Differential Gene Expression of Ca^{2+} Channel-Mediated Signaling Pathways in DPSCs under Hypoxic Condition

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LIST OF ABBREVIATIONS

RNA	Ribonucleic Acid
IRB	Institutional Review Board
NFKB	Nuclear Factor Kappa B
DPSC	Dental Pulp Stem Cell
IL	Interleukin
TNF	Tissue Necrosis Factor
MMP	Matrix Metalloproteinase
DNA	Deoxyribonucleic Acid
LPS	Lipopolysaccharide
PAMPs	Pathogen Associated Molecular Patterns
CSC	Calcium Silicate-based Cements
NET	Neutrophil Extracellular Traps
TLR	Toll Like Receptor
STRO-1	Mesenchymal Stem Cell Marker 1
mRNA	Messenger Ribonucleic Acid
ROC	Receptor-Operated Channel
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
KEGG	Kyoto Encyclopedia of Genes and Genomes
RET	Regenerative Endodontic Therapy
VPT	Vital Pulp Therapy

SUMMARY

Dental pulp stem cell (DPSC) survival, differentiation into odontoblast and deposition of matrix for mineralization is essential for vital pulp therapy (VPT) and regenerative endodontic therapy (RET). Calcium homeostasis in DPSC plays a critical role for its survival, longevity, and differentiation processes. Cells use this external source of signal calcium ions by activating various entry channels with different properties. The objective of this research is to investigate key gene candidates involved with calcium homeostasis in human dental pulp tissue and DPSCs.

DPSCs isolated from human dental pulp were cultured in hypoxic chamber (3%) for 21 days at 37°C in differentiation media and controls were cultured under normal conditions. Later, DPSCs collected were extracted for mRNA isolation. The mRNA was sequenced using NextSeq Illunima and was analyzed using the ERGO^{TM} transcription tool using statistical methods such as Limma (Linear Model) and DESeq2 (Negative Binomial).

There were a total of 24 biomarkers identified with a 6-fold higher over expressed in hypoxic conditions compared to normoxia. These include key genes such as CALM2, MYLK, FGR1, ASPH, PDGFRA, ATP2B4, ATP2B1, PDGFRB, CALM1, and PPP3CA. The genes and pathways were identified in KEGG pathways overlaying expression values.

The release of calcium ions from internal stores and the influx of calcium ions from the environment cause a dramatic and rapid increase in cytoplasmic calcium concentration, which has been exploited for signal transduction. In combination with power statistical analysis such as DESeq2 and Limma, key pathways can be identified that are responsible for calcium homeostasis and calcium channel signaling as key diagnostic markers for VPT and RET.

1.0 INTRODUCTION

1.1 Background

Homeostasis within the dental pulp during pulpitis occurs between inflammatory and regenerative responses and is central to the clinical outcome of vital pulp therapy and regenerative endodontic therapy. As cariogenic bacteria advance toward the pulp, odontoblasts are the first responders to limit the spread of bacteria. However, as disease advances beyond the odontoblastic layer, resident pulpal cells, including immune cells, fibroblasts, endothelial cells, and stem cells, react to the bacterial challenge by expressing a range of pattern recognition receptors (PRRs) that identify pathogen-associated molecular patterns (PAMPs). Consequently, a range of immune cells, which include neutrophils, macrophages, and T and B lymphocytes, are recruited from the vasculature to the diseased site via different cytokine chemotactic gradients (Cooper et al, 2017). There is a delicate balance between persistent pathosis and healing in the root canal system. These cell types have been shown to have a variety of microbial sensors, where among the best characterized are the Toll-like receptor (TLR) family that can detect a myriad of microbial components including cell wall constituents, such as lipopolysaccharides (LPS), to nucleic acids. Upon recognition by host cells, a host of pro-inflammatory cytokines such as interleukin (IL)-1α, IL-1β, tumor necrosis factor alpha (TNF-α), IL-6, and IL-8 as well as pro-resolving cytokines (IL-4 and IL-10) are secreted. Their cumulative effects depend on their levels and temporality and can yield different cellular and tissue outcomes (Smith et al, 2002).

At the molecular level, recognition of bacterial components via PRRs leads to activation of a series of intracellular signaling cascades, which are mediated by p38 mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB) proteins. Translocation of regulatory transcription factors, such as activator protein 1 and NF-κB, to the nucleus from the cytoplasm leads to activation of gene expression of proinflammatory cytokines, most notably IL-1 family, TNF-α, IL-8, and matrix metallopeptidase (MMP)-9. TNF-α is a marker for early inflammation and has a key role in the inflammatory response, affecting osteoclastogenesis and bone formation.

Upon stimulation by TNF-α, IKK complex is activated, which subsequently phosphorylates IkB-a. The phosphorylated IkB-a is degraded, which activates nuclear factor-kappa B (NF-kB) signaling. NF-kB is a master transcription factor that is involved in regulation of different angiogenic factors and proinflammatory cytokines in pulp fibroblasts and oral epithelial cells.

As pulpal inflammation is prolonged and the levels of cytokines increase, the most important cytokines responsible for the chemotactic gradient for immune cells is IL-1. This interleukin is heavily expressed in inflamed pulp and induces synthesis of prostaglandins, most notably PGE_2, which is known to reduce pain threshold in peripheral tissues. The extravasation and antimicrobial responses of the immune cells can result in consequential collateral tissue damage. If allowed to persist chronically, the advancing infection and the corresponding inflammatory response can affect the vitality of the pulp tissue, resulting in extracellular matrix (ECM) breakdown and ultimately tissue necrosis (Cooper et al, 2017).

