014). There are several approaches to regulate its activity, in particular the modulation of the interactions between PP1c and RIPPOs. To do that, the use of small organic molecules derived from natural or organic synthesized compounds and peptides have been developed (D. Zhang et al., 2016). Peptides have emerged as therapeutic molecules and have several advantages, (i) the flexibility allow the adaptation to larger surfaces, (ii) the modularity increases structural diversity, being more selective and potent, (iii) low accumulation in tissues, having low toxicity and side effects in humans (Higueruelo et al., 2013). However, they also present some limitations, as the difficulty to convert them into oral drugs because they are not metabolic stable during hepatic and renal clearance, they cannot cross physiological barriers easily, and they have poor oral bioavailability due to the digestion by enzymes. Luckily, it is possible to modify the synthesized peptides and increase their stability, permeability and bioavailability, controlling the mentioned disadvantages (Howl & Jones, 2015).

Cell-penetrating peptides (CPPs) are relatively short peptides, consisting of less than 40 amino acids in length, with the ability to cross biological membranes and carry cargo intracellularly, that usually are unable to cross an intact lipidic barrier (Figure 8) (Svensen et al., 2012). They can have different origins, such as protein-derived, that represent a part of natural CPPs; chimeric, that have two different origins; and synthetic, that are artificially designed. There are many different CPPs, that can be organized taking in consideration several characteristics. Considering their physicochemical properties, it is possible to organize them into groups: cationic, amphipathic and, hydrophobic (Kristensen et al., 2016). In physiological pH, cationic CPPs are positively charged due to their composition of positively charged amino acids, as arginine and lysine. Amphipathic CPPs are composed of hydrophilic and hydrophobic amino acids that fold in a α-helix with the hydrophilic face and the hydrophobic face, in opposite sides of the structure. Hydrophobic CPPs, as the name suggests, are composed manly by hydrophobic amino acids. However, they need to have some hydrophilic amino acids, otherwise they would be insoluble in body lipids, and they

would be retained in the hydrophobic center of the phospholipid bilayer and would not cross lipidic membranes. There are also anionic peptides that do not form a class of their own and are assigned in the different classes (Milletti, 2012). CPPs can also be cell-specific or non-cell-specific, depending on their ability to target specific cells when delivering the cargo, or if they are not selective and deliver the cargo to any cell (Langel, 2022; Zorko & Langel, 2005). To achieve cell-specificity different strategies were invented as the attachment of antibodies or their fragments, proteins, peptides, carbohydrates, aptamers or small receptor ligands. CPPs have the potential to be used as modulators or vehicles to deliver different biological products into cells as liposomes, cationic peptides, polymers or nanoparticles (Li et al., 2015). In the past years, they have been effectively used in different diseases as cancer, muscular dystrophy, prion disease, cardiology and, viral and bacterial infections (Durzyńska et al., 2015).

A pioneer study has demonstrated that the peptide MSS1 has the ability to disrupt the PP1/AKAP4 complex, increasing PP1 activity and inhibiting sperm motility (J. V. Silva et al., 2021). In this sense, this study is a steppingstone for the application of MSS1 in different tissues to modulate PP1 activity.

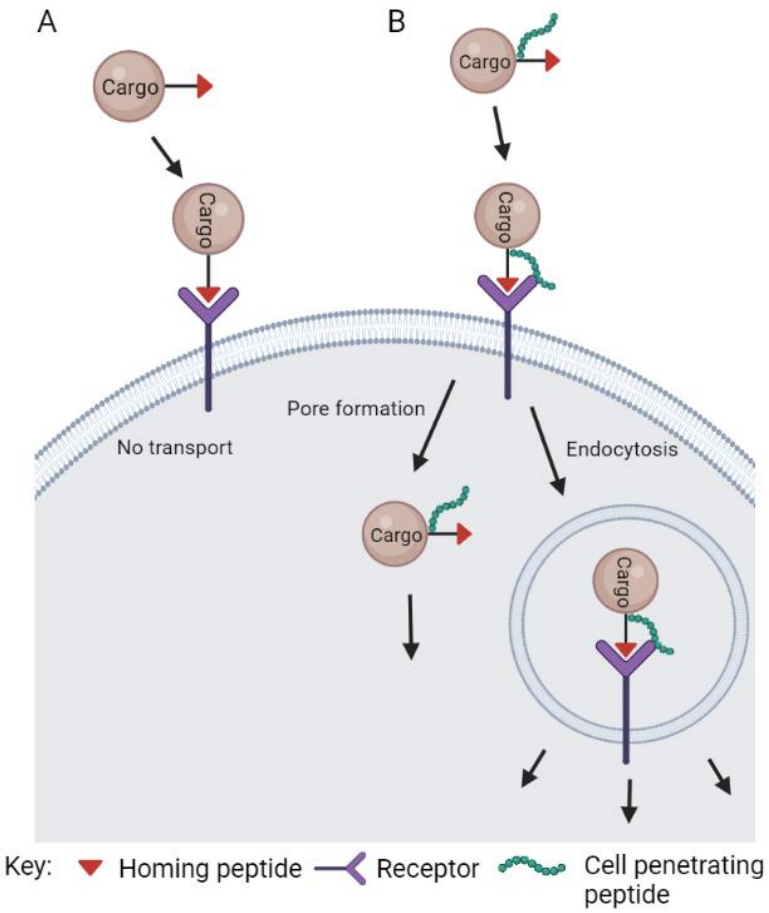

Figure 8: Principle of cell-penetrating peptide targeting and delivery. *(A) A homing peptide (HP) has no inherent internalization properties and only delivers its cargo to specific cell-surface receptors. (B) A HP conjugated with cell-penetrating peptides (HP-CPP) undergoes receptor binding and undergoes cargo internalization via endocytosis or pore formation. Figure was created with BioRender.com Adapted from (Svensen et al., 2012).*

4. Research motivations and Aims

Dental caries represents a global public health challenge with a high prevalence worldwide. The most common treatment for this disease only seeks to substitute the anatomical, aesthetical, and functional settings of the dental structures. Therefore, innovative, more conservative, and regenerative strategies to treat dental caries are majorly envisaged.

The article from Kim et al. (2017) was the first and only reported study to suggested that PP1 activation in odontoblasts may lead to odontogenic differentiation, modulating therefore dental pulp repair and regeneration (Kim et al., 2017). This along with other innovative study, that used MSS1 peptide to disrupt PP1/AKAP4 interaction (J. V. Silva et al., 2021), activating PP1 and concomitantly inhibiting sperm motility served as inspiration for this research. Although the MSS1

study was performed in other biological context, it validated the MSS1 activity and created the basis for this study.

