Universidade de Aveiro Departamento de Ciências Médicas
2021

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Caracterização do interactoma da proteina fosfatase 1 na polpa dentária humana

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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 Sâncio da Cruz Fardilha, Professora Auxiliar com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro e coorientação do Professor Doutor Pedro de Sousa Gomes, Professor Associado da Faculdade de Medicina Dentária da Universidade do Porto.

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palavras-chave Proteína fosfatase 1 (PP1), polpa dentária, regeneração, sinalização celular, diferenciação celular.

resumo

A cárie dentária é a doença infecciosa mais comum em todo o mundo. É uma patologia caracterizada pela destruição (parcial ou total) das estruturas dentárias mineralizadas, devido à atividade de bactérias cariogénicas, podendo evoluir para a afeção dos tecidos pulpares e periapicais. Na sequência do dano, a polpa dentária apresenta um potencial regenerativo e reparador que é ativado em resposta à presença de microrganismos. A proteína serina/treonina fosfatase 1 (PP1) parece desempenhar um papel importante na diferenciação de odontoblastos (células da polpa dentária) e na angiogénese, uma vez que a sua expressão é regulada positivamente nesses processos celulares. A PP1 possui atividade regenerativa nos sistemas cardíaco e nervoso e foi identificada na língua e na polpa dentária.

Com este trabalho pretendemos caracterizar as isoformas da PP1 expressas nas células da polpa dentária e identificar interatores (RIPPOs) da PP1γ, conhecidos por modualrem a atividade reparadora/regenerativas das células da polpa dentária. Após uma análise por SDS-PAGE mostrou-se que as isoformas PP1α, PP1β e PP1γ são expressas em células da polpa dentária, ao contrário da isoforma PP1γ2. Além disso, foi confirmada a expressão de RIPPOs envolvidos nas vias de sinalização que levam a processos regenerativos e de reparação pelas células da polpa dentária, como a AKT, p38 MAPK e MAPK1. Estes resultados preliminares evidenciam que a PP1γ é importante na modulação da ativação da via de sinalização envolvida na reparação/regeneração dentária.

keywords

Protein phosphatase 1 (PP1), dental pulp, regeneration, cell signaling, cell differentiation.

abstract

Tooth's decay is the most common infectious disease worldwide. It is a pathological condition characterized by the (partial or total) breakdown of teeth's mineralized structures, due to the activity of cariogenic bacteria, potentially developing into the affection of pulp and periapical tissues. After damage, the dental pulp has a regenerative and reparative potential that is activated in response to microrganisms. Serine/threonine phosphatase 1 (PP1) seems to play a role in the differentiation of odontoblasts (pulp cells) and in angiogenesis, since its expression is upregulated in these cellular processes. PP1 has regenerative activity in the cardiac and nervous systems and has been identified in the tongue and in the dental pulp.

With this work, we aim to characterize the PP1 isoforms expressed in dental pulp cells and to identify PP1γ interactors (RIPPOs), known to play a role in the modulation of the reparative/regenerative activity of dental pulp cells. We were able to show that PP1α, PP1β and PP1γ isoforms are expressed in dental pulp cells, while PP1γ2 expression was not observed. Also, the expression of RIPPOs involved in the signalling pathways that lead to regenerative and repair processes by dental pulp cells, like AKT, p38 MAPK and MAPK1 was confirmed. Our preliminary results suggest that PP1γ is important in modulating the activation of signalling pathway involved in tooth repair/regeneration.

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List of Abbreviations, symbols, and acronyms

AKT	Protein kinase B
ALP	Alkaline phosphatases
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
BAD	Bcl2-associated agonist of cell death
BMP	Bone morphogenetic protein
BMP-2	Bone morphogenetic protein 2
BMP-4	Bone morphogenetic protein 4
BMP-6	Bone morphogenetic protein 6
BMP-8	Bone morphogenetic protein 8
BSA	Bovine serum albumin
BSP	Bone sialoprotein
cAMP	Cyclic adenosine monophosphate
CO-IP	Co-immunoprecipitation
DFSCs	Dental follicle stem cells
DMEM	Dulbecco's Modified Eagle Medium
DMP-1	Dentin matrix protein-1
DPCs	Dental pulp cells
DPSCs	Dental pulp stem cells
DSP	Dual specificity phosphatase
DSPP	Dentin sialo phosphoprotein
ECM	Fibrillar extracellular matrix
ERK	Extracellular regulated kinases
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FD	Periodontal ligament fibroblasts
FG	Gingival fibroblasts
FGFR	FGF tyrosine kinase receptor
GF	Growth factors
HDPCs	Human dental pulp cells

HIPPIE	Human Integrated Protein Protein Interaction Reference
IL-6	Interleukin 6
IL-8	Interleukin 8
JNK	C-Jun N-terminal kinase
MAPK	Mitogen Activated Protein Kinases
MEK	Mitogen-activated protein kinase kinase
MS	Mass spectrometry
MT	Microtubule
OB	Mesenchymal stromal cells
OCN	Osteocalcin
OI	Osteogenic induced mesenchymal stromal cells
Osx	Osterix
P38	P38 MAP kinases
PA	Apical papilla cells
PBS	Phosphate-buffered saline
PDLSCs	Periodontal ligament stem cells
PI3K	Phosphoinositide 3-kinases
Pi	Inorganic phosphate
PKA	cAMP-dependent protein kinase A regulatory subunit
PKC	Protein Kinase C
PLCγ	Phosphoinositide phospholipase C
PP	Protein phosphatases
PP1	Serine/threonine Protein phosphatase 1
PP1C	Serine/threonine PP1 catalytic subunit
PP1γ	Serine/threonine PP1- gamma catalytic subunit
PP1γ1	Serine/threonine PP1- gamma catalytic subunit isoform 1
PP1γ2	Serine/threonine PP1- gamma catalytic subunit isoform 2
PP2A	Serine/threonine-protein phosphatase PP2A catalytic subunit
PP2B/calcineurin	Serine/threonine-protein phosphatase PP2B catalytic subunit
PP4	Serine/threonine Protein phosphatase 4
PP5	Serine/threonine Protein phosphatase 5
PP6	Serine/threonine Protein phosphatase 6
PP7	Serine/threonine Protein phosphatase 7

PPM	Metallo-dependent protein phosphatase
PPP	Phosphoprotein phosphatase
PP1α	Serine/threonine PP1- alpha catalytic subunit
PPβ	Serine/threonine PP1- beta catalytic subunit
pSer	Phosphoserine
PSP	Protein serine/threonine phosphatase
pThr	Phosphothreonine
PTP	Protein-tyrosine phosphatase
pTyr	Phosphotyrosine
RAS	Ras protein
RIPPO	Regulatory interactors of protein phosphatase one
RT	Room Temperature
Runx2	Runt-related transcription factor 2
SCAP	Stem cells from apical papilla
Ser	Serine
SHED	Stem cells from exfoliated deciduous teeth
Thr	Threonine
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WR	Working Reagent
YAP	Transcriptional coactivator YAP1

1. Introduction

1.1. Teeth – structure and composition

The tooth is one of the hardest structures in all human body. It participates in essential body functions such as speech and chewing, playing also a determinant role in facial aesthetics. In the animal kingdom, teeth have important functions as weapons of attack and defence, so must be composed of hard and resistant constituents, and be well anchored to the jaw bones (1).

Each tooth has a specific location and function, as well as specific morphology and shape that assist on the function fulfilment. Each tooth can be divided into distinct parts: clinical crown (the component exposed into the oral cavity), anatomical crown (the component from the cementum-enamel junction to the cusp), and the root (Figure 1).

From a compositional point of view, each tooth is formed by enamel, dentin, cementum and pulp (Figure 1). The enamel is the outer layer covering the anatomic crown, being the hardest substance in the body and presenting some translucidity. It is recognized as the first line of protection, being able to withstand biting pressure, chemical and microbiological attack, but without the possibility to regenerate, once formed. However, enamel has the ability to remineralize upon the establishment of initial lesions.

Dentin, lies underneath the enamel and the cementum layers, making up the most portion of the tooth structure. The enamel and the dentin meet at the dentoenamel junction. Dentin is compositionally a bone-like tissue that is highly mineralized – despite being not as hard as enamel. It is the main structure of the tooth; it supports enamel and cementum and provides a protective covering for dental pulp. Dentin is synthesized by odontoblasts. Cementum is produced by specialized cells, the cementoblasts, derived from progenitor cells that can also differentiate into fibroblasts of the periodontal ligament or osteoblasts. Cementum is mineralized, avascular and non-innervated tissue; its main functions include covering the dental root and anchoring the fibrous periodontal ligament that anchors the tooth into the alveolar bone socket (1). The enamel and cementum meet at the cementoenamel junction. The enamel, dentin and cementum are hard tissues, and the pulp is a soft tissue with a gelatinous consistency. The pulp embraces the tissue in the pulp chamber (located in the crown) and the pulp canals (located in the roots) and is rich in nerves, blood vessels and connective tissue that are responsible for the nutrition of the tooth

structures. Pulp chamber and canals are initially large but reduce progressively with age, due to the formation of secondary dentin (2).

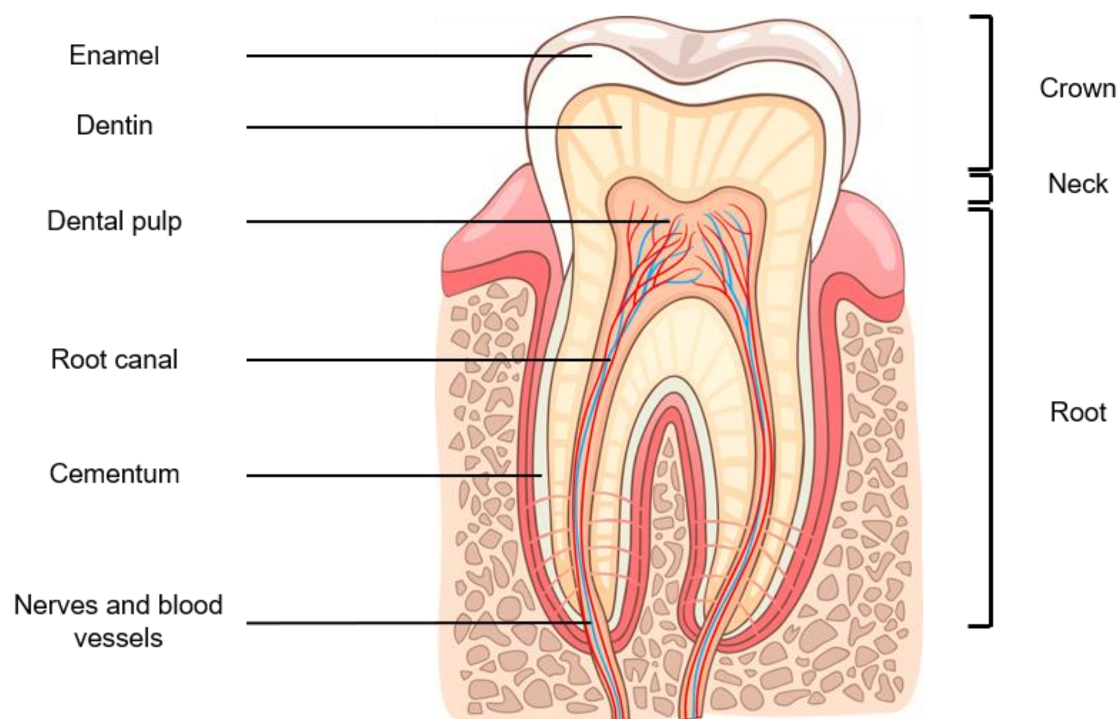

Figure 1 - Tooth morphology. *Definitive human tooth includes 4 types of tissues: enamel, dentin, cementum, and dental pulp that is confined in the pulp chamber and in the root canals. Pulp is composed of connective tissue, nerves and blood vessels that are responsible for the nutritional support of the tooth's structures. Figure was designed by Freepik in freepick.com.*

1.1.1 The dental pulp: composition and function

The development of the tooth, and naturally of the dental pulpal, relies on the bidirectional interaction between oral epithelium and mesenchymal cells, which progressively form the enamel organ and dental papilla, respectively. Pulp development occurs in three stages: the bud, cap, and bell stage. In the bud stage, epithelial cells of the dental lamina proliferate and produce a bud-like projection into the neighbouring mesenchyme. The bud, initially characterized by a round form, progressively enlarges and originates a concave surface – achieving the cap stage. At this phase, oral epithelial cells and mesenchymal cells have developed into the enamel organ and dental papilla, respectively. Subsequently, the structure evolves into the bell stage, characterized by differentiation events at the structural and tissue levels, with the hallmark differentiation of odontoblasts (3).