It is important to note that, despite the proinflammatory nature of these cytokines, they have an inherently protective role in the dental pulp in response to bacteria and bacterial irritants. The inflammatory response is maintained by the actions of pro-resolving mediators synthesized from omega-3 fatty acids, such as resolvins and protectins, which regulate the activities of neutrophils and T cells, respectively. Furthermore, IL-10 produced by Th2 and M2 cells serve to inhibit most of the pro-inflammatory functions of other cytokines. The classic animal study by Tonder and Kvinnsland in 1983 demonstrated that the response of pulpal interstitial pressure to inflammation was limited to the injury site, not widespread throughout the pulp. Within the low-compliant pulpal environment, the pulpal hydrostatic pressure is greatly affected by an increase in blood or interstitial volume. The inflammatory-associated vasodilatation and increased vascular permeability thus increase pulp interstitial fluid pressure, which can compress blood vessels. This unique characteristic of a low-compliant environment attenuates a benefits of high tissue perfusion, which eventually leads to damage to pulp vasculature and circulation (Fouad, 2002). Persistent pulpal inflammation thus leads to attenuated pulp tissue perfusion and a reduction of tissue oxygen supply, or physiologic hypoxia. Initially, the hypoxic condition is a potent local stimulus for

initiation of repair by inducing mesenchymal progenitor cells (i.e., dental pulp stem cells) to directly proliferate and differentiate into various effectors of repair and to indirectly modulate inflammation by secretion of pro-resolving factors (Colombo et al, 2020). Furthermore, dental pulp stem cell (DPSC) survival, differentiation into odontoblast and deposition of matrix for mineralization is essential for regenerative endodontic therapy (RET) (Sui et al, 2020) (Huang et al, 2018). Understanding the pulpal response to the initial hypoxic condition could yield a greater understanding to factors that affect VPT and RET.

1.2 Significance of the Study

Given that the role of calcium channels is known in cell signaling pathways, understanding of Ca^{2+} channel signaling in DPSC might elucidate a role of Ca^{2+}-dependent biomarkers. In cases indicated for VPT or RET, these biomarkers can indicate the severity of pulpitis and can be used as "chair-side" diagnostic armamentarium for more accurate informed treatment planning and for better treatment outcomes.

1.3 Study Aims and Objectives

Our objectives, which are two-fold, are 1) to investigate key gene candidates involved with calcium homeostasis in human dental pulp tissues and DPSCs; and 2) to identify potential Ca^{2+}-dependent biomarkers for teeth that are candidates for VPT or RET.

1.4 Hypothesis

Null hypothesis (H_o): There is no significant difference between the levels of gene expression related to calcium channel signaling in DPSCs between normal and hypoxic conditions.

Alternative hypothesis (H_1): There is a significant difference between the levels of gene expression related to calcium channel signaling in DPSCs between normal and hypoxic conditions.

2.0 REVIEW OF LITERATURE

2.1 Overview of VPT and RET

Traditional pulp sensibility tests such as cold stimulus and electric pulp testing are used to infer the level of pulpal inflammation, with the exaggerated or lingering response to cold indicating activation of Aδ-fiber or C-fiber sensitization and inflammation-induced hypersensitivity. In addition, percussion tests also provide indirect inference of pulpal inflammation, where mechanical allodynia from dental percussion would suggest an irreversibly inflamed pulp. Furthermore, percussion hypersensitivity on the healthy adjacent tooth strongly could be predictive of the overall severity of pain experienced and may reveal a diminished pain threshold and increased pain sensitization due to central sensitization (Erdogan et al, 2021). However, it is believed that clinical test results do not always correlate with histologic findings of pulp status. In fact, there appears to be no histologic evidence that establishes a discrete boundary that would deem the pulp irreversibly inflamed (Lin et al, 2020). From the same study, Ricucci also found that 16% of cases with a clinical diagnosis of irreversible pulpitis demonstrated a histologic diagnosis of reversible pulpitis. Even when cases where the clinical and histological diagnosis of irreversible pulpitis were matching, uninflamed coronal pulp tissue could be identified. When the diagnosis is irreversible pulpitis, it has been shown that DPSCs can persist, evident in the expression of stem cell marker STRO-1. This shows that the clinically compromised dental pulp tissues contain putative stem cells that has the potential for endogenous pulp regeneration (Wang et al, 2010). Given the disconnect between the pulp sensibility tests and the histologic findings, current evidence suggests that VPT should be considered as a viable treatment option for teeth with pulpitis. A recent systematic review and meta-analysis indicated that pulpotomy is a conservative treatment modality and can substitute RCT for irreversible pulpitis in permanent teeth

(Li et al, 2019). The possibility to preserve the vitality of pulps diagnosed with irreversible pulpitis challenges the contemporary nomenclature of irreversible pulpitis. The clinical intraoperative observation under dental operating microscope should dictate the operative decision of whether to perform VPT or RCT, not the preoperative clinical diagnosis.

In a study by Rechenberg et al in 2014, the development of apical periodontitis, as determined by RANKL, can precede irreversible pulpal inflammation and eventual pulpal necrosis, as determined by IL-8. The primary proinflammatory mediator associated with neutrophil chemotaxis was IL-8. Likewise, the mediator associated with bone resorption was the dyad of RANKL/OPG. RANKL was expressed at significantly higher levels in cases with symptomatic irreversible pulpitis (SIP) compared to asymptomatic apical periodontitis (AAP). In contrast, IL-8 levels were significantly lower in SIP compared to AAP. This difference between RANKL and IL-8 expressions demonstrated that when the pulp is acute inflamed, radiographic changes might be more, and one should not rely exclusively on radiographic pathological finding for treatment decision.