Thus, this study aimed to characterize the biological response to the modulation of PP1 protein complexes in differentiating dental pulp cells, grown *in vitro*, using a disrupting peptide, and consequently, activating PP1. To that, we propose to:

- Evaluate the cytotoxic effect of MSS1 in dental pulp stromal cells;
- Characterize the influence of MSS1 in dental pulp stromal cells in a long-term culture through biochemical analysis (cell viability, proliferation, and alkaline phosphatase activity) and osteogenic/odontogenic gene expression.

II. Material and methods

This study was approved by the Ethical Committee of the Faculty of Dental Medicine, U. Porto. Isolation of DPSCs was conducted on selected third molars, extracted by orthodontic reasons, on 3 male patients aged between 20-25 years, without history of caries or periodontal disease, or relevant systemic pathology. Briefly, upon extraction, teeth were abundantly rinsed with sterile saline and cleaned with a chlorhexidine solution. Following, with a turbine bur, the crown was separated at the cementum enamel junction, allowing the isolation of the pulp tissue with an endodontic file. Pulp tissue was minced into small fragments and digested in collagenase type I for 60 minutes, at 37◦C. Upon incubation, a single cell suspension was attained through the passage of the suspension through a 25G needle, and following through a cell strainer, followed by centrifugation. Cells were cultured in alpha-modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (basal medium; all reagents from Gibco, Waltham, MA, USA), in 35 mm cell culture dishes (Corning® 35 mm TC-treated Culture Dish) at 37◦C, 95% humidity, and 5% CO2 atmosphere. Cultures were maintained until a 60-80% confluence and subcultured.

In this study we first did an exploratory analysis to evaluate the potential MSS1 cytotoxicity in dental pulp stromal cells through the evaluation of cells' metabolic activity, for 7 days, at distinct MSS1 concentrations. Following, a detailed culture characterization was conducted with selected MSS1 concentrations.

1. Cell culture

In the exploratory analysis, third passage DPSCs were cultured (10^4 cells/cm^2) in alpha-modified Eagle medium supplemented with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (basal medium; all reagents from Gibco, Waltham, MA, USA) in 96-well plates (Falcon, New York, NY, USA) at 37◦C, 95% humidity, and 5% CO2 atmosphere. After 24 h, the medium was exchanged for fresh basal medium without FBS (control) or exposed to 1, 5 and 10 µM of MSS1 prepared in basal medium without FBS, since MSS1 may interact with some proteins present in the serum. Control and MSS1-exposed cultures were grown for 7 days and were characterized for metabolic activity (resazurin assay) on days 1, 2, 5 and 7.

For the subsequent culture characterization with selected MSS1 concentrations, third passage DPSCs were cultured (5 x 10^3 cells/cm^2) in basal medium in 24-well plates (Falcon, New York, NY,

USA) as described above, for 21 days. After 3 days, the medium was exchanged to fresh basal medium or osteogenic/odontogenic medium, aiming to induce the osteogenic/odontogenic phenotype (Langenbach & Handchel, 2013). The osteogenic/odontogenic medium consists of basal medium supplemented with 10 nM dexamethasone, 50 µg/mL ascorbic acid and 10 mM beta-glycerophosphate (all reagents from Sigma-Aldrich, St. Louis, MO, USA). At days 7, 14 and 21 of culture, cells were exposed to 1 µM of MSS1 prepared in basal medium without FBS. 24 h before the exposure to MSS1, cells were submitted to a 24-hour FBS deprivation period, in order to minimize potential interactions between MSS1 and FBS proteins. After 24 h of exposure to MSS1, the cultures were characterized regarding metabolic activity, alkaline phosphatase activity and DNA quantification.

To evaluate gene expression of osteogenic/odontogenic markers, DPSCs were cultured (5 x 10^3 cells/cm^2) as described above in 6-well plates and grown for 14 days.

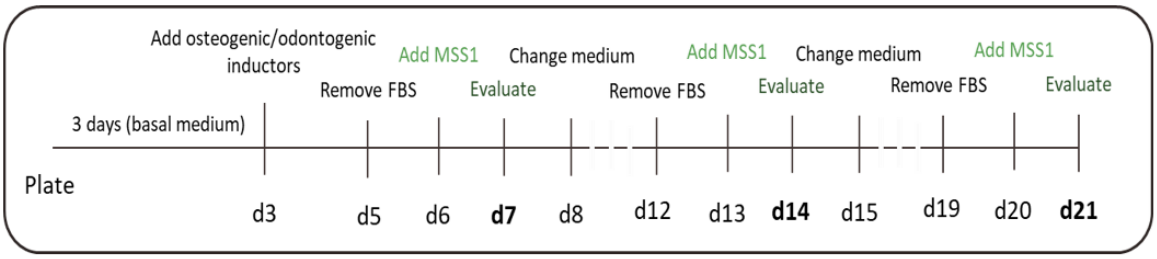

Figure 9: *Schematic representation of the experimental protocol.*

2. Metabolic activity

The metabolic activity of the cultured cells was measured with the alamarBlue® assay (Invitrogen, Carlsbad, CA). It was based on the conversion (reduction) of resazurin to the fluorescent byproduct resorufin, by metabolically active and viable cells (M. M. Silva et al., 2017). Briefly, the culture medium was removed from each well, cultures were rinsed with PBS and incubated with a 1× alamarBlue® solution at 37 °C for 3 h. Resorufin fluorescence was then determined (excitation: 540 nm, emission: 590 nm), in a microplate reader (Synergy HT, Biotek, Winooski, VT, USA).

3. DNA quantification

DNA content was measured using the Quant-iT Picogreen DNA assay (Invitrogen) according to the manufacturer's instructions. Initially, cells were washed with PBS and solubilized with 0.1% (v/v) Triton X-100 solution. Cell lysate was then mixed with the Pico-Green solution and incubated in the dark at room temperature for 5 min. The fluorescence intensity was measured with a

microplate reader at 485 and 528 nm for emission and excitation, respectively (Synergy HT, Biotek, Winooski, VT, USA).

4. Alkaline phosphatase activity

ALP activity of DPSC cultures was evaluated in cell lysates (Triton X-100 0.1%, 30 min) by the hydrolysis of p-nitrophenyl phosphate (p-NPP, 25 mM, Sigma-Aldrich, St. Louis, MO, USA) in an alkaline buffer (pH 10.3, 37◦C, 1 h). The reaction was stopped with NaOH 5 M and the product (p-nitrophenol) was measured at λ = 400 nm in a microplate reader (Synergy HT, Biotek, Winooski, VT, USA). Results were normalized to DNA content and expressed as nano-moles of p-nitrophenol per microgram of protein (nmol/μg protein).

5. Real-time quantitative polymerase chain reaction

Cell cultures were characterized by real-time quantitative polymerase chain reaction (RT-qPCR) to assess the osteogenic/odontogenic differentiation of HDPCs on day 14 of culture. Total RNA was extracted using the TRIzol™ reagent (Invitrogen, Waltham, MA, USA) and reverse-transcribed into complementary DNA (cDNA) with the NZY First-Strand cDNA Synthesis Kit (Nzytech, Lisbon, Portugal), all according to the manufacturer's instructions. The expression of the target genes was quantitatively determined on RTPCR equipment (CFX96, BioRad) using iQTM SYBR® Green Supermix (BioRad, Hercules, CA, USA).