Dental pulp is an unmineralized oral tissue responsible for the nutrition, defence, and sensation within teeth. Besides, it is composed of lymphatic and nervous elements that

occupy the central pulp chamber of each tooth being extended into the radicular canals, and a odontoblastic cell layer that surround all dental pulp tissue (4). The central region of the coronal and radicular pulp is surrounded by a specialized odontogenic area that is divided in 4 layers, (i) pulpal core, the centre of the tooth is rich in cells and vascular supply; (ii) cell-rich zone, containing fibroblasts and undifferentiated mesenchymal cells; (iii) cell-free zone or zone of Weil, rich in reticular fibers, capillaries and nerve networks and (iv) odontoblastic layer, containing odontoblasts that border the pre-dentin (5). Thus, odontoblasts, fibroblasts, stem cells, immune-inflammatory cells (e.g., macrophage, granulocytes, mast cells and plasma cells) are part of dental pulp (1).

The stem cells can differentiate into many types of cells such as chondrocytes, odontoblasts, adipocytes, neuronal cells and osteoblasts, among others (6). These cells are able to form mineralized tissues (7). Until now, distinct types of human dental stem cells have been isolated and characterized from the teeth, substantiating the reparative/regenerative potential of its tissues. These include: dental pulp stem cells (DPSCs) (8,9), stem cells from exfoliated deciduous teeth (SHED) (10), stem cells from apical papilla (SCAP) (11), dental follicle stem cells (DFSCs) (12) and periodontal ligament stem cells (PDLSCs) (13,14).

The fibroblasts present in the dental pulp are responsible for the production of various cytokines and chemokines, as well as inflammatory mediators, such as Interleukin 8 (IL-8), Interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF), further displaying a protective activity within the dental pulp against cariogenic microorganisms (5).

Odontoblastic cells also play a fundamental role in pulp homeostatic maintenance and response to damage. Odontoblasts are highly specialized and form a single layer along the interface between the dental pulp tissue and dentin, displaying an important role in the synthesis of primary and secondary dentin (i.e., dentinogenesis). When the tooth is healthy, the odontoblast single layer acts as a natural barrier between mineralized tissues and provides an extensive sensory network (4,15). When a pathological stimuli occurs, DPSCs seem to migrate to the injured site and differentiate into secondary odontoblasts that will synthesize tertiary dentin, which is characterized by less mineralization, high porosity and fewer tubes, being a higher disorganized tissue (16).

Odontoblasts play a secondary role in dental sensitivity according to Brännström hydrodynamics, which explains that abrupt changes in the fluid within the dentinal tubules, leads to stimulation of nearby odontoblasts causing their afferent myelinated A fibers to send sensory signals to the brain (17), as recently shown by Tazawa et. al study (18).

Odontoblasts are also responsible for secretion of the fibrillar extracellular matrix (ECM) of pre-dentin and also by the subsequent mineralization of the matrix to form dentin, by the secretion of proteolytic enzymes (19). Pre-dentin is an unmineralized matrix that is rich in type I collagen. This matrix is modified first, in the organic deposition phase, by proteolytic enzymes; and in a second phase, by the deposition of minerals in front of the mineralization between pre-dentin and dentin. It results in a constant layer of unmineralized pre-dentin at the dentin-pulp interface, with dentin mineralization corresponding to pre-dentin secretion (20). Odontoblasts are though the primary biologically active cells that maintain the dentin through dentinogenesis and protect the living pulp tissue by deposition of reactionary dentin in response to bacterial invasion, in early stages of dental caries. They are also involved in innate and adaptive immunity (15).

Enamel, dentin and cementum act as a protecting barrier against microorganisms in the external environment. When dental pulp function is compromised, pulp inflammation followed by necrosis may occur, and in severe cases, bone cells may also be affected (21).

Dental pulp is a highly vascularized and innervated region of the tooth, being the site of origin for most pain-related sensations (22). However, the arrangement of the pulp chamber is characterized by minimal collateral blood supply, which makes the immune system response against infections challenging (23). Thus, inflammatory processes in the pulp tissues that are not well addressed can lead to wound and degeneration. Most pulp cells are linked by desmosome-like and gap junctions which implies a deeper and faster communication, however, any negative effect in one cell may affect all others very quickly (23,24).

The main function of the dental pulp is the synthesis of dentin through odontoblasts. However, it also has other important functions, such as, the nutrition of teeth's tissues; sensory functions that allow to recognize differences in temperature and pressure; and defence and repair activity to restore damaged dentin through the synthesis of reparative dentin or tertiary dentin by odontoblasts. Therefore, the integrity of the dental pulp must be maintained to ensure its regenerative properties and to be able to address the damages submitted to the tooth.

1.2. Defence mechanisms after dental damage

The mechanisms of pulp regeneration and dentinogenesis are yet to be fully clarified. After damage, the dental pulp has a regenerative and reparative potential that is activated in order to prevent injury development in the mineralized tissues (enamel and dentin) and

within the dental pulp itself (25). Dental pulp can form calcified tissues in response to environmental factors such as dental caries, trauma and injury (26). It is also known that denervated teeth have their dentin repair responses compromised, as well as a reduced biomechanical protection (27).

Upon injury, the immune system acts on the pulp-dentin complex through dendritic cells, macrophages, lymphocytes, endothelial cells, and various epithelial and mesenchymal cells. When the integrity of enamel and dentin is compromised, signals are released to activate T-lymphocytes, which in turn orchestrate other immunocompetent cells to assemble the local immune defence of dental pulp (28).

Regenerative response of the dental pulp depends mostly on stem cells, which are cells characterized by a self-renewing capacity and ability to differentiate into distinct lineages of adult cell populations (29). Thus, humans, during aging, reveal a decreased stem cell niche in the teeth, which leads to hampering changes in the regenerative capacity of the pulp (5,25).

Fibroblasts also play an important role in managing pulp repair/regeneration and within the response to injury (30). They are responsible for synthesizing growth factors involved in the restoration of blood vascularisation, nerve sprouting and regeneration of pulp-dentin complex, recruiting stem cells and directing its migration and sprouting into the injury site (31,32). Moreover, fibroblasts synthesize proteins that are involved in the production of complementary bioactive fragments process (33). Bioactive fragments lead to a regenerative response in pulp tissue and nerve, but are also efficient in destroying cariogenic bacteria (32,34).

Through a clinic perspective, necrotic tissue resulting from pulp infection – through the penetration of cariogenic bacteria along mineralized teeth structures - and cell death, must be removed. Current strategies rely on the elimination and replacement by non-viable synthetic materials, particularly when the regenerative potential of the tissue is exhausted. Therefore, one of the main focuses on dental medical research is to identify and improve new approaches and techniques that focus to increase dental pulp regeneration and maintenance, avoiding tooth devitalization. The most recurrent approach against infection and irreparable damage in the pulp, relying on its removal and substitution for a biocompatible material (35).

Transplantation of mesenchymal stem cells into endodontically-treated root canals, is one of the main research focuses. This technique aims to regenerate the damaged pulp-dentin complex. Although most of the research conducted on stem cell-mediated regenerative

endodontics used animal models, initial human clinical data are becoming available (36). However, much of the actual research also involves using scaffolds and biomodulators to tailor the appropriate growth and differentiation of cells (37). These scaffolds aim to imitate the natural pulp microenvironment, providing all kinds of signals, molecules and allowing cell matrix and cell-cell interactions, aiming to provide a better regenerative potential (38). In the last years, dental pulp stem cells became very important tool to study repairing mechanisms. It will allow to better understand the role of these cells in dental-pulp and dentin repair, further embracing tissue engineering strategies and stem cell/scaffold constructs (39,40).

1.3. Proteomics of human dental pulp

The main objective of proteomic research is to understand protein expression and function through the identification and analysis of the total proteins in a certain setting. This seeks to characterize and structure the functions of proteins, as well as interactions between proteins, proteins-nucleic acids, proteins-lipids and enzymes-substrates and also analyse post-translational modifications, proteins processing and activation, renewal and protein synthesis and its isoforms, thus being able to draw conclusion regarding the function of certain cell or tissue type, at a given time (41).

Until now, only three complex studies described the proteome of human dental pulp (42–44). In the first work Pääkkönen et al. (2005) (43) two-dimensional (2D) gel electrophoresis technique and mass spectrometry (MS/MS) analysis were used to identify the proteins expressed in dental pulp. The authors identified 96 proteins in the human dental pulp tissue (43). The second study was conducted by Wei X et al. (2008) (42), it was based in 2D differential gel electrophoresis to study the proteome of human dental pulp during the odontoblasts-like differentiation of dental pulp cells, *in vitro* (42). Only 23 proteins were mapped by MS and related to the differentiation phase of odontoblasts. The third study Loureiro C et al (2020) (44) carried out a quantitative proteomic analysis of dental pulp samples using nano ACQUITY UPLC-Xevo QTof MS system. A total of 465 human proteins were identified in samples of normal, inflamed, and necrotic pulp (44).

The regeneration of dental pulp is characterized by the activation of cascades that will promote cell adhesion, proliferation, migration, angiogenesis and differentiation of dental pulp cells (45). Growth factors (GF) have the ability to control the activity of stem cells, regulating the rate of proliferation, differentiation, including the ability to stimulate cells to synthesize mineralized matrices (45,46). It has been shown that human dental pulp cells

(HDPC) secrete GF like basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) and their concentration increases after tooth injury (47).

Basic FGF is expressed in almost every tissues (48). This GF is involved in cell proliferation, cell differentiation, cell migration, angiogenesis, and cell survival under physiological and pathological conditions (48,49). When bFGF is present in the extracellular matrix, it is able to connect to the FGF 1-4 tyrosine kinase receptors (FGFR1-4) of the pulp cells' membrane, leading to the activation of the RAS-MAPK, PI3K-AKT and PLCγ-PKC pathways (Figure 2) and consequently, resulting in cell proliferation and cell survival (48). Studies showed that bFGF has an effect on dental injuries healing in a processes recapitulating the odontoblastic differentiation *in vitro* and *in vivo* (50), including the tertiary dentin formation (51). Basic FGF is also related to signalling pathways involved in DPSCs proliferation (52), as well as in DPSCs differentiation and recruitment events (53). Sagomonyants et. al (2015) found that bFGF mediates dental pulp odontoblastic early differentiation by activation of FGFR-MEK-MAPK1-2 and BMP-BMPR signalling pathways, resulting in the upregulation of odontoblastic differentiation markers, such as bone sialoprotein (BSP), osteocalcin (OCN), dentin matrix protein-1 (DMP-1), and dentin sialo phosphoprotein (DSPP) (54). Basic FGF has a role in DPSCs differentiation since it induces neural differentiation of DPSCs through MAPK and AKT signalling pathways (55). Besides that, a study showed that bFGF induced a positive cytokine regulation in dental pulp mediated by PKC, PI3K-AKT and MAPK signal pathway as indicated by the increase in the phosphorylated form of p38 and c-Jun N-terminal kinase (JNK), regulated by MAPK1-2, PKC and AKT.

Basic FGF stimulates angiogenesis *in vivo* and plays an important role in neovascularization of injured tissue (56). It is also known that bFGF synergizes with VEGF, secreted from pulp fibroblasts, regulating stem cells differentiation (56,57).

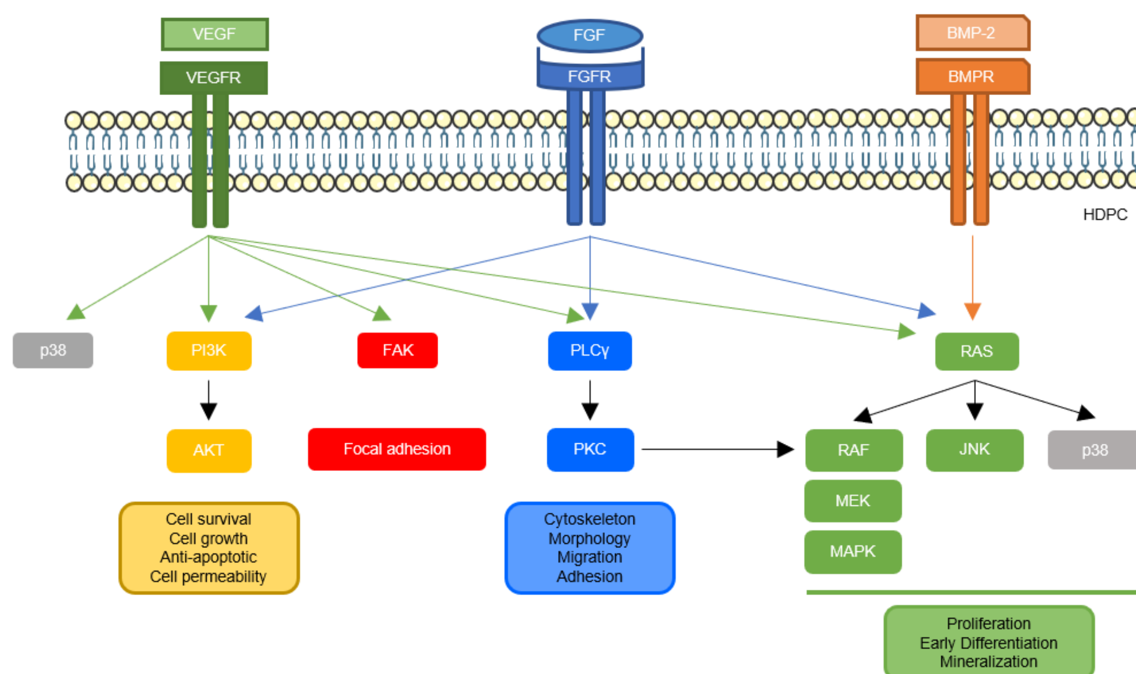

Figure 2 - Main signaling pathways in HDPCs that modulate regenerative and repair processes. *Representative scheme of fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP) activity in HDPCs. Growth Factors when bound to the membrane receptors activate signaling pathways mediated mostly by kinases. Protein kinase B (AKT) and phosphoinositide 3-kinase (PI3K) form the important signaling pathway for the regulation of the cell cycle, acting on cell survival and growth mainly. Protein kinase C (PKC) and phospholipase C gamma (PLCγ) signal pathways are involved in cell migration and adhesion, and can activate MAPK signal pathway. MAPK signal pathway includes mitogen activated protein kinase (MAPK), p38 MAP kinases (p38), c-jun N-terminal kinase (JNK), mitogen activated protein kinase kinase (MEK), RAF proto-oncogene serine/threonine-protein kinase (RAF) and RAS protein (RAS). When activated, this signal pathway is responsible for the processes of cell proliferation and differentiation in HDPCs in early stage of dental pulp injuries. Image was based on Vaseenon et al 2020* (56) *and was made using SMART - servier medical art database (https://smart.servier.com).*