Historically, the main approach toward treatment of an immature tooth presenting with a necrotic pulp and apical periodontitis was apexification with either $Ca(OH)_2$ or an apical plug with MTA. The main disadvantage of this technique was that it does not promote further root development, especially in root dentin thickness, and making the tooth susceptible to horizontal root fracture. In the 1960s, Frank proposed long-term calcium hydroxide use to induce an apical barrier, or apexification, of an immature tooth with pulpal necrosis before placement of an obturation material. However, long-term $Ca(OH)_2$ contact with dentin decreases the mechanical strength of dentin. Torabinejad and Chivian (1999) suggested the replacing long-term $Ca(OH)_2$ apexification with MTA plug as an alternative technique. Yet, this MTA plug at the apical region

likely would not promote additional root development. Cvek (1992) reported a greater incidence of root fracture at the cervical region in root canal treated immature teeth in comparison to mature teeth, suggesting that maintaining pulp vitality of immature teeth is of great importance. Nygaard-Ostby (1961) demonstrated in a classic study the presence of a vascular tissue that is formed at the apical third, in the unfilled portion of the root canal, of a mature root presented with a necrotic pulp and a periapical lesion. It was demonstrated that the "pulp-like" connective tissue was able to grow several millimeters into the canal space after removal of necrotic tissue. This was often considered as the origin for regenerative endodontics, where a treatment modality for an immature tooth with necrotic pulp could be *de novo* formation of a highly vascularized tissue within the canal space that has the capacity of inducing normal root development and maturation. Iwaya et al (2001) and Banchs & Trope (2004) demonstrated successful outcomes for RETs in immature permanent mandibular premolars presented with the diagnosis of pulpal necrosis and with periapical infection. Other contributing factors have been the expansion of stem cell research, in particular the discovery of MSCs with the potential to differentiate into odontogenic-like cell lines and the potential for therapeutic applications of tissue engineering.

Regenerative endodontic procedures have been defined by the AAE as "biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex." The ideal treatment goals are to promote regeneration of pulp tissue and the continuation of root development. This is achieved by having an appropriate environment inside the root canal system, which is void of bacteria and necrotic pulp tissue and the presence of an appropriate scaffold as well as a timely coronal seal. The objectives of RET include the following: primarily, resolution of symptoms and clinical and radiographic evidence of healing; secondly, increased root thickness and length; and lastly, positive response to

sensibility testing. Three important treatment factors were proposed for successful outcomes: disinfection of the root canal, placement of a matrix in the canal conductive to cellular proliferation and differentiation, and a bacterial tight seal of the access opening. Successful healing is typically observed between 6-12 months after treatment where apical radiolucency is resolved. Increased root width and length typically occur 12-24 months after treatment. In a retrospective case series done at San Antonio, the study identified significant predictors affecting outcomes of RET. The success rate was 84% with etiology of pulpal necrosis, NaOCl concentration, type of medicament, and age being significant predictors of failure. Root development was observed in over 90% of cases with etiology of pulpal necrosis (caries), type of medicament (DAP), age (younger), sex (female), NaOCl concentration (full strength), and apical diagnosis (AAA) being significant predictors for radiographic root area (RRA) changes. The recommended age for RET was 8-18 years. A young patient population may have a wider apical foramen, a factor strongly associated with revascularization, and possess stem cells with a greater regenerative potential than older patients. Greater RRA change was also associated with positive pulp sensibility responses (Chrepa et al, 2020). According to Lovelace et al (2011), the evoked-bleeding step used in RET resulted in stem cell migration into the root canal systems, evident by the 600-fold increase of the levels of their molecular markers CD73 and CD105 compared to equivalent levels found in the systemic blood collected from the same patients. This study showed that the stem cells arise from the local tissues near the root apex. The goal of this evoked-bleeding step is to release undifferentiated stem cells from the periapical tissues via direct manipulation and deliver them into the root canal system. These undifferentiated cells have not yet committed to either osteoblastic or odontoblastic lineages. A likely source of these stem cells was the apical papilla (SCAPs) because it contains an enriched population of mesenchymal stem cells (Lovelace et al, 2011).

2.2 Brief Overview of MTA and CSC

MTA was the first calcium silicate-based cement (CSC) introduced as root canal repair material in the early 1990s by Torabinejad. MTA forms a reactionary interface with dentin, which mimics the structure of hydroxyapatite upon setting in the presence of calcium ions and tissue fluids. MTA exhibits crystal formation within dentinal tubules responsible for microbial entombment and neutralization. High pH (12.5) is microbiocidal and encourages extraction of growth factors in adjacent dentin that contribute to calcific bridging. MTA has hygroscopic properties and hardens when exposed to moisture, making the material ideal to be used in direct contact with blood or tissue fluids. The small particle size and adaptation dentin of CSCs allow them to impede microleakage and bacterial growth. The gradual release of calcium encourages hard tissue formation as well as promoting signaling molecules and stimulating growth factors from dental pulp (Torabinejad & Parirokh, 2010).

The components of new calcium silicate-based cements include dicalcium silicate and tricalcium silicate, both of which are essential constituents of MTA and hydraulic calcium silicate cements. Due to their hydraulic properties, CSCs attain immediate strength on hydration and upregulate angiogenic and transcription factors as well as pulp fibroblasts and stem cells. The bioinductive capacities of CSCs are evident in that they can positively induce hard tissue barriers, influence cell differentiation and proliferation, while producing minimal inflammation. Removal of calcium sulfate in original MTA formulation allows for a shorter setting time of 10 minutes in many new CSCs, making them suitable for one-visit VPT or RET procedures (Camilleri, 2020).