All genes were normalized to the reference gene (GADPH, BioRad) and are described in Table 1.

Table 1: *Genes and respective primers assay ID (BioRad) for RT-qPCR.*

Gene	Gene name	Assay ID
Reference	Glyceraldehyde-3-phosphate dehydrogenase (GADPH)	qHsaCED0038674
Osteogenic	Alkaline phosphatase (ALPL)	qHsaCED0045991
	Bone morphogenic protein-2 (BMP-2)	qHsaCID0015400
	Collagen type I alpha I chain (Col1α1)	qHsaCED0043248
Odontogenic	Dentin sialo phosphoprotein (DSPP)	qHsaCED0002962
	Integrin binding sialoprotein (IBSP)	qHsaCED0002933
	Matrix extracellular phosphoglycoprotein (MEPE)	qHsaCED0045573

6. Statistical analysis

All experiments were performed in triplicate as independent experiments, all data was expressed as mean values ± standard deviation. Statistical analysis was performed using the IBM® SPSS® Statistics 25. Data normality was assessed by the Shapiro– Wilk test. One-way analysis of variance (ANOVA) was performed, followed by the post hoc Tukey test for multiple comparisons. To

compare two different groups t-test was performed. For both, p-values ≤ 0.05 were considered significant.

III. Results

1. Metabolic activity of DPSCs when exposed to different concentrations of MSS1

Dental pulp stromal cells were exposed to different MSS1 concentrations (1 µM, 5 µM and 10 µM) and the metabolic activity of the cultures was assayed through the resazurin assay, upon 1, 2, 5 and 7 days of exposition, Figure 10. Control cultures were grown in the absence of the peptide.

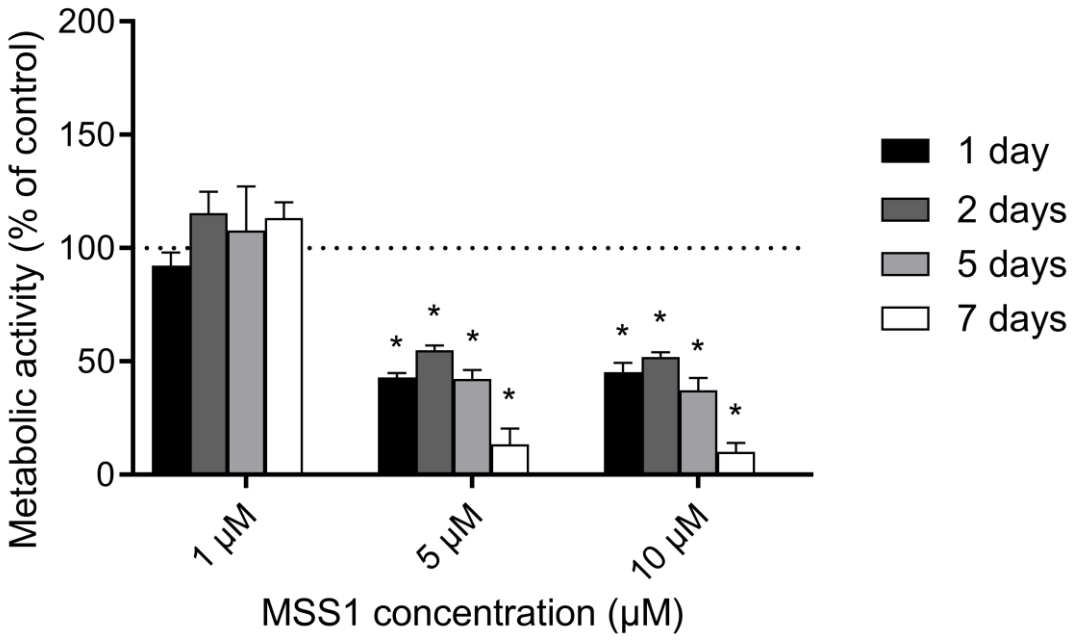

Figure 10: *Metabolic activity of dental pulp stromal cells cultured in basal medium, and exposed to MSS1 peptide at 1, 5 and 10 µM for 1, 2, 5 and 7 days. *Significantly different from the control group, p<0,05.*

The exposure of dental pulp stromal cells to 1 µM of MSS1, for 1, 2, 5 and 7 days, did not affected culture's metabolic activity, when compared with the control. Contrarily, the exposure of dental pulp stromal cells to higher concentrations, 5 and 10 µM of MSS1, significantly decreased metabolic activity, from day 1 onwards and over time, in comparison with the control.

2. Characterization of DPSCs when exposed to 1 µM of MSS1

Dental pulp stromal cells were cultured for a maximum period of 21 days and at 6, 13 and 20 days of culture, cells were exposed to 1 µM of MSS1 for 24 hours in basal and osteogenic/

odontogenic-inducing conditions and different assays were performed at 7, 14 and 21 days of culture, for detailed culture characterization. Controls were performed in the absence of peptide and FBS (FBS -/-), and a positive control was performed in the presence of FBS – marked as the dashed line at 100% in Figure 11, 12 and 13 and at 1 in Figure 14 and 15.