VEFG is a key regulatory factor of vascular permeability and angiogenesis (58). VEGF gene is expressed by human pulp fibroblast and can be induced by pro-inflammatory cytokines, expressed by dental pulp as a response to damage and inflammation (59). VEFG is upregulated in macrophages, odontoblast-like cells and DPSCs (60). The VEGF produced by HDPCs acts directly on them in an autocrine way but can further act on endothelial cells, which signal pathways are known, and seem to activate the cell proliferation and/or differentiation. The activation of this processes is due to the connection of VEGF to the vascular endothelial growth factor receptor (VEGFR) present in the dental pulp cells' membrane (61). VEGFR activation can regulate PI3K-AKT pathway leading to cell survival, cell proliferation, anti-apoptotic pathway and cell permeability (62). Another important target

of VEGF is PLCγ-PKC and downstream induction of the MAPK and other PKC-dependent pathways to induce cell proliferation and vaso-permeability (63). Activation of p38 MAPK and FAK on endothelial cells, through VEGF signal pathway, will be important in endothelial cell migration (Figure 2) (64,65).

Growth factors, like bone morphogenetic protein 2 (BMP-2), BMP-4, BMP-6 and BMP-7 have an important role in the biology of pulp cells. BMP-2 is increased during terminal differentiation of odontoblasts (66,67). BMP-2 is required for the differentiation of SHED into odontoblasts (68) and the same happen on DPSCs differentiation into odontoblast (69). It is known that inorganic phosphate (Pi) regulates BMP-2 expression via cAMP/PKA and ERK1/2 pathways in HDPCs (70). BMP-2 also plays an important role in the regulation of early and late odontoblast differentiation through non-canonical BMP signaling, involving JNK, MAPK and p38 MAPK signaling pathways (Figure 2) (71–73). It was also shown that DPCs treated with BMP-2 reveal an induced late-stage odontoblastic differentiation through the activation of JNK. When inhibition of JNK occurs, it does not affects the activation of MAPK1 - which induces the early differentiation, but late differentiation is not assured (74). Studies have also shown that odontoblasts are capable of BMP-2 production and secretion, promoting odontoblast and other HDPCs' differentiation (72,75,76).

Alkaline phosphatases (ALP) activity is relevant in odontoblast differentiation and dentin formation (77). ALP induces the formation of mineralized nodules that can indirectly represent the mineralization capacity and differentiation ability of odontoblasts cells. BMP-2, when stimulating HDPCs, increases the formation of mineralized nodules what may evidence the ALP activity in these cells (71).

It was also suggested that bFGF activity in odontoblast differentiation may induce the expression of Dmp1 and Dspp (mediated by FGFR/MEK/MAPK1 signaling) and increase the expression of BMP-2 and consequently the activation of BMP signal pathway (54,78,79). However, there are evidences that bFGF has two effects on odontoblast differentiation, having a positive role during proliferation, growth and early differentiation phase and an inhibitory effect during the late differentiation/mineralization phase, showing the importance of BMP-2 signal pathway activity (78).

It is known that when DPSCs are treated with bFGF there is an inhibition in mineralization due to a decrease in ALP activity (80). With this information, bFGF can play an important role in an initial state of differentiation, as well as in the activation of BMP signal pathway by acting positively on odontoblasts. However, its inactivation is essential in late stages of differentiation and mineralization since the late is characterized by an intense activity of ALP

that is inhibited if the DPCs are under the constant effect of bFGF. Once bFGF regulates the BMP-2 activity, that are activated during late differentiation/mineralization phase, it is possible to conclude that during an initial phase of dental injury, bFGF is released to promote the proliferation and HDPCs survival and start differentiation of HDPSCs in odontoblast-like cells and that there is a decrease of bFGF, at a later stage, to promote mineralization and later stage of differentiation in mature odontoblast cells (81).

Thus, the activity of kinases in bFGF, VEGF and BMP signal pathway are important to trigger a defensive response as well as the proliferation and differentiation of DPSCs in odontoblasts that will carry out mineralization and will repair damaged dentin.

1.4. Protein phosphatase 1 (PP1)

Protein phosphatases have the opposite function of protein kinases, they are responsible for the removal of the phosphate group (PO_4^{3-}) from phosphoproteins by hydrolyzing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl (-OH) group (82).

Protein phosphatases and protein kinases play a fundamental role in cell regulation since they are responsible for mediating signal transduction of many proteins, thus changing their functional state according to their phosphorylation state. Since more than 20% of all proteins can interact with kinases and phosphatases, they become extremely important in cell regulation and can quickly modify the function of enzymes, molecule structures, proteins, receptors and ion channels without having to modify their expression. This duality can produce long-term alteration in cellular transcription, translation and complement of proteins expressed in the cell (83). Until now, there are approximately 226 known protein phosphatases (84) which are classified into 3 families: phosphoprotein phosphatase (PPP) family, metallo-dependent protein phosphatase (PPM) family and protein-tyrosine phosphatase (PTP) family (85). The PPP family, that includes serine/threonine protein phosphatase 1 (PP1), and PPM are responsible for most dephosphorylation reactions of phosphoserine and phosphothreonine (pSer/pThr) and also phosphotyrosine (pTyr) (86,87).

PP1 belongs to the phosphoprotein phosphatases (PPP) superfamily, responsible for more than 90% of dephosphorylation reactions in a cell. PPP includes PP1, PP2A, PP4, PP6, PP2B/calcineurin, PP5 and PP7 and they are widely distributed Ser/Thr which regulate multiple cellular processes including cell division and proliferation (88). All proteins of this

superfamily share a high homology in the catalytic domains between each order, diverging only at their N- and C- terminal (89).

PP1 is composed by a catalytic subunit (PP1C) and a regulatory subunit, known as regulatory interactors of protein phosphatase one (RIPPOs). The connection of the PP1C subunit to RIPPO subunit allows the formation of numerous forms of PP1, which have distinct substrate specificities, restricted subcellular locations and diverse regulation (90). PP1 was first described in 1940s as being responsible for dephosphorylates glycogen phosphorylase a, reforming the inactive glycogen phosphorylase b, but among the years it was associate to many other functions, such as glycogen metabolism, muscle contraction, cell division and progression, apoptosis, meiosis, protein synthesis neuronal activities, RNA splicing and regulation of membrane receptors and channels (91–93).

In mammals, the catalytic subunit PP1C is encoded by 3 separated genes (*PPP1CA*, *PPP1CB* and *PPP1CC*) with slight differences in N- and C- terminal. *PPP1CC* gene suffers alternative splicing giving rise to isoform PP1γ1 and a testis-enriched and sperm-specific isoform, PP1γ2 (94). This two PP1γ isoforms only diverge in their C-terminal (95). Therefore, the localization and substrate specificity of these isoforms depends on oligomeric complexes formation and the regulatory subunits RIPPOs.

With the use of specific antibodies for each PP1 isoform it was possible to verify that all 4 are expressed in most mammalian cells and each one has its own location and characteristics (96). Taking in consideration the PP1γ, the PP1γ1 isoform has an higher expression in the nucleoli in association with RNA, and in mitosis, since this isoform is associated with microtubules (97).

1.4.1 Role of Protein Phosphatase 1 in pulp regeneration

PP1 expression in the tooth was discovered in 2017 by Kim et. al, showing that PP1 is mainly expressed in pre-odontoblasts, odontoblasts, endothelial cells, and dental pulp cells of the developing mouse pulp tissue. This study raised the probability of PP1 regulating the development of odontoblasts phenotype by modulation of dental extracellular matrix proteins and odontogenic factors. It was identified that PP1 expression levels are lower during the tooth formation and at the 13th day, the levels of PP1 increase during the state of differentiation and mineralization. The increase of PP1 levels indicate that PP1 may have an important role in the maturation and mineralization of dental pulp cells (98). PP1 is an *in vitro* target for natural ceramides that are associated with the biological differentiation process (99). C2 ceramide is an activator of PP1 (100). Kim et al. showed that C2 ceramide increased the expression of early marks of osteogenesis such as alkaline phosphatase

(ALP) activity and later markers such as alizarin red staining and the up-regulation of dentin matrix protein 1 (DMP-1), and dentin sialo phosphoprotein (DSPP) (98). Human dental pulp cells have to secrete high levels of angiogenic factors like endothelial growth factors and angiopoietin 1 (101), in order to induce the capillary tube formation (102). Thus, C2 ceramide increases the expression of angiopoietin 1 and promotes migration and capillary tube formation of endothelial cells (98). This study also showed that expression of BMP-2, Runx2 and Osx - that are the main transcription factors required for the activation of osteoblast differentiation (103) - was enhanced by PP1 activation and inhibited by PP1 silencing. It was also reported that BMP-Runx2 pathway might be partially activated by PP1 during odontoblastic differentiation (98).

1.5. Aims

PP1 is expressed in dental pulp cells, mainly in odontoblats, with higher expression levels during the late differentiation and mineralization processes. Odontoblats intervene directly with the regeneration and repair processes of the pulp, suggesting that PP1 can be a molecular target for pulp repair and regeneration.

In order to address the molecular signaling during pulp injury and repair/regeneration, aiming to foster inovative therapeutic approaches, this study aims to identify PP1γ interactome and discover potential RIPPOs that are related with the repair and regeneration processes, and that may present a therapeutic potential on dental pulp regeneration. To accomplish this general goal the following aims were proposed:

Aim 1: To Identify RIPPOs in dental pulp using a bioinformatic aproach;

Aim 2: To identify the PP1 expression pattern in human dental cell cultures;

Aim 3: To identify the PP1γ RIPPOs enrolled in dental pulp regeneration.

2. Material and methods

The experimental procedures were performed at the Signal Transduction Laboratory, Institute for Biomedicine (iBiMED), University of Aveiro (Aveiro, Portugal). All the details need to complete the methods information may be found in Supplementary Data.

2.1. Bioinformatic

2.1.1 Data collection

Characterization of experimentally detected PP1γ RIPPOs in human were retrieved from the Human Integrated Protein Interaction Reference HIPPIE public database (consulted, Jan 23th, 2020) using PP1G_HUMAN UniProt ID reference. From this search, 385 entries were obtained. Moreover, the proteome of the human dental pulp was collected by combining the data from Pääkkönen V et al (2005) (43) and Loureiro C et al (2020) (44). Pääkkönen V et al (2005) (43) provides information on 98 proteins present in human dental pulp. Finally, information from Loureiro C et al (2020) (44) was used, which provides information on 211 proteins founded in human dental pulp.

2.1.2 Data analysis and illustration

To characterize the PP1γ RIPPOs present in human dental pulp, a Venn diagram analysis was performed using the Venny 2.1 tool (https://bioinfogp.cnb.csic.es/tools/venny/index.html) (104). Briefly, the human dental pulp data from Pääkkönen V et al (2005) (98 proteins) (43) and Loureiro C et al (2020) (211 proteins) (44) were combined with the PP1γ RIPPOs human dataset. The output from HIPPIE database resulted in the identification of 385 PP1γ interactors, however 12 proteins were excluded since they contained a score of 0, and a total of 373 PP1γ RIPPOs were selected for the Venn diagram analysis. The crossing and analysis of the 3 lists was based on UniProt ID; in each list 1 protein was not assigned by the UniProt ID and these 3 proteins were compared in both lists manually, and the correspondence was not verified.