2.3 Roles and sources of Ca^{2+}

The dynamic calcium (Ca^{2+}) dependent signaling system has been reported to be important for DPSCs (Rathinam et al, 2021). Calcium channels have long been known to be involved in

signaling pathways for cell proliferation, as well as playing an important role in dentin and dental pulp regeneration (Durham & Walton, 1982). Normal cells require high external calcium concentrations. The most likely mechanism by which Ca^{2+} ions exert an impact on cell behaviors is by activating chimeric Ag receptor T cells, thus promoting cell differentiation and proliferation. Specifically, Ca^{2+} ions have a role as an intracellular second messenger that is involved with variety of diverse processes such as cell proliferation, apoptosis, differentiation, activation of excitatory cell types, gene transcription, and protein expression.

Sources of Ca^{2+} for the cell include both extracellular Ca^{2+} and intracellular Ca^{2+}, with extracellular Ca^{2+} being the principal source of signal Ca^{2+}. For example, calcium hydroxide is a by-product of hydration of calcium silicate-based cements (CSCs), which disassociates into hydroxyl and calcium ions that increase their levels extracellularly. Elevated extracellular Ca^{2+} can briefly increase intracellular Ca^{2+} concentration due to activation of calcium-sensing receptor (CaSR). Cells use this external source of signaling Ca^{2+} by activating various entry channels with different properties, such as store-operated channels (SOCs), second-messenger-operated channels (SMOCs), and receptor-operated channels (ROCs). An example is the N-methyl-D-aspartate receptors (NMDARs) that interact with glutamate. Most notably, the voltage-gated calcium (CaV) channels, found in excitable cells, can generate rapid Ca^{2+} fluxes, which control many cellular processes. When the extracellular Ca^{2+} is taken up by the cells, the cellular Ca^{2+} uptake occurs by Ca^{2+} binding to proteins and also at the endoplasmic reticulum (ER) and mitochondria. The ER-mitochondrial Ca^{2+} axis is a major determinant of cell survival and cell death (Riddle et al, 2006). These internal Ca^{2+} stores serve as another principal source of Ca^{2+} for signaling, in which ryanodine receptors (RYRs) or inositol-1,4,5-trisphosphate receptors (IP3Rs) regulate the release of Ca^{2+}. IP3 is generated by pathways using different isoforms of phospholipase C (PLCβ, δ, ε, γ and ζ) and regulates IP3Rs. Cyclic ADP-ribose (cADPR) releases Ca^{2+} via RYRs. There are separate acidic Ca^{2+} stores that is utilized by the nicotinic acid adenine dinucleotide phosphate (NAADP) to release Ca^{2+} release via the NAADP-sensitive mechanism, which may feedback onto either IP3Rs or RYRs. NAADP and cADPR are generated by CD38. This enzyme itself can be

inhibited by NADH and ATP and may be sensitive to cellular metabolism. Ca^{2+} sensitive enzymes convert the dynamic changes in extracellular and intracellular Ca^{2+} concentration into well-defined cell bioactivities. Specifically, intracellular Ca^{2+} dynamics have a duality of roles in both initiating and mediating stem cell differentiation; in addition, Ca^{2+} homeostasis in DPSC plays a critical role for its survival, longevity, and differentiation processes (Sohn et al, 2015). In-depth knowledge of the role of Ca^{2+} dynamics in stem cell differentiation into a tissue-specific lineage can lead to better utilization of the unique properties of DPSCs. A clinical translation is the use of CSCs in VPT and RET, which can trigger Ca^{2+} dynamics and affect stem cell-related gene expression, cellular differentiation, and mineralization potential.

3.0 MATERIALS AND METHODS

3.1 Study Design

This *in vitro* study was designed to challenge human dental pulp stem cells (hDPSCs) in either normoxic (normal) or hypoxic conditions. With all the samples, the total mRNA was extracted and sequenced. For the differential gene expression analysis, the results of mRNA were analyzed, and statistically significant under- and over-expressed genes were compared.

3.2 Sample Collection and DPSC Isolation

Pulpal tissue samples derived from clinically healthy human third molar teeth were obtained from the Oral Surgery clinic at the University of Illinois at Chicago (UIC) College of Dentistry. The teeth were extracted for oral surgical reasons (i.e. impaction) in patients aged 15-30 years old [IRB approval (2011-0129)]. As part of the IRB protocol, written informed consent to donate dental pulp tissue for research purposes was obtained from the subject or his/her legal guardian.

Within an hour postmortem, from the extracted pulp tissue samples, hDPSCs were isolated following Gronthos and Shi protocol (2000), by introducing the pulp tissue samples to an enzyme solution for 1 hour at 37°C, followed by 30 minutes of vortexing. Upon removal of large cell aggregates, cells were passed through a 70μm strainer to obtain single-cell suspensions. To maintain and refresh the cells until confluent, a 10% fetal bovine serum (FBS) was used as a basal medium in a α-MEM (Life Technologies, NY, USA). The viability and characterization of DPSCs was done via flow cytometry analysis and observed 87% purity of DPSCs as indicated by CD105 and CD29 (Boyle et al, 2014). hDPSCs were cultured in 20% serum-containing media at 37°C.

3.3 Hypoxia Exposure

The samples were divided into two equal groups. The normoxia half of the hDPSC samples were cultured in 20% serum containing media at 37°C in room air, consisting of 21% oxygen. The hypoxia half of the hDPSC samples were cultured in 1% serum containing media in a tri-gas incubator (Thermo Fisher Scientific, MA, USA) for up to 21 days at 37°C in optimal hypoxic conditions (3% oxygen). The 3% oxygen exposure was identified as the most optimal hypoxic condition out of the 1.5%, 3% and 5%, especially since 1.5% (tissue hypoxic condition) does not lead to optimal DPSC cell growth. As previously mentioned, the oxygen level was critical since it has to be low enough to induce the physiologically stressed condition while being high enough as to be conducive to survivability of hDPSCs. From the hDPSCs collected, mRNA and proteins were extracted for quantitative real time PCR (RT-PCR) and Bioplex (Luminex xMap) analysis, respectively, to investigate the levels of genes as listed below (Figure 1).