2.1. Metabolic activity

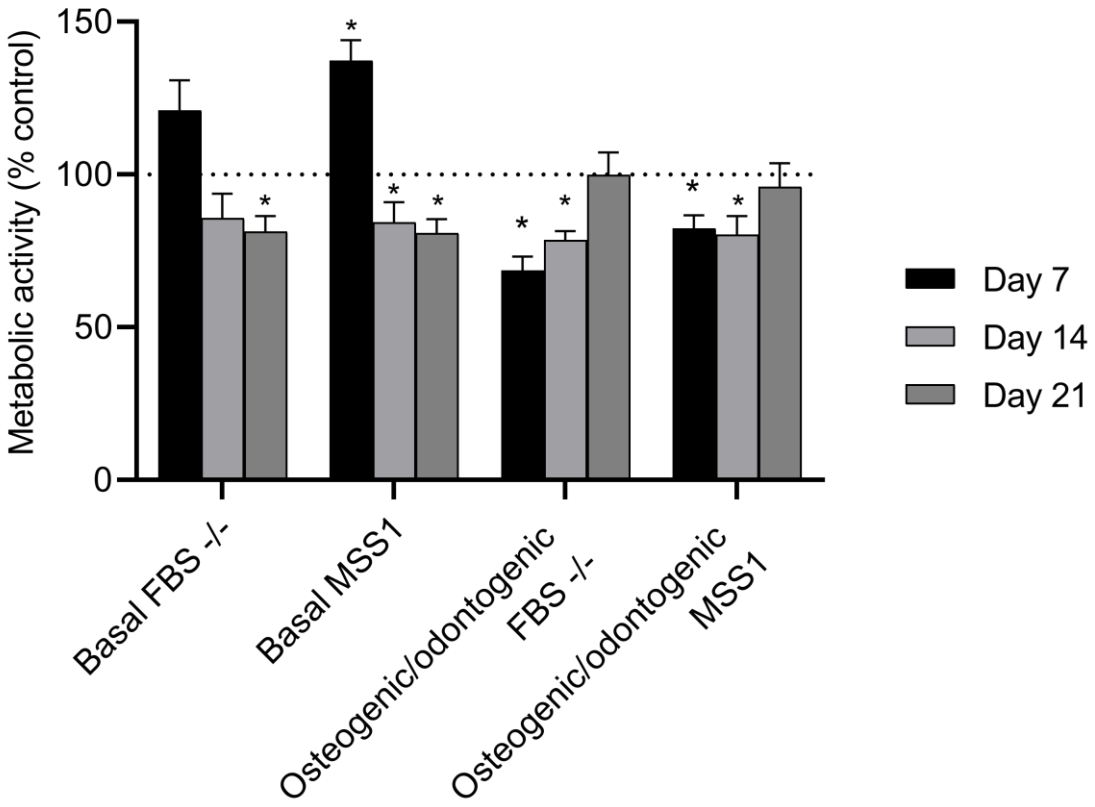

***Figure 11**: Metabolic activity of dental pulp stromal cells cultured in basal and osteogenic/odontogenic conditions, with and without FBS, and exposed to MSS1 peptide at 1 µM for 7, 14 and 21 days. *Significantly different from the positive control group, p<0,05. Basal FBS -/- , basal FBS-free medium; Basal MSS1, basal medium with MSS1; Osteogenic/odontogenic FBS -/-, osteogenic/odontogenic FBS-free medium; Osteogenic/Odontogenic MSS1, osteogenic/odontogenic medium with MSS1.*

In basal conditions, the exposure to MSS1 significantly increased metabolic activity of dental pulp stomal cells, after 7 days of culture, in comparison to the positive control - Figure 11. However, a trend for a significant decrease was verified for the remaining culture time, in a similar way to the respective control (FBS -/-). In the other hand, in osteogenic/odontogenic conditions, the addition

of MSS1 decreased the metabolic activity after 7 and 14 days, similarly to the absence of serum condition (FBS -/-). No significant differences were found at 21 days of culture.

2.2. Cell proliferation

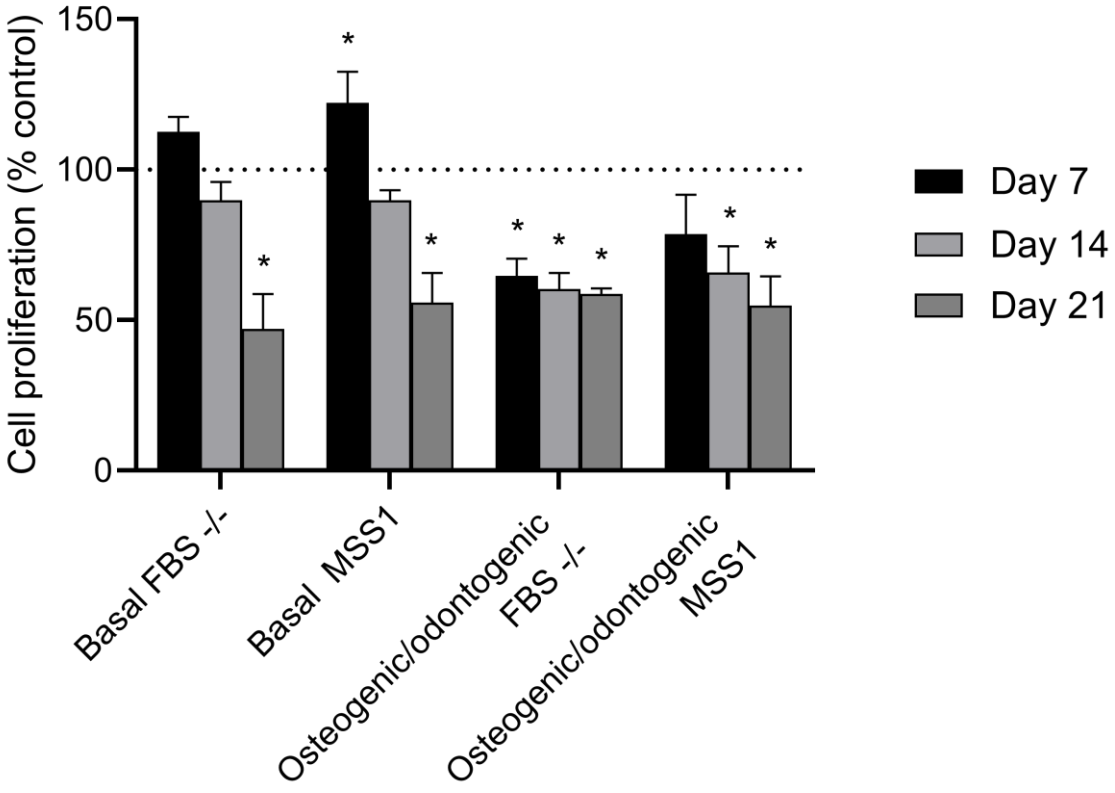

Figure 12: *Cell proliferation of dental pulp stromal cells cultured in basal and osteogenic/odontogenic conditions, with and without FBS, and exposed to MSS1 peptide at 1 µM for 7, 14 and 21 days. *Significantly different from the positive control group, p<0,05. Basal FBS -/- , basal FBS-free medium; Basal MSS1, basal medium with MSS1; Osteogenic/odontogenic FBS -/-, osteogenic/odontogenic FBS-free medium; Osteogenic/odontogenic MSS1, osteogenic/odontogenic medium with MSS1.*

Cell proliferation was significantly increased, when dental pulp stromal cells were exposed to 1 µM of MSS1 after 7 days of culture, at basal conditions- Figure 12. Whether no significant differences were attained at day 14, after 21 days, cell proliferation was significantly reduced with MSS1, similarly to FBS-free conditions, as compared to the positive control. In osteogenic/odontogenic MSS1 conditions, at day 7, cell proliferation was not affected, however, over time, it decreased significantly, similarly to FBS-free conditions.