2.2. Cell culture

In this work, human cell cultures established from primary human tissues were used. Briefly, mesenchymal stromal cells (OB), osteogenic induced mesenchymal stromal cells (OI), periodontal ligament fibroblasts (FD), gingival fibroblasts (FG), apical papilla cells (PA), and

dental pulp cells (DPCs) cultures were obtained from BoneLab - Faculty of Dental Medicine U. Porto. The cells were maintained in alpha-MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (1% penicillin/streptomycin mixture). Osteogenic differentiation of the precursor cells occurred with the addition of 10 mM β-glycerophosphate, 10^{-8} M dexamethasone and 50 µg/mL ascorbic acid. All cell lines were grown on T75 flasks at 37ºC in a 5% CO2 humidified incubator.

2.3. Cell extracts preparation

Cell culture medium was removed from T75 flasks and cells were washed with 5mL of ice-cold 1x PBS two times. For cell lysis, 500 uL of 1% SDS lysis buffer was used and the content transferred to 1.5 mL microcentrifuge tubes and incubated for 30min at 4ºC with constant agitation. Sonication was performed for 10 seconds 3 times and the content placed in new microcentrifuge tubes. The samples were centrifuged at 4ºC for 20 min at 16,000 g. The supernatant was transferred to new microcentrifuge tubes and kept in ice.

2.4. Bicinchoninic acid assay

Extracts were mass normalized using the bicinchoninic acid (BCA) assay (Pierce BCA kit, Fisher Scientific, Portugal). Samples were prepared in duplicate by adding 25 µl of each sample in a 96-well plate. Standard protein concentrations were prepared as described in Supplementary table 1. The Working Reagent (WR) was prepared under the manufacture's indications: briefly the BCA reagent A was mixed with BCA reagent B in a proportion of 50:1. Then, 200 µl of WR were added to each well (standards and samples) and the plate was incubated for 30 min at 37ºC. Once the 96-well cooled to room temperature (RT) the absorbance was measured at 562 nm using an Infinite® 200 PRO (Tecan, Switzerland). A standard curve was obtained by plotting BSA standard absorbance vs BSA concentration and used to determine the total protein concentration of each sample.

2.5. Western Blotting

The protein extracts were resolved by a 10% SDS-polyacrylamide gel electrophoresis run and the proteins were electrotransferred to 0.45 µm nitrocellulose membranes. The gel was run at 200 V and electrotransferred at 200 mA for 2 hours. For loading control, the membrane was incubated with Ponceau S. solution (Sigma Aldrich, 0.1% [w/v] in 5% acetic acid) for 15 min at RT with slow agitation. The membranes were washed 3 times with distilled water until the protein bands were well defined and analysed using densitometer

GS-800 BioRad (USA). To remove the staining, the membrane was washed twice with 1x TBS-T. Then, non-specific protein-binding sites on membrane were blocked with 5% non-fat dry milk solution in TBS-T 1x or 5% bovine serum albumin (BSA) in 1x TBS-T for 1 hour at RT. For the PP1 isoform characterization in the protein extracts, the membranes were incubated with primary antibody for 1 hour at RT with rabbit anti-PP1α and mouse anti-PP1β or rabbit anti-PP1γ, diluted 1:2500, 1:1000 and 1:5000 respectively in 5% non-fat dry milk solution in 1x TBS-T). To identify if the proteins involved in signal pathways in the HDPCs were expressed, the membranes were incubated with mouse anti-AKT1(1:1000) and rabbit anti-p38 MAPK (1:1000), in 5% non-fat dry milk solution in 1x TBS-T overnight at 4ºC, and with rabbit anti-RAF1(1:1000) in 5% non-fat dry milk solution in 1x TBS-T and mouse anti-MAPK1 (1:1000) in 5% bovine serum albumin (BSA) in 1x TBS-T for 1 hour at RT.

After the incubation, the membranes were washed 3 times for 10 min with 1x TBS-T. Then, the membranes were incubated with secondary antibody for 1 hour at RT (IRDye®680RD anti-rabbit or IRDye®800CW anti-mouse diluted 1:20000 in 5% non-fat dry milk solution in 1x TBST or 5% bovine serum albumin (BSA) in 1x TBS-T). After the incubation, the membranes were washed twice with 1x TBS-T for 10 min and once with 1x Tris-buffered saline (TBS) and immunodetected using Odyssey Infrared Imaging System (LI-COR® Biosciences, US).

The pixel intensity was quantified using Quantity One ® software (BioRad, USA). The intensity from an empty lane within each membrane was subtracted from all signals and all data was normalized to the Ponceau S. control.

3. Results and Discussion

The work performed aimed to identify PP1γ interactome and potential RIPPOs enrolled in dental pulp cells' regenerative processes. To accomplish such goal, a bioinformatic analysis was carried out by crossing the human dental pulp proteome and the PP1γ RIPPOs. The next section presents a detailed description of the results.

3.1. Identification of PP1γ interactome in dental Pulp using a Bioinformatics approach

To obtain the PP1γ interactome in human dental pulp, a search was performed in the HIPPIE database. This research provides a confidence score and functionally annotated human protein-protein interactions (PPIs). Searching PP1G_HUMAN lead to an output that comprised 385 annotated PP1γ interactions called RIPPOs. However, 12 proteins were removed since their score was 0.00, which translates into a lack of concrete evidence regarding the veracity of this interaction. Thus, 373 proteins were used for the study and the protein with the lowest score was 0.49, which translates into an interaction with low level of confidence. The average confidence reached was at 0.63 and the highest confidence at 0.73. Of the 373 proteins, 14 had scores between 0.49 and 0.62 (low confidence), 242 proteins between 0.63 and 0.72 (medium confidence) and 117 between 0.73 and 0.98 (high confidence).
With the merge of Pääkkönen V et al. (2005) (43) data and from Loureiro C et al (2020) (44) data, and after the elimination of the duplicates, a total of 289 proteins were obtained. These proteins can be considered the human dental pulp proteome, so far known.

Crossing this information with the HIPPIE database output, through Venny 2.1 (104), it was possible to know which RIPPOs were present in HDPCs. From this analysis, 20 proteins were obtained which are known to interact with PP1γ and are found in human dental pulp proteome (Figure 3). Of these, 4 had scores >0.73 (high confidence), 15 had scores between 0.73-0.63 (medium confidence) and 1 had a score <0.63 (low confidence). The proprieties and functions of these proteins can be found in (Table 1).

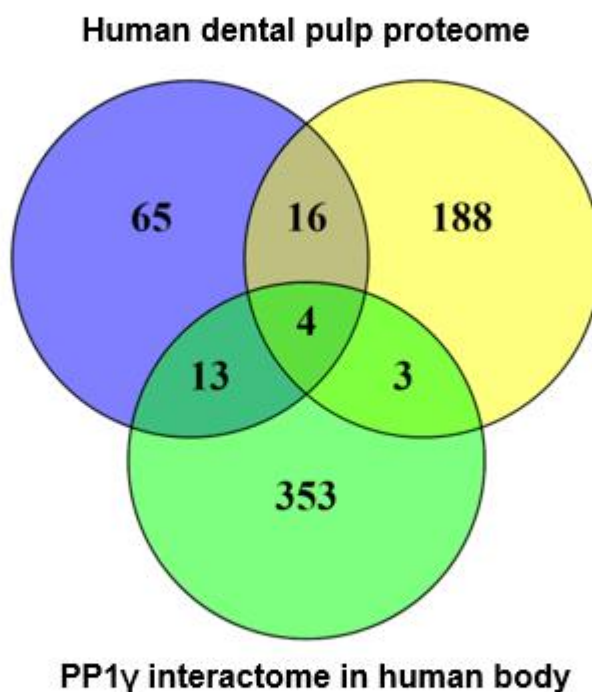

Figure 3 - RIPPOs present in human dental pulp. *Venn diagram illustrating the proteins that are present in the human dental pulp and that at the same time are known to interact with PP1γ. This illustration was drawn through Venny 2.1 interactive tool for comparing lists (https://bioinfogp.cnb.csic.es/tools/venny/index.html).*

In this way, a total of 78 proteins were obtained from Pääkkönen V et al. (2005) (43), 191 from Loureiro C et al (2020) (44), being 20 common to both works. This is explained since the articles used different techniques of MS to obtain and characterize the dental pulp cells' proteins. From Pääkkönen V et al. (2005) (43) 13 of 78 proteins are PP1γ RIPPOs and from Loureiro C et al (2020) (44) 3 of 191 proteins are RIPPOS. From the 20 common proteins in both works 4 proteins are PP1γ RIPPOs. Therefore, a total of 20 proteins were obtained which are PP1γ RIPPOs and are present in human dental pulp.

Table 1 – *PP1γ RIPPOs identified in Human dental pulp through bioinformatics analysis.*

UniProt KB	Protein name	Molecular function	Biological process	Score
P31946	14-3-3 protein beta/alpha	Receptor signalling complex scaffold activity	Signal transduction	0.75
P27348	14-3-3 protein theta	Receptor signalling complex scaffold activity	Signal transduction; Cell communication	0.63
P63104	14-3-3 protein zeta/delta	Receptor signalling complex scaffold activity	Regulation of cell cycle	0.55
P60709	Actin, cytoplasmic 1	Structural constituent of cytoskeleton	Cell growth and/or maintenance	0.82
P63261	Actin, cytoplasmic 2	Component of the cytoskeleton	Cell motility	0.86
P06576	ATP synthase subunit beta, mitochondrial	Transporter activity	Metabolism; Energy pathways	0.72
Q16555	Dihydropyrimidinase-related protein 2	Cytoskeletal protein binding	Cytoskeleton organization; Signal transduction	0.63
Q9BY44	Eukaryotic translation initiation factor 2A	Cadherin, tRNA and ribosome binding	Regulation of translation; Ribosome assembly	0.63
P09104	Gamma-enolase	Promotes neuron cell survival	Neurotrophic and Neuroprotective	0.63
P02792	Ferritin light chain	Storage protein	Transport	0.63
P02545	Prelamin-A/C	Structural molecule activity	Cell growth and/or maintenance	0.73
P07196	Neurofilament light polypeptide	Structural constituent of cytoskeleton; Protein C-terminus binding; Identical protein binding	MAPK cascade; Axonal transport of mitochondrion	0.63
O00299	Chloride intracellular channel protein 1	Intracellular ligand-gated ion channel activity	Transport	0.72
Q06323	Proteasome activator complex subunit 1	Endopeptidase activator activity	Positive regulation of endopeptidase activity; Regulation of proteasomal protein catabolic process; Antigen processing and presentation.	0.63
Q9Y6H5	Synphilin-1	Identical protein binding	Cell death; Cellular protein metabolic process	0.88
P78371	T-complex protein 1 subunit beta	Chaperone activity	Protein metabolism	0.72
P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta	Transmembrane signalling systems	Signal transduction	0.63
P06753	Tropomyosin alpha-3 chain	Cytoskeletal protein binding	Cell growth and/or maintenance	0.72
P67936	Tropomyosin alpha-4 chain	Structural constituent of cytoskeleton	Cell growth and/or maintenance	0.72
P07437	Tubulin beta chain	Structural constituent of cytoskeleton	Cell growth and/or maintenance	0.72

The 14-3-3 proteins family encodes several isoforms and PP1γ can interact with beta/alpha, theta, and zeta/delta (105). These proteins bind with proteins like RAF1 (106,107), PKC (108), PI3K (109), Bcl2-associated agonist of cell death (BAD) (110), transcriptional coactivator YAP1 (YAP) (111), RAS (112) and MEK (113). 14-3-3 proteins are key players in several steps of some signal pathways, activating or inactivating many proteins. The interaction between 14-3-3 and PI3K (114) activates the AKT signal pathway, leading AKT to inactive BAD and YAP, 14-3-3 interactors (111,115) by phosphorylation. It results in an anti-apoptotic effect in cells (116). Moreover, 14-3-3 proteins can also interact with RAF1 and MEK, in MAPK signal pathways, and activate these proteins when binding and inhibiting their dephosphorylation (107,113). Subsequently, 14-3-3 proteins were shown to inhibit the PKC (117). Thus, 14-3-3 activates PI3K and inactivates BAD and YAP. Since PP1γ binds to 14-3-3 proteins and also dephosphorylate AKT (118), PP1γ may inhibit PI3K-AKT signal pathway and induce cell death (Figure 4).

In the MAPK signal pathway, 14-3-3 proteins may activate RAF-1 and MEK and lead to cell proliferation and differentiation (119). In this case, since PP1γ interacts with 14-3-3 and RAF1, PP1γ needs to be inactive to let MAPK signal pathway to be active and to promote proliferation and differentiation of HDPCs (Figure 4).

In PLCγ-PKC signal pathway, it is known that 14-3-3 protein inhibit PKC protein (117). Since 14-3-3 is a RIPPO, the activation of PP1γ may be important to allow the activation of PKC signal pathway through its interaction with 14-3-3. With this, PKC signal pathway becomes important as it will allow the regulation of the morphology of several HDPCs, as well as the regulation of the migration of HDPSCs to the damage sites and of endothelial cells' migration and sprouting within blood vessels' formation.