3.4 RNA Isolation

Total mRNA was extracted from hDPSC tissue samples using the Maxwell® 16 LEV simply RNA Tissue Kit (Promega, Madison, WI, USA) and a Promega Maxwell 16 instrument. Both mRNA quality and quantity were analyzed using automated RNA electrophoresis via an RNA ScreenTape assay, which was implemented on a TapeStation2200 automated electrophoresis device (Agilent Technologies, Santa Clara, CA). The RNA HS Assay Kit with a Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY, USA) was used for further quantification.

Within the Illumina sequencing platform-compatible libraries, RNA-seq libraries were prepared with the Lexogen QuantSeq 3’ mRNA sequencing library preparation kit. The novel approach from this protocol is unique in that it can be used for more accurate determination of gene expression values, shorter sequencing length, and lower total reads per sample. The KAPA

real-time PCR Library Quantification Kit was utilized to assess and quantify library quality. All libraries were pooled and ran on an Illumina Nextseq500 instrument using high-output kit (75 cycles), and sequences were for 1x80 bases. The library was tested, and bar coding was performed for equal pooling. Sequencing was performed on Illumina Hi Seq 200 to get 1 x 100 base reads. We obtained around 80 million reads with four samples for each group in this study.

3.5 Bioinformatics and Statistical Analysis

The sequence reads were evaluated for quality using FastQC methods and end-trimmed reads were used for normalization with both DESeq2 (Mortazavi et al, 2008) (Varet et al, 2016) and Limma (Ritchie et al, 2015). After the reads were quantified, they were aligned using Tophat2 algorithm for accuracy. Feature Counts algorithm provided a fast and accurate set of read summary tools. Normalization of the RNA seq reads was carried out using trimmed mean of M-values (TMM) and applied voom transformations for counts (log-cpm), in which the values were used to detecting differential gene expression (DGE). For the DGE, once the normalizations of the reads were performed using the procedure log2 transformations, Spearman rank clustering was performed. Fold change and standard errors were estimated by fitting a separate linear model for each gene, followed by empirical Bayes that were used to rank genes in the order of evidence for differential expression. In order to capture specific genes that were over- or under-expressed in normoxic cells when compared to hypoxic cells, the RNA was isolated and sequenced using NextSeq Illumina. The RNA-Seq reads were processed for normalization, using DESeq2 and Limma. The count data was imported into R package for analysis and sequence mapping to the appropriate gene. Both statistically significantly under-expressed and over- expressed genes were computed using both DESeq2 and Limma. After sequencing and statistical analysis, the mRNA

was analyzed using the ERGO transcription tool, specifically, linkages and expression-based clustering (Overbeek et al, 2003).

4.0 RESULTS

Using log2 as a cut off and a p-value of <0.05, a total of 224 genes (Linear model: Limma) and 112 (Negative Binomial: DESeq2) over-expressed genes and 68 (Limma) and 130 (DESeq2) under-expressed genes were identified. Between these statistical techniques, there were 44 over-expressed genes and 20 under-expressed genes common to both statistics methods that differed in normoxia compared to hypoxia conditions. We identified 24 potential gene candidates for biomarkers with a 6.0-fold higher over expressed in hypoxia conditions compared to normoxia, these include genes such as CALM2, MYLK, FGR1, ASPH, PDGFRA, ATP2B4, ATP2B1, PDGFRB, CALM1, and PPP3CA. The representative gene functions are listed in Table 1.

Gene	Gene Function
CALM2 (calmodulin 2)	Calcium binding protein that primarily has a role in signaling pathways, cell cycle proliferation and progression
MYLK (myosin light chain kinase)	Calcium/calmodulin dependent enzyme – phosphorylates myosin regulatory light chains to facilitate myosin interaction with actin filaments to produce contractile activity
FGFR1 (fibroblast growth factor receptor 1)	Interacts with FGFs leading to cascade of downstream signals to influence mitogenesis and differentiation
ASPH (aspartate beta-hydroxylase)	Involves in calcium homeostasis by regulating calcium release from sarcoplasmic reticulum
PDGFRA (platelet derived growth factor receptor alpha)	Has a role in organ development, wound healing, and tumor progression

ATP2B4 (ATPase plasma membrane Ca2+ transporting 4)	Isoform 4 – plays a critical role in intracellular calcium homeostasis and removes bivalent calcium ions from eukaryotic cells against very large concentration gradients
ATP2B1 (ATPase plasma membrane Ca2+ transporting 1)	Isoform 1 – plays a critical role in intracellular calcium homeostasis and removes bivalent calcium ions from eukaryotic cells against very large concentration gradients
PDGFRB (platelet derived growth factor receptor beta)	Aids in rearrangement of the actin cytoskeleton and is essential for development of the cardiovascular system
CALM1 (calmodulin 1)	Calcium-induced activation of calmodulin regulates and modulates the function of cardiac ion channels
PPP3CA (protein phosphatase 3 catalytic subunit alpha)	Enables several functions, including calmodulin binding activity and calmodulin-dependent protein phosphatase activity. Involved in response to calcium ion

Table 1. Differential Gene Expression of DPSCs in Hypoxic Conditions. The top ten genes that were most over-expressed in hypoxia.