2.3. ALP activity

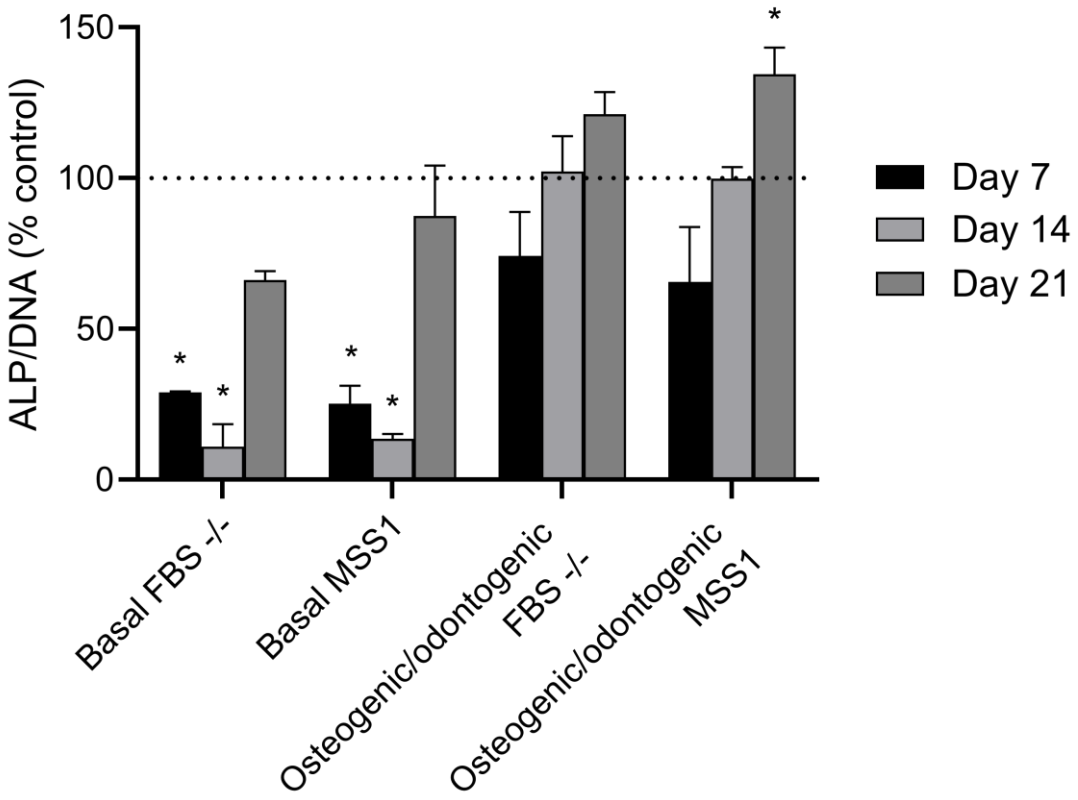

Figure 13: *ALP activity of dental pulp stromal cells cultured in basal and osteogenic/odontogenic conditions, with and without FBS, and exposed to MSS1 peptide at 1 µM for 7, 14 and 21 days. *Significantly different from the positive control group, p<0,05. Basal FBS -/-, basal FBS-free medium; Basal MSS1, basal medium with MSS1; Osteogenic/odontogenic FBS -/-, osteogenic/odontogenic FBS-free medium; Osteogenic/Odontogenic MSS1, osteogenic/odontogenic medium with MSS1.*

ALP activity was significantly diminished, after exposition of dental pulp stromal cells to 1 µM of MSS1, after 7 and 14 days of culture, at basal conditions- Figure 13. After 21 days, no significant differences were observed in basal MSS1, similarly to FBS-free conditions. Globally, there were no significant differences between MSS1 and the FBS -/- control. In osteogenic/odontogenic conditions at 7 and 14 days of culture, MSS1 exposure did not significantly modify ALP activity; after 21 days, it increased it. A similar trend was observed in FBS-free conditions.

2.4. Osteogenic/odontogenic gene expression

2.4.1. Basal medium

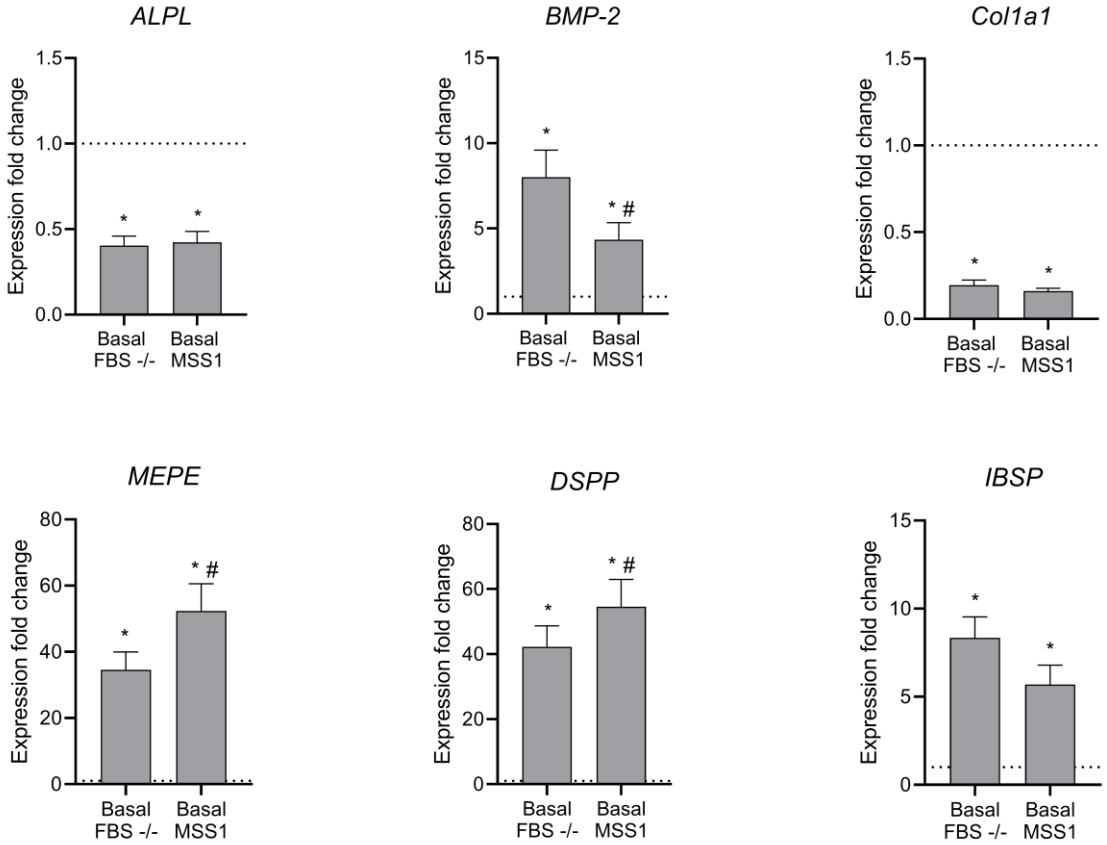

Figure 14: *Expression of osteogenic/odontogenic genes in dental pulp stromal cells in basal conditions, with and without FBS, and exposed to MSS1 peptide at 1 µM at 14 days. *Significantly different from the respective control group, #Significantly different from the cultures exposed to basal FBS-free medium, p<0,05. Basal FBS -/-, basal FBS-free medium; Basal MSS1, basal medium with MSS1; ALPL- Alkaline phosphatase; BMP-2- Bone morphogenic protein-2; Col1a1- Collagen type I alpha I chain; MEPE- Matrix extracellular phosphoglycoprotein; DSPP- Dentin sialo phosphoprotein; IBSP- Integrin binding sialoprotein.*