Therefore, in a normal HDPCs activity, without tooth damage, it is expected that PP1γ is active in the MAPK signal pathway to prevent the activation of MAPK1 and 14-3-3, in order to ensure RAS and MEK inactivation. Besides, in AKT signal pathway, PP1γ needs to be inactivated to prevent cells to activate apoptosis processes and to ensure their survival. By these PP1γ/AKT interaction, the YAP and BAD phosphorylation will be avoided, and cells will suffer apoptosis. In normal tooth it is also expected that PP1γ will be inactivated in PKC signal pathway, only staying active to inhibit 14-3-3 protein and promote cell morphology rearrangement, migration and cytoskeleton regulation (120).

In case of tooth damage, PP1γ will need to be inactivated in MAPK and AKT signal pathway to increase HDPCs proliferation and early differentiation in odontoblast-like cells and to allow cells survival and growth (Figure 4).

Basic FGF promotes early differentiation and inhibits late differentiation of odontoblast-like cells (121). Basic FGF activate MAPK signal pathway and this pathway needs to be stopped, allowing BMP-2 activation to induce late differentiation of odontoblast-like cells into mature odontoblast cells. PP1γ may have an important role to end MAPK signal pathway since PP1γ can inhibit 14-3-3 and MAPK1 directly, hampering early odontoblast differentiation (98). With the inhibition of FGF, downregulation of BMP will also happen, and it is known that this signal pathway has positive roles at the late stages of differentiation (121). However, the activation of PP1γ on HDPCs will activate partially BMP-2 and Runx2 pathways and Osx, transcription factors required for the activation of osteoblast differentiation, that will lead odontoblast-like cells to the terminal differentiation in mature odontoblast and start mineralization processes.

Taking it into consideration, we can suggest that PP1γ needs to be inactive when tooth injuries occur, allowing bFGF to induce MAPK signal pathway and to ensure the cell proliferation and early differentiation. In later stages of odontoblast differentiation PP1γ needs to be active to stop MAPK signal pathway activity, promoting BMP-2, Runx2 and Osx signal pathways. When it occurs, BMP-2 activates JNK and p38 MAPK and leads the cell to a late stage of odontoblastic differentiation (74,122). In summary, we can infer that PP1γ can be responsible for the ending of MAPK signal pathway, induced by bFGF in early stages of odontoblast differentiation, promoting the activation of BMP-2 signal pathway and late differentiation of odontoblast-like cells.

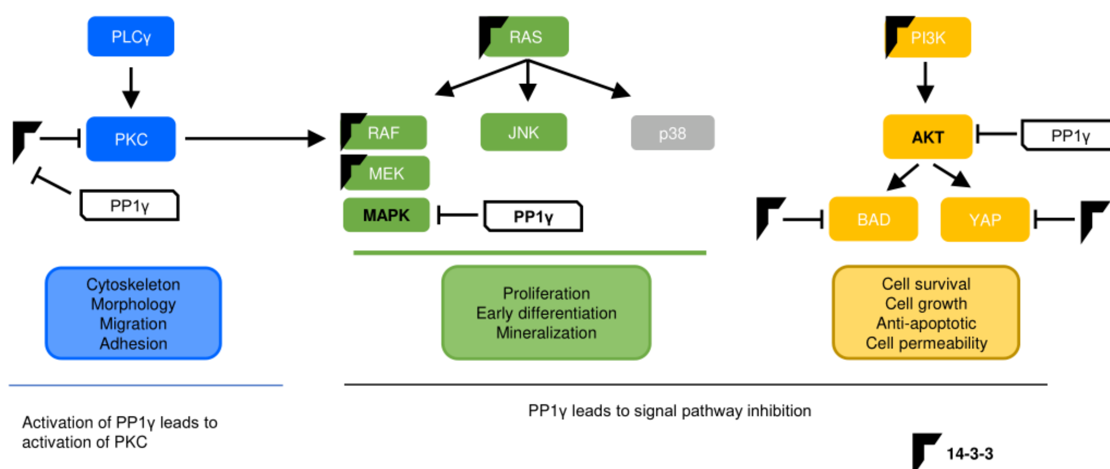

Figure 4 - PP1γ role in PKC, MAPK and AKT signal pathways. *The activation of protein kinases is essential for the activation of signal pathways in HDPCS. In PKC signal pathway, since PP1γ interacts with 14-3-3 proteins that inhibit PKC activity, the role of PP1γ will be necessary to prevent PKC to be inhibited leading to cell migration and adhesion. In MAPK signal pathway, 14-3-3 protein will activate RAF and MEK ensuring the progression of signal pathway. In this case, the activation of PP1γ will prevent the RAS and MEK binding to 14-3-3 protein breaking down the activation of the pathway. The same can be concluded since PP1γ interacts directly with MAPK (ERK2), dephosphorylating it. Lastly, in AKT signal pathway, PP1γ can dephosphorylate AKT and inhibit the 14-3-3 protein activity that activates PI3K and inactivates BAD and YAP proteins. This leads to the disruption of the signal pathway and the accumulation of BAD and YAP proteins in HDPC promoting apoptosis. Bold and black are represented RIPPOs present in HDPCs.*

GF and proteins important in the response to tooth injuries are highly expressed and secreted in HDPCs (123). For example, HDPCs can secrete VEGF and angiopoietin 1 (101) to induce capillary tube formation (102). It is known that the activation of PP1 up regulates angiopoietin 1 and promotes migration of endothelial cells (103). PP1γ activation can promote BMP-2 activation and 14-3-3 proteins inhibition that are known to inactivate PCK protein, which is essential to cell migration and adhesion. In this case, activation of PP1γ may be necessary to promote PKC signal pathway and promote endothelial cells and HDPSCs migration and adhesion (Figure 4). It is also important to highlight that BMP-2 and VEGF, when they co-exist, promote a synergistic effect on osteogenesis (124). However, another study proves that the inhibition of PP1 can enhance endothelial migration (125). These results can be different because of the different targets of phosphatases in cells, and the distinctive expression of GF on these cell populations.

PP1γ can also play an important role in the regulation of the neuronal dental pulp cells through its interaction with certain proteins of the **Table *1*.** Actin, β-tubulin, tropomyosin

alpha-3, tropomyosin alpha-4, neurofilament light and gamma-enolase are related to the regulation of morphology and plasticity, survival and differentiation of neuronal dental pulp cells in human body (126–131). The formation of complexes through their interaction with PP1γ can lead to a possible repair/regenerative potential. This regulation may be important to trigger a repair and regenerative response in neuronal dental pulp cells when dental pulp is damaged. However, there is a need for further studies to better understand PP1γ role in interacting with these proteins and how PP1γ regulates them.

3.2. PP1 isoforms are expressed in tooth cell lines

To characterize the expression of PP1 isoforms in human mesenchymal stromal cells (OB), osteogenic induced mesenchymal stromal cells (OI), periodontal ligament fibroblasts (FD), gingival fibroblasts (FG), apical papilla cells (PA), and dental pulp cells (DPCs) an SDS-PAGE was performed (Figure 5). The protein extracts were run in a 10% SDS-PAGE gel. The blots were incubated with primary antibodies, anti-PP1α, anti-PP1β and anti-PP1γ and of anti-PP1γ2 for 1 hour. As positive control, HeLa (HeLa) and spermatozoa (Spz) extracts were used.

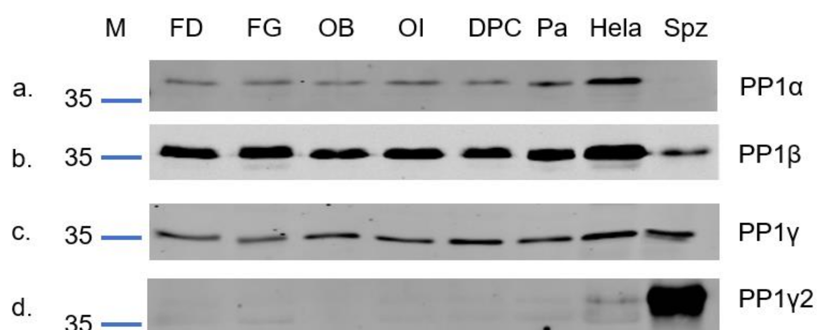

Figure 5 - PP1 isoforms are present in several dental cells in culture. *Confirmation of the presence of PP1 isoforms in the various cell line types of the tooth. Hela and sperm cells were used as controls. PP1α (a), PP1β (b) and PP1γ (c) are found in all cell lines and PP1γ2 (d) is only present in sperm cells.*

Endogenous PP1 isoforms, if expressed, should have a molecular weight around ~38 kDa (PP1α), ~37 kDa (PP1β), ~37 kDa (PP1γ) and ~39 kDa (PP1γ2). In (Figure 5) a., b. and c. it is possible to observe that endogenous PP1α, PP1β and PP1γ are expressed in all the dental cell lines. As expected, PP1γ2 expression was not verified in dental cells (Figure 5.d.), since it is a testis enriched protein and spermatozoa specific protein. Thus, these results prove the presence of PP1α, PP1β and PP1γ in HDPCs and not the expression of

PP1γ2. Taking it in consideration the PP1γ detected may be related to the expression of PP1γ1 isoform since PP1γ2 expression was not observed in HDPCs.

3.2.1 PP1 isoforms quantification

PP1 expression was quantified using Quantity One ® software and Ponceau S. staining was used as loading control. As already described on section 3.2 the PP1α, PP1β, PP1γ are expressed in the HDPCs and in other cells populations from the oral environment. We showed that the 3 PP1 isoforms have different patterns of expression in the different cell lines (Figure 6).

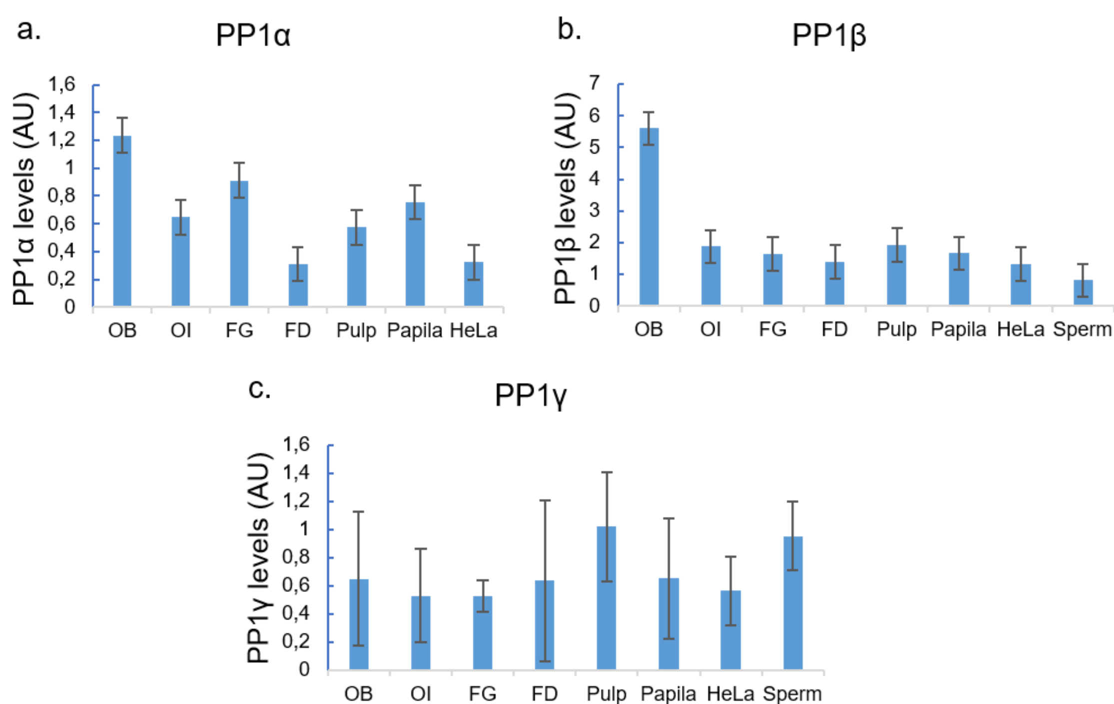

Figure 6 - PP1 isoforms quantification. *Quantification of PP1α, PP1β and PP1γ. PP1α has a high level of expression in OB, OI, FD, Pulp and Papila (a.). PP1β has an increased expression in all dental pulp cells, as comparing to control (b.). PP1γ has an increased expression in pulp cells (c.). Hela was used as control.*

3.3. PP1γ RIPPOs expression in dental pulp cells

Several kinases are important in some signal pathways that ensure the survival, proliferation, and differentiation of HDPCs like AKT1, p38 MAPK, MAPK1(ERK2) and RAF1. To confirm the expression of these proteins in the DPCs, an SDS-PAGE was performed.

The protein extracts were run in a 10% SDS-PAGE gel. The membranes were incubated with primary antibodies, anti-AKT1, anti-p38 MAPK, anti-RAF1 and anti-MAPK1. HeLa extracts were used as a positive control.