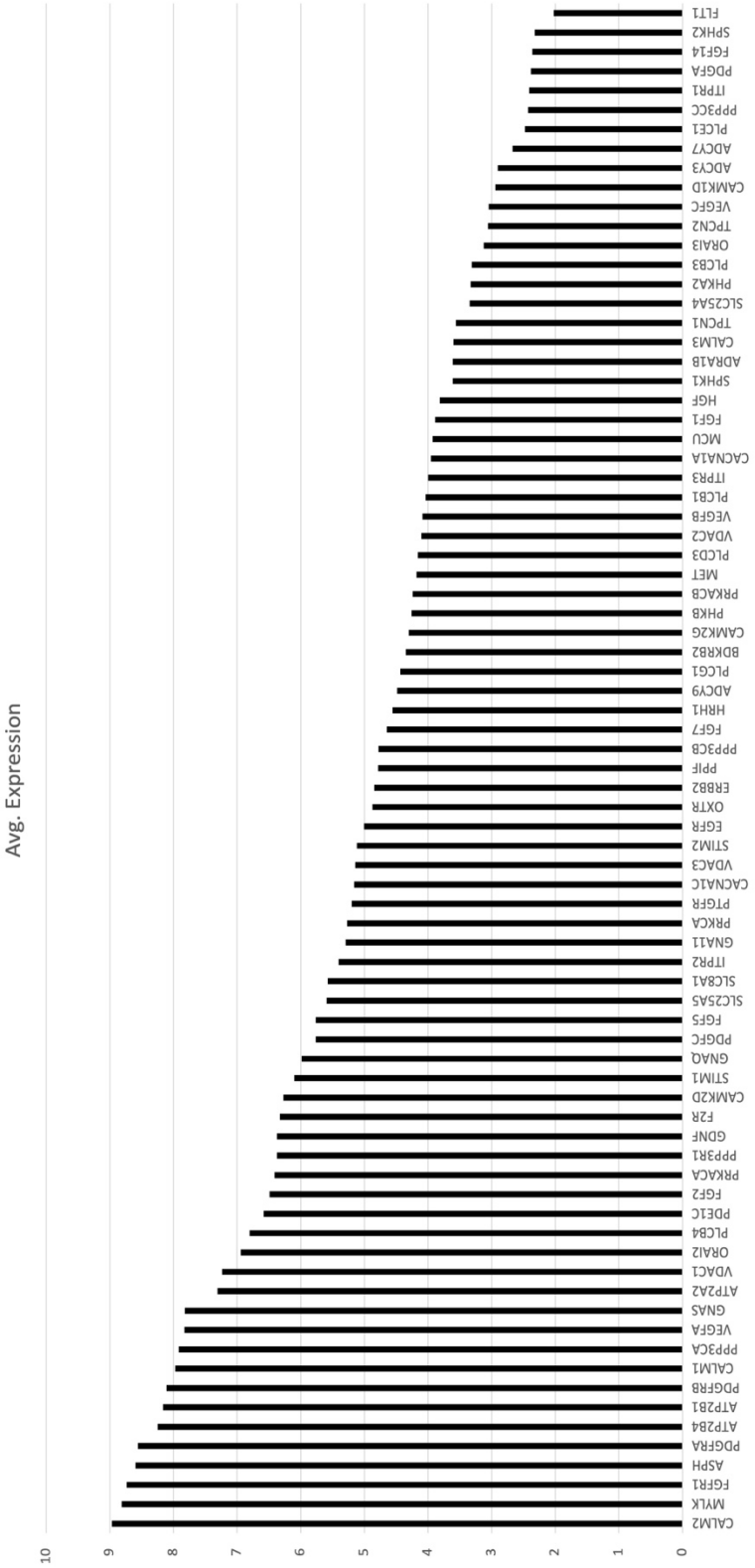

Figure 1. Over-expressed Genes Related to Calcium Channel Signaling in Hypoxia. 44 over-expressed genes common to both Limma and DESeq2.