Dental pulp stomal cells cultured in basal free FBS medium and basal medium with 1 µM of MSS1 were compared with basal conditions for the expression of several osteogenic/odontogenic genes, Figure 14. Cultures grown in the presence of MSS1 presented similar expression of *ALPL* and *Col1a1* compared with cultures grown in the absence of FBS, being those levels significantly inferior to those of the positive control. The expression of *BMP-2* in cells exposed to MSS1 was reduced when compared with FBS-free conditions, although it is around 5 folds increased to the

positive control group. Exposure to MSS1 significantly increased the expression of *MEPE* and *DSPP*, as compared to cultures grown in the absence of FBS. MSS1 increased gene expression about 50 folds, while the FBS -/- group increases around 40 folds, as compared to the positive control. The exposure to MSS1 increased *IBSP* expression, to a level similar to the FBS-free condition, with the expression being significantly higher than in the positive control group.

2.4.2. Osteogenic/odontogenic medium

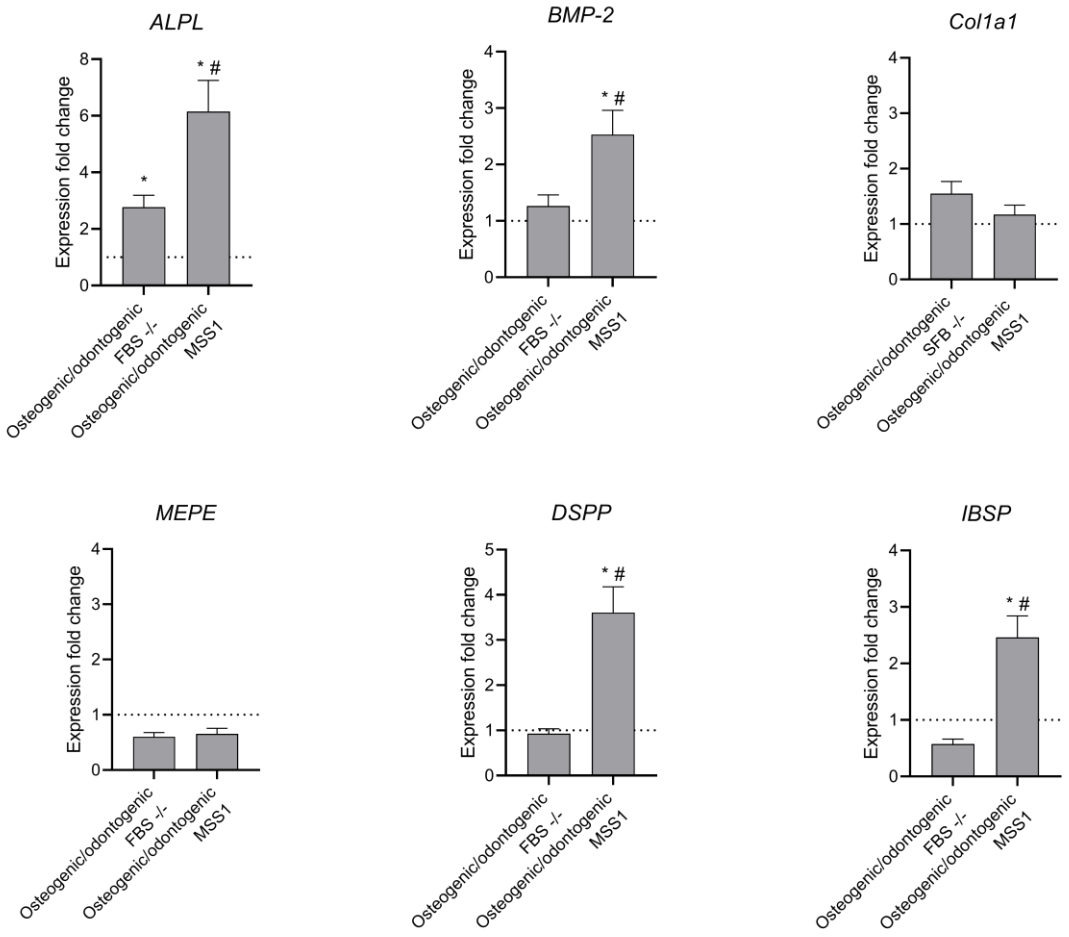

Figure 15: *Expression of osteogenic/odontogenic genes in dental pulp stromal cells in osteogenic/odontogenic conditions, with and without FBS, and exposed to MSS1 peptide at 1 µM at 14 days. *Significantly different from the respective control group, #Significantly different from the cultures exposed to basal free FBS medium, p<0,05. Basal FBS -/-, basal FBS-free medium; Basal MSS1, basal medium with MSS1; ALPL- Alkaline phosphatase; BMP-2- Bone morphogenic protein-2; Col1a1- Collagen type I alpha I chain; MEPE- Matrix extracellular phosphoglycoprotein; DSPP- Dentin sialo phosphoprotein; IBSP- Integrin binding sialoprotein.*

Expression of osteogenic and odontogenic genes were evaluated in cells cultured in osteogenic/odontogenic free FBS medium and osteogenic/odontogenic MSS1 (1 µM) medium and compared with cells cultured in osteogenic/odontogenic medium with FBS (positive control), Figure 15. The exposure to MSS1 lead to the increased expression of *ALPL* and *BMP-2* genes, when compared with either cells cultured in FBS-free medium, or with the positive control. The odontogenic *DSPP* and *IBSP* genes were significantly increased in cultures exposed to MSS1 than in those grown in osteogenic/odontogenic medium with (positive control) or without FBS. The expression of *Col1a1* and *MEPE* genes was similar in all experimental conditions and controls.

IV. Discussion

Dental pulp consists of a delicate connective tissue with blood vessels, lymphatics, nerve fibers and stem cells that is protected by dentin. Dentin is very susceptible to mechanical, thermal, electrical damage, chemical irritants and traumatic injuries that can lead to pulpal damage (Hernández et al., 2015). According to epidemiological studies dental caries is the most frequent etiological factor for the appearance and development of pulp diseases (Delgado et al., 2012). Dental caries is a chronic disease that affects not only children but also all age groups with high prevalence levels worldwide (Rathee & Sapra, 2022). It is associated with high morbidity and extensive healthcare costs (Kazeminia et al., 2020).