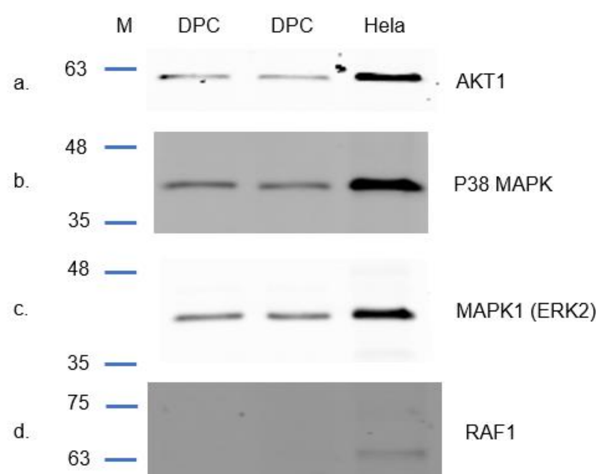

Figure 7 - PP1γ RIPPOs are expressed in human dental pulp cells. *Confirmation of several kinases that are RIPPOs, in human dental pulp cells. Kinases AKT1 and MAPK1 interact with PP1γ and its presence can be confirmed in HDPCs. RAF1 expression in HDPCs cannot be confirmed. The expression of proteins was analyzed in Human dental pulp cells (HDPCs) and Hela cells that were used as positive control. The marker (M) unities are kDA.*

The proteins expressed in HDPCs should present a molecular weight of ~56 kDa (AKT1), ~41 kDa (p38 MAPK), ~41 kDa (MAPK1) and ~73 kDa (RAF1). We demonstrated that AKT (a.), p38 MAPK (b.) and MAPK1 (c.) are expressed in HDPCs and can now be considered part of the dental pulp proteome. The presence of AKT1 and MAPK1 in HDPCs is important to be studied since these proteins are RIPPOs. The regulation of AKT and MAPK signal pathways by PP1γ suggests the modulation of these pathways leading the HDPCs to survive and grow or to differentiate and proliferate, respectively. Therefore, these two kinases can complete the information from Table 1 with these two new proteins in (**Table 2**).

Table 2 - *PP1γ RIPPOs identified in Human dental pulp through Western blot.*

UniProt KB	Protein name	Molecular function	Biological process	Score
P31749	RAC-alpha serine/threonine-protein kinase	14-3-3 protein binding; Protein kinase activity; PKC biding	Cell proliferation, migration, 'erentiation, and survival	0.63
P28482	Mitogen-activated protein kinase 1	MAP kinase activity; Phosphatase biding;	MAPK cascade; Positive regulation of gene expression	0.63

The expression of p38 MAPK in HDPCs can be supported by these results however this is not a RIPPO.

RAF1 cannot be confirmed in HDPCs by these results (d.). These results lead us to believe that the amount of RAF1 protein was not enough to be detected by the western blot.

In summary, the bioinformatic analysis provided us with 20 proteins identified as PP1γ RIPPOs that are present in human tooth pulp, allowing to infer PP1γ possible function in HDPCs, through its interaction with those RIPPOs. PP1γ may have a regulatory role in morphology, differentiation, and survival regulation of pulp cells through its interaction with actin, β-tubulin, tropomyosin alpha-3, tropomyosin alpha-4, neurofilament light and gamma-enolase.

PP1γ interaction with several isoforms of proteins 14-3-3 infer that it may play an important role in their regulation. 14-3-3 proteins interact in PKC, MAPK and AKT signal pathways. The regulation of the signal pathways is the way to lead the fate of HDPCs, once activation or inactivation of these signal pathways can result in HDPCs survival, proliferation, growth, and differentiation. The protein expression results allowed us to confirm the presence of AKT and MAPK in HDPCs and showed that several are the possible ways how PP1γ can regulate, directly or indirectly HDPCs fate.

4. Conclusions and Future perspectives

4.1. Conclusions

This work aimed to identify the expression pattern of PP1γ in HDPCs, as well as to identify possible functions of PP1γ through its interaction with RIPPOs present in HDPCs, further identifying the main role of PP1γ in HDPCs functionality, and influence in angiogenesis and repair/regenerative processes in injuries of the teeth.

The bioinformatic approach allowed us to identify 20 proteins that are known PP1γ RIPPOs and found to be expressed in HDPCs. From the 20 PP1γ RIPPOs, 5 are isoforms of 14-3-3 that interact with PP1γ. Three main signal pathways - AKT, MAPK and PKC, modulated by PP1γ and respective RIPPOs, were identified and discussed in terms of repair/regenerative potential, as well as regarding proliferative, growth, survival, differentiation, migration, and adhesion cellular events. Since 14-3-3 isoforms can activate RAS, RAF, MEK and PI3K and inactivate PKC, BAD and YAP, 14-3-3 regulation by PP1γ makes this protein a key for the indirect regulation of the AKT, MAPK and PKC signal pathways. This 14-3-3/ PP1γ interaction may indicate that PP1γ then actively inhibits the AKT and MAPK signal pathway. The inhibition of MAPK pathway mediated by bFGF can be important since this GF has either a positive effect on early HDPCs differentiation and a negative effect in late stages of mature odontoblast differentiation. Moreover, PP1γ can directly and indirectly end MAPK signal pathway by interacting with several proteins of MAPK signal pathway and 14-3-3 proteins. PP1γ also induces BMP-2, Runx2 and Osx that are transcription factors required for the activation of osteoblast/terminal odontoblastic differentiation. Activated PP1γ will promote BMP-2 activation and the expression of p38 and JNK that are mainly responsible for late odontoblastic differentiation.

In conclusion, PP1γ will play an inhibitory role in the MAPK signal pathway responsible for inducing early odontoblast differentiation and at the same time will promote the BMP-2 signal pathway responsible for the late differentiation of odontoblasts. Mature odontoblasts will initiate mineralization that is the best response to tooth injuries.

PP1γ may also have an important role in regulating the morphology, adhesion, and migration of HDPCs. With the interaction of PP1γ with 14-3-3, it can be inferred that it plays an activating role in the PKC signal pathway since it connects with 14-3-3, which is known to inhibit PKC. This PP1γ /14-3-3 interaction may prevent inhibition of the PKC pathway and promote cell migration, adhesion, and morphology of HDPCs.

PP1γ may also have a role in the regulation of neuronal dental pulp cells (127,129) by interacting with actin, β-tubulin, tropomyosin alpha-3, tropomyosin alpha-4, neurofilament light and gamma-enolase. This interaction may be an evidence that PP1γ is involved in morphology, cell survival and differentiation of neuronal dental pulp cells.

In conclusion, the results show a panoply of regulatory processes to be ensured by PP1γ in HDPCs. When inhibited PP1γ may have a role in the cell survival, proliferation and early HDPCs differentiation through MAPK and AKT signal pathway. When activated PP1γ may be the key to stop the MAPK signal pathway and induce late transcript factors like BMP-2 to induce late odontoblast differentiation and posterior mineralization activity. Also, PP1γ probably plays a role in the inhibition of 14-3-3 proteins in order to promote PKC signal pathway in endothelial cells and HDPSCs. This PP1γ /14-3-3 interaction may be crucial to promote cell migration and adhesion, but also to promote differentiation of these cells since PKC is able to activate MAPK signal pathway. PP1γ interaction with actin, β-tubulin, tropomyosin alpha-3, tropomyosin alpha-4, neurofilament light and gamma-enolase, may also be evidence of PP1γ's involvement in regulation of morphology, survival and differentiation of neuronal dental pulp cells.

4.2. Future perspectives

The future work in this project should focus on the identification of a complete interactome of PP1γ in dental pulp cells and tissues by a co-immunoprecipitation of PP1γ in HDPCs and its identification by MS. With this analysis we may be able to understand clearly the RIPPOs present in HDPCs, and consequently the main role of PP1γ in the regeneration of dental pulp when tooth damage occurs. Two proteins that are expected to be identified are RAS and RAF1, known to be RIPPOs, involved in MAPK signal pathway but their presence in HDPCs is yet to be confirmed. Lastly, could be identified identify PP1γ complex involved in dental pulp regeneration suitable for therapeutic intervention.

5. References

1. Nanci A, Cate AR Ten. Ten Cate's Oral Histology: Development, Structure, and Function. Structure of the Oral Tissues. 2012. pp. 79-107.
2. Honda M, Sato M, Toriumi T. Characterization of Coronal Pulp Cells and Radicular Pulp Cells in Human Teeth. J Endod. 2017 Sep;43(9):S35–9.
3. Yamazaki H, Hayashi SI. Contribution of Neural Crest Cells in Tooth Development and the Possibility of Tooth Regeneration. J Oral Biosci. 2004;46(6):509–18.
4. Goldberg M, Hirata A. The Dental Pulp: Composition, Properties and Functions. JSM Dent. 2017;
5. Nakanishi T, Takegawa D, Hirao K, Takahashi K, Yumoto H, Matsuo T. Roles of dental pulp fibroblasts in the recognition of bacterium-related factors and subsequent development of pulpitis. Vol. 47, Japanese Dental Science Review. Elsevier Ltd; 2011. p. 161–6.
6. Iohara K, Zheng L, Ito M, Tomokiyo A, Matsushita K, Nakashima M. Side Population Cells Isolated from Porcine Dental Pulp Tissue with Self-Renewal and Multipotency for Dentinogenesis, Chondrogenesis, Adipogenesis, and Neurogenesis. Stem Cells. 2006;
7. Wang J, Ma H, Jin X, Hu J, Liu X, Ni L, et al. The effect of scaffold architecture on odontogenic differentiation of human dental pulp stem cells. Biomaterials. 2011;
8. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000 Dec;97(25):13625–30.
9. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. J Dent Res. 2002;
10. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, et al. SHED: Stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A. 2003 May;100(10):5807–12.
11. Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, et al. Mesenchymal stem cell-mediated functional tooth regeneration in Swine. Csete M, editor. PLoS One. 2006 Dec;1(1):e79.
12. Morsczeck C, Götz W, Schierholz J, Zeilhofer F, Kühn U, Möhl C, et al. Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. Matrix Biol. 2005 Apr;24(2):155–65.
13. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet. 2004 Jul;364(9429):149–55.
14. Ivanovski S, Gronthos S, Shi S, Bartold PM. Stem cells in the periodontal ligament. Vol. 12, Oral Diseases. 2006. p. 358–63.

15. Yumoto H, Hirao K, Hosokawa Y, Kuramoto H, Takegawa D, Nakanishi T, et al. The roles of odontoblasts in dental pulp innate immunity. Vol. 54, Japanese Dental Science Review. Elsevier Ltd; 2018. p. 105–17.

16. Casagrande L, Cordeiro MM, Nör SA, Nör JE. Dental pulp stem cells in regenerative dentistry. Vol. 99, Odontology. 2011. p. 1–7.

17. Nweeia MT, Eichmiller FC, Hauschka P V., Donahue GA, Orr JR, Ferguson SH, et al. Sensory ability in the narwhal tooth organ system. Anat Rec. 2014 Apr;297(4):599–617.

18. Tazawa K, Ikeda H, Kawashima N, Okiji T. Transient receptor potential melastatin (TRPM) 8 is expressed in freshly isolated native human odontoblasts. Arch Oral Biol. 2017 Mar;75:55–61.

19. Butler WT. Dentin matrix proteins and dentinogenesis. Connect Tissue Res. 1995 Jan;33(1–3):59–65.

20. Sloan AJ. Biology of the Dentin-Pulp Complex. In: Stem Cell Biology and Tissue Engineering in Dental Sciences. Elsevier Inc.; 2015. p. 371–8.

21. Yu C, Abbott P. An overview of the dental pulp: its functions and responses to injury. Aust Dent J. 2007;

22. Schuh CMAP, Benso B, Aguayo S. Potential novel strategies for the treatment of dental pulp-derived pain: Pharmacological approaches and beyond. Vol. 10, Frontiers in Pharmacology. Frontiers Media S.A.; 2019. p. 1068.

23. Huang GTJ. Pulp and dentin tissue engineering and regeneration: Current progress. Regenerative Medicine. 2009.

24. Bjørndal L, Mjör IA. Pulp-dentin biology in restorative dentistry. Part 4: Dental caries--characteristics of lesions and pulpal reactions. Quintessence Int. 2001;

25. Balic A. Biology explaining tooth repair and regeneration: A mini-review. Gerontology. 2018.

26. Ikeda H. Sensory experiences in relation to pulpal nerve activation of human teeth in different age groups. Arch Oral Biol. 2003;

27. Byers MR, Taylor PE. Effect of Sensory Denervation on the Response of Rat Molar Pulp to Exposure Injury. J Dent Res. 1993;

28. Jontell M, Okiji T, Dahlgren U, Bergenholtz G. Immune defense mechanisms of the dental pulp. Critical Reviews in Oral Biology and Medicine. 1998.

29. Bakopoulou A, Leyhausen G, Volk J, Tsiftsoglou A, Garefis P, Koidis P, et al. Comparative analysis of in vitro osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP). Arch Oral Biol. 2011 Jul;56(7):709–21.