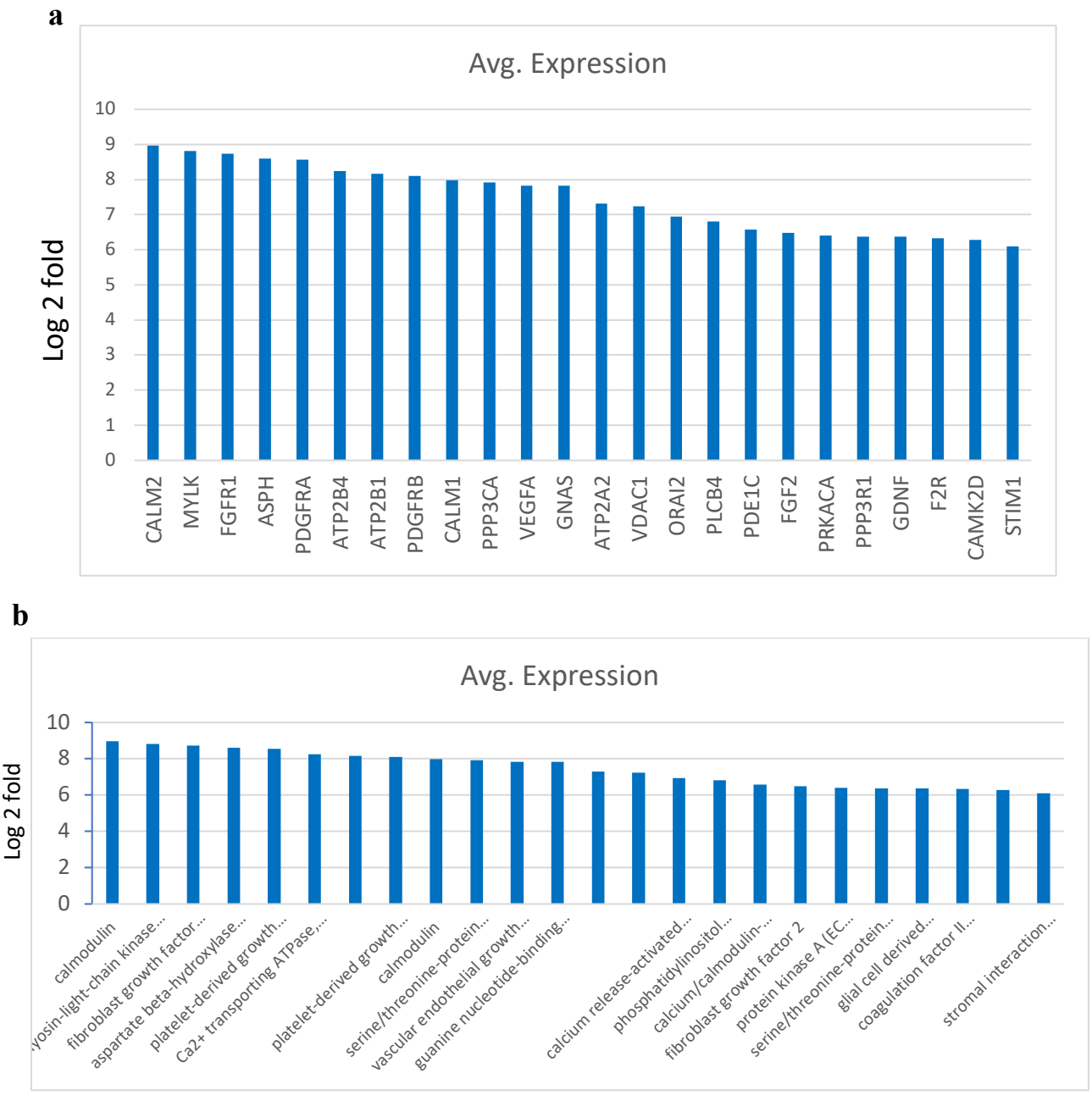

Figure 2. Total Gene Expression. 24 Most Over-Expressed Genes (**a**) and Corresponding Gene Functions (**b**) in Hypoxia

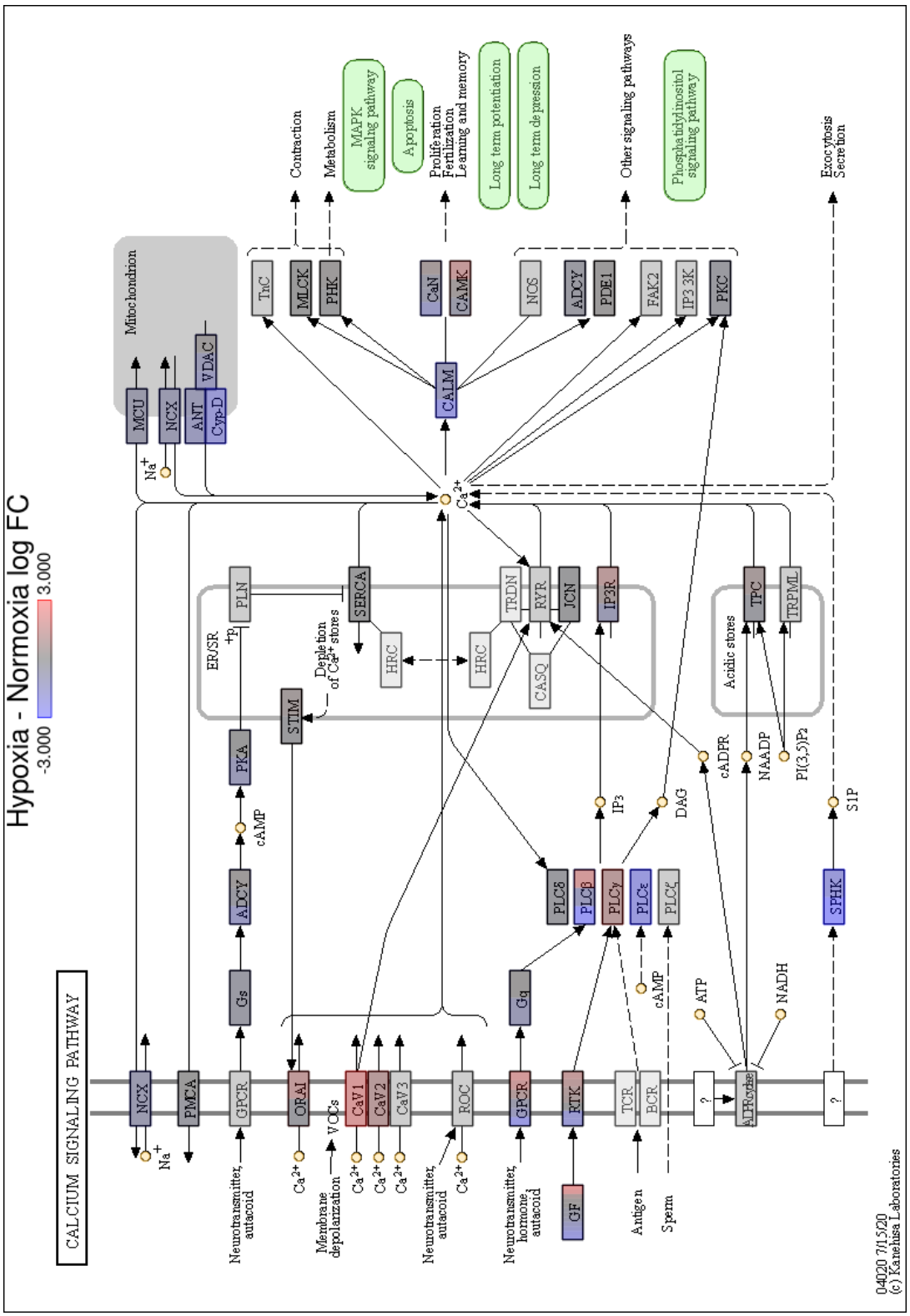

Figure 3. KEGG Pathway Map of Calcium Channel Signaling. Interplay between genes affecting calcium entry channel and genes influencing internal calcium release and their effects.