Current therapeutic strategies for pulpal diseases involve tooth devitalization (and as the last resource, tooth removal) that despite presenting high success rates, aim for a symptomatic relive and are frequently associated with subsequent complications, as secondary dental tissue infection, tooth fragility, and increased fracture risk (El karim et al., 2021). These strategies aim for a reparative outcome that do not allow the regeneration of the lost tissues. Currently, there are no practical treatments, in clinic, that allow the regeneration of the lost dental pulp (Xu et al., 2021).

Upon pulp damage a regenerative response in the form of tertiary dentinogenesis is observed, activating signalling cascades that promote cell adhesion, proliferation, migration, angiogenesis and differentiation of dental pulp cells (Bjørndal & Darvann, 1999). El Karim et al. divided the reparative process of dental pulp damage into three different phases: inflammation, proliferation and remodeling. The first one is characterized by the production of pro-inflammatory cytokines that assist on damage elimination; it is followed by inflammation control and resident cell proliferation; in the last one, cell differentiation is dominant (El karim et al., 2021). In the past years, several approaches have emerged as potential therapies in dental pulp repair and regeneration, which seem to rely on the modulation of complex signalling cascades to promote cell adhesion, proliferation, migration and differentiation of distinct cell populations, majorly centered on dental pulp stromal cells (Sloan & Smith, 2007). Some of these approaches include transplantation of DPSCs into injured incisors (Xuan et al., 2018), and pulp capping with biomaterials that induce the functionality of dental pulp cells (Chang et al., 2016; Yun et al., 2016). For instance, Yun et al. treated DPSCs with growth hormone and mineral trioxide aggregate, which promoted cell proliferation and differentiation, with prospective clinical transnationality (Yun et al., 2016).

PP1 has been shown to be a key regulator of several biological processes, being associated with the development of different organs/tissues, as the lung (Tazawa et al., 2017), and the eye (Butler, 1998). Its association with the differentiation of cells into the neuronal (Alastair J. Sloan, 2015) and myogenic (Yu & Abbott, 2007) pathways was also reported, suggesting its repair and regenerative potential. More recently, Kim et al. (2017) demonstrated that PP1 activation in odontoblasts modulates the odontogenic differentiation and may consequently be considered within the frame of the dental pulp regeneration.

In this study we used an innovative strategy, a disrupting peptide – MSS1, to promote dental pulp regeneration through the modulation of PP1 functionality. The activity of this peptide was discovered in a distinct biological context – Silva et al. used the MSS1 peptide to disrupt PP1 interactions, increasing its activity and thus inhibiting sperm motility (Silva et al., 2021). We believe that its ability to disrupt PP1 complexes and making PP1 active is maintained despite the tissue (Silva et al., 2021). In this sense, there are no previous studies evaluating the efficacy of MSS1 in dental pulp stromal cells, and for that reason, an initial exploratory analysis was performed. For that purpose, we tested different concentrations of the peptide to evaluate its potential cytotoxic effect. Our results demonstrated that MSS1, at 5 and 10 µM, reduced cultures' metabolic activity and may hinder the functional activity of the assayed cells. On the other hand, the exposure of dental pulp stromal cells to 1 µM of MSS1 did not induce significant alterations. These results are in accordance with other studies that used similar concentrations - under 10 µM - of different compounds to activate PP1 (Chatterjee et al., 2012; Fischer et al., 2018; Kim et al., 2017). Silva et al. tested the cytotoxicity of MSS1 in sperm cells and cells viability was only affected at 20 µM MSS1. To inhibit sperm motility, they only used 500 pmol/mL, demonstrating the potent effect of the peptide. Thus, the remaining experiments were performed using 1 µM of MSS1.

Dental pulp stromal cells were cultured in basal and osteogenic/odontogenic conditions for 21 days. Osteogenic/odontogenic medium was supplemented with molecules routinely used to induce odontogenic differentiation, namely dexamethasone, ascorbic acid and β-glycerophosphate. Hamidouche et al. (2008) demonstrated that dexamethasone induces osteogenic differentiation of cells by activation of Wnt/β-catenin signalling dependent *Runx2* expression (Hamidouche et al., 2008). Ascorbic acid is a cofactor for enzymes that hydroxylate proline and lysine in pro-collagen. Ascorbic acid has been demonstrated to induce osteogenic differentiation by increasing the secretion of collagen type 1 that leads to the phosphorylation of ERK MAPK signalling pathway, that translocates into the nucleus, binds to *Runx2* and induces the

expression of osteogenic proteins (Vater et al., 2011). β-glycerophosphate serves as a phosphate source for mineralization and also induces osteogenic differentiation through ERK phosphorylation (Langenbach & Handchel, 2013). Thus, these supplements promote osteogenic/odontogenic gene expression (Coelho & Fernandes, 2000; Kim et al., 2012; Kyllönen et al., 2013). Cell culture in basal medium is expected to mimetic early stages of repair and regeneration process, with an environment with low osteogenic/odontogenic stimuli, while cultures induced into the osteogenic/odontogenic lineage, are expected to mimic more advanced stages of the tissue repair and regeneration, in which cells are strongly induced to differentiate into the designated odontogenic lineage.

Metabolic activity was evaluated, and the results demonstrated that MSS1 increased metabolic activity in basal conditions, at 7 days of culture, being significantly different from the control, while the cultures grown in the absence of FBS do not show differences to the control. Thus, MSS1 seems to compensate the absence of FBS and increase the metabolic activity of the cells. Cell proliferation shows a pattern similar to that of the metabolic activity, in basal conditions. There are many factors that may influence the metabolic activity of the cultures, as increased metabolic activity is not synonymous to increased cell proliferation (DeBerardinis et al., 2008). However, when cells are proliferating, this may be further transduced as an increased metabolic activity, as it is observed in our results. Exposure of DPSCs to MSS1 increased metabolic activity and cell proliferation at 7 days of culture. These results suggest that MSS1 has therapeutic interest to induce cell proliferation at early stages of dental pulp repair and regeneration – throughout the proliferative stage, increasing the cell number. In osteogenic/odontogenic conditions MSS1 exposure induced similar metabolic activity in free-FBS and MSS1 conditions, suggesting that the negative effect is due to the absence of FBS. In terms of cell proliferation, the free-FBS and MSS1 are also similar, despite that at day 7, MSS1 was able to induce the cell proliferation, being in accordance with its capacity to induce initial culture proliferation.