30. Jeanneau C, Lundy FT, El Karim IA, About I. Potential Therapeutic Strategy of Targeting Pulp

Fibroblasts in Dentin-Pulp Regeneration. J Endod. 2017 Sep;43(9):S17–24.

31. Mathieu S, Jeanneau C, Sheibat-Othman N, Kalaji N, Fessi H, About I. Usefulness of controlled release of growth factors in investigating the early events of dentin-pulp regeneration. J Endod. 2013 Feb;39(2):228–35.

32. Chmilewsky F, About I, Chung SH. Pulp Fibroblasts Control Nerve Regeneration through Complement Activation. J Dent Res. 2016 Jul;95(8):913–22.

33. Chmilewsky F, Jeanneau C, Laurent P, About I. Pulp fibroblasts synthesize functional complement proteins involved in initiating dentin-pulp regeneration. Am J Pathol. 2014 Jul;184(7):1991–2000.

34. Jeanneau C, Rufas P, Rombouts C, Giraud T, Dejou J, About I. Can pulp fibroblasts kill cariogenic bacteria? Role of complement activation. J Dent Res. 2015 Dec;94(12):1765–72.

35. Jung C, Kim S, Sun T, Cho YB, Song M. Pulp-dentin regeneration: current approaches and challenges. J Tissue Eng. 2019 Jan;10:204173141881926.

36. Fawzy El-Sayed KM, Jakusz K, Jochens A, Dörfer C, Schwendicke F. Stem cell transplantation for pulpal regeneration: A systematic review. Vol. 21, Tissue Engineering - Part B: Reviews. 2015. p. 451–60.

37. Fawzy El-Sayed KM, Ahmed GM, Abouauf EA, Schwendicke F. Stem/progenitor cell-mediated pulpal tissue regeneration: a systematic review and meta-analysis. Vol. 52, International Endodontic Journal. 2019. p. 1573–85.

38. Eramo S, Natali A, Pinna R, Milia E. Dental pulp regeneration via cell homing. Vol. 51, International Endodontic Journal. 2018. p. 405–19.

39. Pramila R, Muthu M. Regeneration potential of pulp-dentin complex: Systematic review. Journal of Conservative Dentistry. 2012.

40. Zivkovic P, Petrovic V, Najman S, Stefanovic V. Stem cell-based dental tissue engineering. TheScientificWorldJournal. 2010.

41. Westont AD, Hood L. Systems Biology, Proteomics, and the Future of Health Care: Toward Predictive, Preventative, and Personalized Medicine. J Proteome Res. 2004 Apr;3(2):179–96.

42. Wei X, Wu L, Ling J, Liu L, Liu S, Liu W, et al. Differentially Expressed Protein Profile of Human Dental Pulp Cells in the Early Process of Odontoblast-like Differentiation In Vitro. J Endod. 2008;

43. Pääkkönen V, Ohlmeier S, Bergmann U, Larmas M, Salo T, Tjäderhane L. Analysis of gene and protein expression in healthy and carious tooth pulp with cDNA microarray and two-dimensional gel electrophoresis. Eur J Oral Sci. 2005;

44. Loureiro C, Buzalaf MAR, Pessan JP, de Moraes FRN, Pelá VT, Ventura TMO, et al. Comparative analysis of the proteomic profile of the dental pulp in different conditions. A pilot study. Braz Dent J.

2020 Jun;31(3):319–36.

45. Martin I, Padera RF, Vunjak-Novakovic G, Freed LE. In vitro differentiation of chick embryo bone marrow stromal cells into cartilaginous and bone-like tissues. J Orthop Res. 1998 Mar;16(2):181–9.

46. Stevens MM, Marini RP, Schaefer D, Aronson J, Langer R, Shastri VP. In vivo engineering of organs: The bone bioreactor. Proc Natl Acad Sci U S A. 2005 Aug;102(32):11450–5.

47. Tran-Hung L, Laurent P, Camps J, About I. Quantification of angiogenic growth factors released by human dental cells after injury. Arch Oral Biol. 2008 Jan;53(1):9–13.

48. Ornitz DM, Itoh N. The fibroblast growth factor signaling pathway. Wiley Interdiscip Rev Dev Biol. 2015 May;4(3):215–66.

49. Müller AK, Meyer M, Werner S. The roles of receptor tyrosine kinases and their ligands in the wound repair process. Vol. 23, Seminars in Cell and Developmental Biology. 2012. p. 963–70.

50. Kim YS, Min KS, Jeong DH, Jang JH, Kim HW, Kim EC. Effects of fibroblast growth factor-2 on the expression and regulation of chemokines in human dental pulp cells. J Endod. 2010 Nov;36(11):1824–30.

51. Kim JC, Park JC, Kim SH, Im GI, Kim BS, Lee JB, et al. Treatment of FGF-2 on stem cells from inflamed dental pulp tissue from human deciduous teeth. Oral Dis. 2014 Mar;20(2):191–204.

52. Yuan X, Cao X, Yang S. IFT80 is required for stem cell proliferation, differentiation, and odontoblast polarization during tooth development. Cell Death Dis. 2019 Feb;10(2):63.

53. Suzuki T, Lee CH, Chen M, Zhao W, Fu SY, Qi JJ, et al. Induced migration of dental pulp stem cells for in vivo pulp regeneration. J Dent Res. 2011 Aug;90(8):1013–8.

54. Sagomonyants K, Kalajzic I, Maye P, Mina M. Enhanced dentinogenesis of pulp progenitors by early exposure to FGF2. J Dent Res. 2015 Nov;94(11):1582–90.

55. Zhang J, Lian M, Cao P, Bao G, Xu G, Sun Y, et al. Effects of Nerve Growth Factor and Basic Fibroblast Growth Factor Promote Human Dental Pulp Stem Cells to Neural Differentiation. Neurochem Res. 2017 Apr;42(4):1015–25.

56. Vaseenon S, Chattipakorn N, Chattipakorn SC. The possible role of basic fibroblast growth factor in dental pulp. Vol. 109, Archives of Oral Biology. Elsevier Ltd; 2020. p. 104574.

57. Sakai VT, Zhang Z, Dong Z, Neiva KG, MacHado MAAM, Shi S, et al. SHED differentiate into functional odontoblasts and endothelium. J Dent Res. 2010 Aug;89(8):791–6.

58. Ferrara N. Vascular endothelial growth factor: Basic science and clinical progress. Vol. 25, Endocrine Reviews. 2004. p. 581–611.

59. Chu SC, Chung HT, Yang SF, Huang FM, Su YF, Hsieh YS, et al. Induction of vascular endothelial growth

factor gene expression by proinflammatory cytokines in human pulp and gingival fibroblasts. J Endod. 2004 Oct;30(10):704–7.

60. Telles PDS, Hanks CT, Machado MAAM, Nör JE. Lipoteichoic acid up-regulates VEGF expression in macrophages and pulp cells. J Dent Res. 2003 Jun;82(6):466–70.

61. Matsushita K, Motani R, Sakuta T, Yamaguchi N, Koga T, Matsuo K, et al. The role of vascular endothelial growth factor in human dental pulp cells: Induction of chemotaxis, proliferation, and differentiation and activation of the AP-1-dependent signaling pathway. J Dent Res. 2000 Aug;79(8):1596–603.

62. Koch S, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. Vol. 2, Cold Spring Harbor Perspectives in Medicine. 2012. p. a006502–a006502.

63. Simons M, Gordon E, Claesson-Welsh L. Mechanisms and regulation of endothelial VEGF receptor signalling. Vol. 17, Nature Reviews Molecular Cell Biology. 2016. p. 611–25.

64. Zhao X, Guan JL. Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. Vol. 63, Advanced Drug Delivery Reviews. 2011. p. 610–5.

65. Sawada J, Li F, Komatsu M. R-Ras Inhibits VEGF-Induced p38MAPK Activation and HSP27 Phosphorylation in Endothelial Cells. J Vasc Res. 2016;52(5):347–59.

66. Nakashima M. Induction of dentine in amputated pulp of dogs by recombinant human bone morphogenetic proteins-2 and -4 with collagen matrix. Arch Oral Biol. 1994 Dec;39(12):1085–9.

67. Nakashima M, Reddi AH. The application of bone morphogenetic proteins to dental tissue engineering. Nat Biotechnol. 2003 Sep;21(9):1025–32.

68. Casagrande L, Demarco FF, Zhang Z, Araujo FB, Shi S, Nör JE. Dentin-derived BMP-2 and odontoblast differentiation. J Dent Res. 2010 Jun;89(6):603–8.

69. Demarco FF, Casagrande L, Zhang Z, Dong Z, Tarquinio SB, Zeitlin BD, et al. Effects of morphogen and scaffold porogen on the differentiation of dental pulp stem cells. J Endod. 2010 Nov;36(11):1805–11.

70. Tada H, Nemoto E, Foster BL, Somerman MJ, Shimauchi H. Phosphate increases bone morphogenetic protein-2 expression through cAMP-dependent protein kinase and ERK1/2 pathways in human dental pulp cells. Bone. 2011 Jun;48(6):1409–16.

71. Yang J, Ye L, Hui TQ, Yang DM, Huang DM, Zhou XD, et al. Bone morphogenetic protein 2-induced human dental pulp cell differentiation involves p38 mitogen-activated protein kinase-activated canonical WNT pathway. Int J Oral Sci. 2015 Jun;7(2):95–102.

72. Saito T, Ogawa M, Hata Y, Bessho K. Acceleration effect of human recombinant bone morphogenetic protein-2 on differentiation of human pulp cells into odontoblasts. J Endod. 2004 Apr;30(4):205–8.

73. Qin W, Lin ZM, Deng R, Li DD, Song Z, Tian YG, et al. p38a MAPK is involved in BMP-2-induced odontoblastic differentiation of human dental pulp cells. Int Endod J. 2012 Mar;45(3):224–33.

74. Qin W, Liu P, Zhang R, Huang S, Gao X, Song Z, et al. JNK MAPK is involved in BMP-2-induced odontoblastic differentiation of human dental pulp cells. Connect Tissue Res. 2014 Jun;55(3):217–24.

75. Iohara K, Nakashima M, Ito M, Ishikawa M, Nakasima A, Akamine A. Dentin regeneration by dental pulp stem cell therapy with recombinant human bone morphogenetic protein 2. J Dent Res. 2004 Aug;83(8):590–5.

76. Yang W, Harris MA, Cui Y, Mishina Y, Harris SE, Gluhak-Heinrich J. Bmp2 is required for odontoblast differentiation and pulp vasculogenesis. J Dent Res. 2012 Jan;91(1):58–64.

77. Kido J ichi, Ishida H, Nagata T, Hamasaki A, Nishikawa S, Wakano Y. Effects of parathyroid hormone, 1,25-dihydroxyvitamin D3, and prostaglandin E2 on alkaline phosphatase activity in cultured dental pulp and gingiva cells of bovine calf. J Endod. 1991 Apr;17(4):161–4.

78. Sagomonyants K, Mina M. Biphasic effects of FGF2 on odontoblast differentiation involve changes in the BMP and Wnt signaling pathways. In: Connective Tissue Research. 2014. p. 53–6.

79. Hyun SY, Lee JH, Kang KJ, Jang YJ. Effect of FGF-2, TGF-β-1, and BMPs on teno/ligamentogenesis and osteo/cementogenesis of human periodontal ligament stem cells. Mol Cells. 2017 Aug;40(8):550–7.

80. Osathanon T, Nowwarote N, Manokawinchoke J, Pavasant P. BFGF and JAGGED1 regulate alkaline phosphatase expression and mineralization in dental tissue-derived mesenchymal stem cells. J Cell Biochem. 2013 Nov;114(11):2551–61.

81. Osathanon T, Nowwarote N, Pavasant P. Basic fibroblast growth factor inhibits mineralization but induces neuronal differentiation by human dental pulp stem cells through a FGFR and PLCγ signaling pathway. J Cell Biochem. 2011 Jul;112(7):1807–16.

82. Hunter T. Protein kinases and phosphatases: The Yin and Yang of protein phosphorylation and signaling. Vol. 80, Cell. 1995. p. 225–36.

83. Schulman H. Intracellular Signaling. In: Fundamental Neuroscience: Fourth Edition. Elsevier; 2013. p. 189–209.

84. Liu Y, Chance MR. Integrating phosphoproteomics in systems biology. Vol. 10, Computational and Structural Biotechnology Journal. 2014. p. 90–7.

85. Korrodi-Gregório L, Esteves SLC, Fardilha M. Protein phosphatase 1 catalytic isoforms: Specificity toward interacting proteins. Transl Res. 2014;164(5):366–91.

86. Shi Y. Serine/Threonine Phosphatases: Mechanism through Structure. Cell. 2009 Oct;139(3):468–84.

87. Das AK, Helps NR, Cohen PTW, Barford D. Crystal structure of the protein serine/threonine

phosphatase 2C at 2.0 Å resolution. EMBO J. 1996 Dec;15(24):6798–809.

88. Cohen P. The origins of protein phosphorylation. Nat Cell Biol. 2002;4(5).

89. Silva J, Freitas M, Fardilha M. Phosphoprotein Phosphatase 1 Complexes in Spermatogenesis. Curr Mol Pharmacol. 2015;

90. Wu D, De Wever V, Derua R, Winkler C, Beullens M, Van Eynde A, et al. A substrate-trapping strategy for protein phosphatase PP1 holoenzymes using hypoactive subunit fusions. J Biol Chem. 2018 Sep;293(39):15152–62.

91. Cohen PTW. Protein phosphatase 1 - Targeted in many directions. J Cell Sci. 2002 Jan;115(2):241–56.

92. Bollen M, Stalmans W. The structure, role, and regulation of type 1 protein phosphatases. Crit Rev Biochem Mol Biol. 1992;

93. MacDougall LK, Jones LR, Cohen P. Identification of the major protein phosphatases in mammalian cardiac muscle which dephosphorylate phospholamban. Eur J Biochem. 1991 Mar 28;196(3):725–34.

94. Da Cruz e Silva EF, Fox CA, Ouimet CC, Gustafson E, Watson SJ, Greengard P. Differential expression of protein phosphatase 1 isoforms in mammalian brain. J Neurosci. 1995;

95. Fardilha M, Esteves SLC, Korrodi-Gregório L, da Cruz e Silva O a B, da Cruz e Silva FF. The physiological relevance of protein phosphatase 1 and its interacting proteins to health and disease. Curr Med Chem. 2010;17(33):3996–4017.

96. Ceulemans H, Bollen M. Functional Diversity of Protein Phosphatase-1, a Cellular Economizer and Reset Button. Physiol Rev. 2004 Jan;84(1):1–39.

97. Andreassen PR, Lacroix FB, Villa-Moruzzi E, Margolis RL. Differential subcellular localization of protein phosphatase-1 α, γ1, and δ isoforms during both interphase and mitosis in mammalian cells. J Cell Biol. 1998;141(5):1207–15.

98. Kim J-Y, Kim D-S, Auh Q-S, Yi J-K, Moon SU, Kim E-C. Role of Protein Phosphatase 1 in Angiogenesis and Odontoblastic Differentiation of Human Dental Pulp Cells. J Endod. 2017 Mar;43(3):417–24.

99. Chalfant CE, Kishikawal K, Mumby MC, Kamibayashi C, Bielawska A, Hannun YA. Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid. J Biol Chem. 1999 Jul;274(29):20313–7.

100. Knapp KM, English BK. Ceramide-mediated stimulation of inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF) accumulation in murine macrophages requires tyrosine kinase activity. J Leukoc Biol. 2000 May;67(5):735–41.

101. Kim JJ, Kim SJ, Kim YS, Kim SY, Park SH, Kim EC. The role of SIRT1 on angiogenic and odontogenic potential in human dental pulp cells. J Endod. 2012 Jul;38(7):899–906.

102. Bae WJ, Jue SS, Kim SY, Moon JH, Kim EC. Effects of sodium tri- and hexametaphosphate on proliferation, differentiation, and angiogenic potential of human dental pulp cells. J Endod. 2015 Jun;41(6):896–902.

103. Iacobazzi D, Garaeva I, Albertario A, Cherif M, Angelini GD, Caputo M, et al. Protein phosphatase 1 beta is modulated by chronic hypoxia and involved in the angiogenic endothelial cell migration. Cell Physiol Biochem. 2015;36(1):384–94.

104. Oliveros JC. VENNY. An interactive tool for comparing lists with Venn Diagrams [Internet]. bioinfogp.cnb.csic.es/tools/venny/index.html. 2007. Available from: http://bioinfogp.cnnb.csic.es/tools/venny/index.html

105. Rosenquist M, Sehnke P, Ferl RJ, Sommarin M, Larsson C. Evolution of the 14-3-3 protein family: Does the large number of isoforms in multicellular organisms reflect functional specificity? J Mol Evol. 2000 Nov;51(5):446–58.

106. Fantl WJ, Muslin AJ, Kikuchi A, Martin JA, MacNicol AM, Grosst RW, et al. Activation of Raf-1 by 14-3-3 proteins. Nature. 1994 Oct;371(6498):612–4.

107. Freed E, Symons M, Macdonald SG, McCormick F, Ruggieri R. Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. Science (80-). 1994 Sep;265(5179):1713–6.

108. Robinson K, Jones D, Patel Y, Martin H, Madrazo J, Martin S, et al. Mechanism of inhibition of protein kinase C by 14-3-3 isoforms. 14-3-3 isoforms do not have phospholipase A2 activity. Biochem J. 1994 May;299(3):853–61.

109. Pozuelo-Rubio M. Regulation of autophagic activity by 14-3-3 proteins associated with class III phosphatidylinositol-3-kinase. Cell Death Differ. 2011 Mar;18(3):479–92.

110. Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB, et al. 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell. 2000 Jul;6(1):41–51.

111. Basu S, Totty NF, Irwin MS, Sudol M, Downward J. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. Mol Cell. 2003 Jan;11(1):11–23.

112. Light Y, Paterson H, Marais R. 14-3-3 Antagonizes Ras-Mediated Raf-1 Recruitment to the Plasma Membrane To Maintain Signaling Fidelity. Mol Cell Biol. 2002 Jul;22(14):4984–96.

113. Fanger GR, Widmann C, Porter AC, Sather S, Johnson GL, Vaillancourt RR. 14-3-3 proteins interact with specific MEK kinases. J Biol Chem. 1998 Feb;273(6):3476–83.

114. Neal CL, Xu J, Li P, Mori S, Yang J, Neal NN, et al. Overexpression of 14-3-3ζ in cancer cells activates PI3K via binding the p85 regulatory subunit. Oncogene. 2012 Feb;31(7):897–906.

115. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell. 1996 Nov;87(4):619–28.

116. Xing H, Zhang S, Weinheimer C, Kovacs A, Muslin AJ. 14-3-3 Proteins block apoptosis and differentially regulate MAPK cascades. EMBO J. 2000 Feb;19(3):349–58.

117. Toker A, Ellis CA, Sellers LA, Aitken A. Protein kinase C inhibitor proteins. Purification from sheep brain and sequence similarity to lipocortins and 14-3-3 protein. Eur J Biochem. 1990 Jul 31;191(2):421–9.

118. Thayyullathil F, Chathoth S, Shahin A, Kizhakkayil J, Hago A, Patel M, et al. Protein phosphatase 1-dependent dephosphorylation of Akt is the prime signaling event in sphingosine-induced apoptosis in Jurkat cells. J Cell Biochem. 2011 Apr;112(4):1138–53.

119. Sun Q, Wang W. Structures of BRAF–MEK1–14-3-3 sheds light on drug discovery. Signal Transduct Target Ther. 2019 Dec;4(1):59.

120. Keenan C, Kelleher D. Protein kinase C and the cytoskeleton. Vol. 10, Cellular Signalling. 1998. p. 225–32.

121. Sagomonyants K, Kalajzic I, Maye P, Mina M. FGF Signaling Prevents the Terminal Differentiation of Odontoblasts. J Dent Res. 2017 Jun;96(6):663–70.

122. Hu Y, Chan E, Wang SX, Li B. Activation of p38 mitogen-activated protein kinase is required for osteoblast differentiation. Endocrinology. 2003 May;144(5):2068–74.

123. Nakashima M, Iohara K. Regeneration of dental pulp by stem cells. Adv Dent Res. 2011 Jul;23(3):313–9.

124. Wang T, Guo S, Zhang H. Synergistic Effects of Controlled-Released BMP-2 and VEGF from nHAC/PLGAs Scaffold on Osteogenesis. Biomed Res Int. 2018 Sep;2018:1–12.

125. Gabel S, Benefield J, Meisinger J, Petruzzelli GJ, Young MRI. Protein phosphatases 1 and 2A maintain endothelial cells in a resting state, limiting the motility that is needed for the morphogenic process of angiogenesis. Otolaryngol - Head Neck Surg. 1999 Oct;121(4):463–8.

126. Blitzer RD, Connor JH, Brown GP, Wong T, Shenolikar S, Iyengar R, et al. Gating of CaMKII by cAMP-regulated protein phosphatase activity during LTP. Science (80-). 1998 Jun;280(5371):1940–3.

127. Oliver CJ, Terry-Lorenzo RT, Elliott E, Bloomer WAC, Li S, Brautigan DL, et al. Targeting Protein Phosphatase 1 (PP1) to the Actin Cytoskeleton: the Neurabin I/PP1 Complex Regulates Cell Morphology. Mol Cell Biol. 2002 Jul;22(13):4690–701.

128. Gaudet P, Livstone MS, Lewis SE, Thomas PD. Phylogenetic-based propagation of functional annotations within the Gene Ontology consortium. Brief Bioinform. 2011 Sep;12(5):449–62.

129. Terry-Lorenzo RT, Inoue M, Connor JH, Haystead TAJ, Armbruster BN, Gupta RP, et al. Neurofilament-

L is a protein phosphatase-1-binding protein associated with neuronal plasma membrane and post-synaptic density. J Biol Chem. 2000 Jan;275(4):2439–46.

130. Alessi A, Bragg AD, Percival JM, Yoo J, Albrecht DE, Froehner SC, et al. γ-Syntrophin scaffolding is spatially and functionally distinct from that of the α/β syntrophins. Exp Cell Res. 2006 Oct;312(16):3084–95.

131. Foster LJ, Zeemann PA, Li C, Mann M, Jensen ON, Kassem M. Differential Expression Profiling of Membrane Proteins by Quantitative Proteomics in a Human Mesenchymal Stem Cell Line Undergoing Osteoblast Differentiation. Stem Cells. 2005 Oct;23(9):1367–77.

6. Supplementary data

Supplementary table 1 – Standards for BCA assay

Tube Name	Volume of BSA	From Tube	Volume Lysis Buffer	Final [BSA]
A	30 µL	BSA stock	0 µL	2000 µg/mL
B	37.5 µL	BSA stock	12.5 µL	1500 µg/mL
C	32.5 µL	BSA stock	32.5 µL	1000 µg/mL
D	17.5 µL	B	17.5 µL	750 µg/mL
E	32.5 µL	C	32.5 µL	500 µg/mL
F	32.5 µL	D	32.5 µL	250 µg/mL
G	32.5 µL	E	32.5 µL	125 µg/mL
H	10 µL	F	40 µL	25 µg/mL
I	0 µL	-	40 µL	0 µg/mL

Supplementary table 2 – Experimental solutions for Western blot

Western blot		
Running gel 10% (2 gels, 1.5 mm thickness)	ddH2O	7.720 mL
	Tris 1.5 M pH8.8	5.000 mL
	Acrylamide 40%	4.920 mL
	Bisacrylamide 2%	1.960 mL
	SDS 10%	0.200 mL
	APS 10%	0.100 mL
	TEMED	0.020 mL
Stacking gel 4% (2 gels, 1.5 mm thickness)	ddH2O	4.736 mL
	Tris 0.5 M pH6.8	2.000 mL
	Acrylamide 40%	0.784 mL
	Bisacrylamide 2%	0.320 mL
	SDS 10%	0.080 mL
	APS 10%	0.040 mL
	TEMED	0.008 mL
Tris-HCl 1.5 M pH 8.8 buffer	For 1 L, dissolve 181.5 g Tris in 800 mL deionized water. Adjust pH at 8.8 with HCl and make up to 1 L with deionized water.	

Tris-HCl 0.5 M pH 6.8 buffer	For 1 L, dissolve 60 g Tris in 800 mL deionized water. Adjust pH at 6.8 with HCl and make up to 1 L with deionized water.
10% APS (ammonium persulfate)	For 10 mL of deionized water add 1 g of APS.
10% SDS (sodium dodecilsulfate)	For 500 mL of deionized water dissolve 50 g of SDS.
Loading gel buffer	For 10 mL, add 44 mL glycerol, 250 µL Tris-HCl 0.5 M pH 6.8 buffer, 0.8 g SDS, 0.2 mL β-mercaptoethanol and 3.3 mL deionized water. Add bromophenol blue (a small amount). Keep it at RT for short periods or at 4ºC for longer periods.
Running buffer 1x	For 1 L, add 800 mL deionized water, 100 mL Tris-Gly 10x and 10 mL 10% SDS. Make up to 1 L with deionized water.
Transfer buffer 1x	For 1 L, add 100 mL Tris-Gly 10x to 700 mL of deionized water and 200 mL methanol.
10x Tris buffered saline (TBS) (stock)	For 0.5 L, dissolve 6.055g Tris in deionized water and adjust pH at 8.0. Add 43.8325 g NaCl and make up to 500 mL with deionized water.
1x TBST (TBS + Tween 20)	For 1 L, add 100 mL TBS 10x and 500 µL Tween-20 to 900 mL of deionized water.
Ponceau S.	For 0.1 L, dissolve 0.1 g Ponceau S (0.1%) in 5 mL acetic acid (5%) and make up to 100 mL with deionized water.