From the several biological processes that PP1 has been associated with, it has been further found to be determinant on the regulation of cell differentiation (Chalfant et al., 1999). Taking this into consideration and the fact that ALP activity is used as a biochemical marker of osteogenic/odontogenic differentiation, we evaluated ALP activity to assess the capability of MSS1 to disrupt PP1 complexes, making PP1 active and consequently induce osteogenic/odontogenic differentiation. Cultured cells in basal conditions showed significant decreased ALP activity after 7 and 14 days of culture. This may be due to the absence of FBS, since it is verified in both conditions, free FBS and MSS1. In the remaining culture times, ALP activity

seems to increase around 30%, when exposed to MSS1 than when culture in FBS-free conditions. Thus, MSS1 seems to be able to compensate FBS absence, demonstrating its potential to induce osteogenic/odontogenic differentiation. In osteogenic/odontogenic conditions, MSS1 increased ALP activity after 21 days of culture, being consistent with the observed reduction in cell proliferation. Cell proliferation and differentiation have an inverse relationship, in other words, when differentiation is induced, cells exit from the division cycle and proliferation reduces (Ruijtenberg & van den Heuvel, 2016). The RT-qPCR results, obtained at day 14, further suggest the potential modulation of MSS1 in DPSCs odontogenic differentiation. In basal conditions, MSS1 does not seem to significantly affect *ALP* expression, as *ALP* expression levels are similar to free-FBS conditions. However, in osteogenic/odontogenic conditions, MSS1 significantly increased its expression. Cells in osteogenic/odontogenic conditions are exposed to high differentiation stimuli and MSS1 seems to induce even more the osteogenic/odontogenic differentiation of dental pulp stroma cells, demonstrating MSS1 potential to induce dental pulp regeneration.

In parallel, we assessed the osteogenic/odontogenic differentiation capacity of dental pulp stromal cells, in the presence of MSS1, through the evaluation of other differentiation markers, as *BMP-2*, *Col1a1*, *MEPE*, *DSPP* and *IBSP*. The listed markers are widely used to assess osteogenic/odontogenic differentiation (Baldión et al., 2018; Sabbagh et al., 2020; Wu et al., 2019). *BMP-2* is involved in the SMAD pathway that has been reported to have an important role in tertiary dentinogenesis and has shown to have osteoinductive capacity and to induce osteogenic/odontogenic differentiation in different cell types, including dental pulp stromal cells. *Col1a1* is the predominant collagen in dentin, and it is fundamental for cellular proliferation, migration and mineralization, being expressed by odontoblastic cells (Sandberg et al., 1988; Xuechao Yang et al., 2007). Teti et al. demonstrated that during odontogenic differentiation of DPSCs treated with a hybrid hydrogel, the expression of *Col1a1* was significantly increased. *MEPE* is a member of bone matrix protein family, and it was found in dental tissues. Liu et al. have demonstrated that *MEPE* regulates odontogenic induction of DPSCs (Liu et al., 2004). *IBSP* has been shown to have a key role during development and regulating the mineralization of bone and dentin tissues (Leme et al., 2022). The increased expression of this gene has been associated with the formation of mineralized matrix, being a mid-differentiation marker (Leme et al., 2022). *DSPP* is known to play a major role during early odontoblastic differentiation and late dentin mineralization (Butler et al., 1992). He et al. demonstrated that in DPSCs differentiations, when *BMP-2* expression increases, the expression of *DSPP* also increases (He et al., 2008). It has also

been demonstrated that MAPK pathway promotes *DSPP* expression and promotes odontogenic differentiation in DPSCs (P. Silva et al., 2009).

In basal conditions, the cells exposure to MSS1 did not influence the expression of osteogenic genes *ALPL* and *Col1α1,* since the results are similar to the cultures in FBS-free medium. The absence of FBS within the culture milieu may have a predominant effect on this assessment. Although the *BMP-2* expression of cells cultured with the peptide is decreased when compared with the cells cultured without FBS, it is increased when compared with the positive control. Relatively to odontogenic genes, MSS1 stimulates the upregulation of *MEPE* and *DSPP* genes, demonstrating its capacity to induce odontogenic differentiation. In sum, MSS1 in basal conditions, enhanced the expression of odontogenic markers of differentiation- *MEPE* and *DSPP*, with minor effects on osteogenic markers- *ALPL* and *Col1α1*.

In osteogenic/odontogenic conditions, MSS1 significantly increased *ALPL* and *BMP-2* osteogenic expression, being consistent with Kim et al. results. The exposure to the peptide also promoted the overexpression of *DSPP* and *IBSP*, demonstrating the strong capacity of MSS1 to induce odontogenic differentiation, since cells were cultured with potent inductors of these genes – dexamethasone, ascorbic acid and β-glycerophosphate. MSS1 was still able to further increase their expression. Siggelkow et al. have demonstrated that *MEPE* is mainly active during proliferative and early matrix maturation stages (Siggelkow et al., 2004). Exposure to MSS1 did not induce alterations in *MEPE* expression, maybe due to the fact that cells were already in mid/late mineralization stages. Thus, our results demonstrated the MSS1 capacity to induce the osteogenic/odontogenic expression in dental pulp stromal cells cultured with osteogenic/odontogenic inductors.

V. Conclusions and future perspectives

The main objective of this thesis was to modulate PP1 complexes and, consequently, dental pulp regeneration using a cell-penetrating peptide that specifically disrupts PP1 complexes. To achieve this goal, dental pulp stromal cells were incubated in the presence of MSS1 peptide for 21 days and culture characterization was assessed through the evaluation of different parameters, as metabolic activity, cell proliferation, ALP activity and osteogenic/odontogenic gene expression.

The exposure of dental pulp stromal cells to MSS1 showed that the peptide was able to induce metabolic activity and cell proliferation, in early stages of dental pulp repair and regeneration, compensating the absence of FBS and increasing cell number. Evaluation of ALP activity allowed us to understand the effect of MSS1 in late stages of dental pulp repair and regeneration, demonstrating its potential to inducing osteogenic/odontogenic differentiation of cells. Additionally, other osteogenic/odontogenic markers were assessed and MSS1, in basal conditions - that mimic an early stage of tissue development/healing, enhanced the expression of odontogenic markers; while in osteogenic/odontogenic-induced conditions – mimicking a more advanced stage of development/healing – MSS1 induced the expression of the osteogenic/odontogenic markers, even in the presence of exogenous inducers, demonstrating its high capability to induce the odontogenic differentiation of dental pulp precursor cells.

On the continuation of this work, it would be interesting to understand how does PP1 induce osteogenic/odontogenic differentiation and identify which molecular pathways it is involved. Then, posteriorly do an *in situ* study to test the clinical applicability of MSS1 in the treatment of pulp diseases, and to understand if its effect is maintained in physiological conditions, in which the peptide is subjected to several interactions.

Concluding, in this work we demonstrated the potential therapeutic effect of MSS1 in disrupting PP1 complexes, in dental pulp stromal cells, potentially activating PP1 and therefore promoting dental pulp repair and regeneration. Thus, MSS1 might be useful to treat dental caries in a more conservative way than the current strategies, stimulating cell proliferation and osteogenic/odontogenic differentiation, focusing on a regenerative outcome.

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