5.0 DISCUSSION

5.1 Clinical Relevance

In both the cytoplasm and the nucleus, the calcium dynamics can activate signaling pathways, most notably the mitogen-activated protein kinase (MAPK) cascade, that affect many downstream cellular functions. The presence of Ca^{2+} transients are essential for differentially controlling the activation of different genes and directing cells to specific developmental pathways. As seen in the KEGG pathway (figure 3), upon calcium entry into the cytoplasm through the entry channels like the voltage-gated (CaV) channels or ROC, different isoforms of phospholipase C (PLC), such as the β and ε isoforms, can be activated. IP3 is generated from PLC, which can bind to IP3R in the endoplasmic or sarcoplasmic reticulum and release the internal stores of Ca^{2+}. Furthermore, additional intracellular Ca^{2+} can be released from acidic stores via the NAADP-sensitive mechanism. The high cytoplasmic concentration of Ca^{2+} can interact with CALM (calmodulin) to influence a variety of significant cellular processes, which include mediating cell survival and death, encompassing the mechanisms of cell cycle, apoptosis, and autophagy. Of particular importance is the activation of the MAPK cascade, which is a highly conserved module involved in various cellular functions, which includes cell migration, differentiation, and proliferation.

The AAE Position Statement on Vital Pulp Therapy (2021) states that, VPT procedures for permanent teeth diagnosed with SIP or AIP yielded 85-100% success rates at 1-2 years when MTA and other CSCs are utilized. Similarly, a recent systemic review and meta-analysis on the use of RET on treatment of pulp necrosis in mature teeth showed 95% success at 12 months, as indicated by high periapical lesion reduction and moderately positive responses to pulp sensibility tests (Scelza et al, 2021). While the majority of histologic analyses on teeth that had undergone RET

showed that true regeneration of the pulp-dentin complex was not achieved, a recent case report demonstrated the possibility of regeneration of the normal pulp-dentin complex in an immature maxillary central incisor diagnosed with symptomatic irreversible pulpitis. The standard protocol was slightly modified to only amputate the pulp up to a few millimeters (5mm) short of the apex (Sabeti et al, 2021). Rathinam found that DPSC uptake of Ca^{2+} released from CSC did not result in cell death. Specifically, one of the CSCs, Biodentine®, was found to favorably affect intracellular Ca^{2+} dynamics in DPSCs stimulated with CSCs, which consequently resulted in the mineralization potential, differential gene expression, and cellular differentiation (Rathinam et al, 2021). Figure 4 demonstrates clinical cases where initial pulpal diagnoses ranged from irreversible pulpitis to pulpal necrosis, where young teeth with immature apices were treated following the protocols of VPT or RET. It provided the rationale for using CSCs to modulate inflammatory response within the pulp, which allows for SCAPs to differentiate into root odontoblasts to resume and complete root development and closure.

Figure 5 demonstrates our proposed model for calcium entry to DPSC in response to inflammation-induced hypoxia. In an immature apex subjected to bacteria or trauma-induced inflammation and followed by hypoxia, hypoxia-inducible factor-1alpha (HIF-1α) is translocated into the cytoplasm and leads to production of inhibitor of nuclear factor kappa B protein (Iκ-Bα), which inactivates the nuclear factor kappa B (NF-kB) transcription factor in the nucleus of DPSC. Therefore, hypoxia induced HIF-1α leads to transcriptions of genes that affect calcium transport mechanisms. Under normoxia, HIF-1α is subjected to degradation. However, in hypoxia, HIF-1α is stabilized. Thus, the effect of hypoxia on the expression of calcium channel signaling pathway in DPSC is evident. As DPSC requires calcium mineralization to differentiate and proliferate, the

influence of inflammation appears to be of significant importance in calcium homeostasis as well as in the fate of DPSC.

In summary, calcium is well known to be a determinant for cellular functions and hard tissue formation. Levels of intracellular calcium plays a significant role in mediating the mechanisms essential for cellular functions. Studies have shown a wide variety of plasma membrane localized calcium entry channels that contribute to cellular functions. To our body of knowledge, there were no studies hitherto identified the calcium channels expressed in dental pulp cells. Considering the fact that dental pulp cells are highly dependent on calcium mineralization for it to differentiate, we believe that addressing the unique functional of calcium channel in dental pulp stem cells are imperative. Hence, in continuation to our investigations on the transcriptional signaling and metabolic imbalances in dental pulp regeneration, we also studied the role of calcium entry channels in dental pulp stem cells.

5.2 Study Limitations

There are a few limitations of this study, mostly stemming from its study design and *in vitro* nature. Pulp tissue samples cultured under normoxia were not matched to those cultured under hypoxia as related to tooth type, location (maxillary or mandibular), gender, and age. It is also important to note that while hypoxia is a potent stimulus for DPSCs under inflammatory conditions, DPSCs can react to a variety of inflammatory mediators and many other cell signaling molecules. This study cannot capture this complexity and diversity of the DPSC response.

5.3 Future Directions

Looking ahead, with the genes and KEGG pathway identified in this study, future research should be able to map out the pathway to identify biomarkers. Additional roles for calcium-sensing receptor (CaSR) expression can be determined. Gene knockouts can be performed on animal

models. The objective is to generate miRNA to target inflammatory markers and ultimately a chair-side model that can be used to efficiently identify biomarkers in teeth that are candidates for VPT or RET. The content in this study can be further expanded in a future post-doc or PhD-level research.

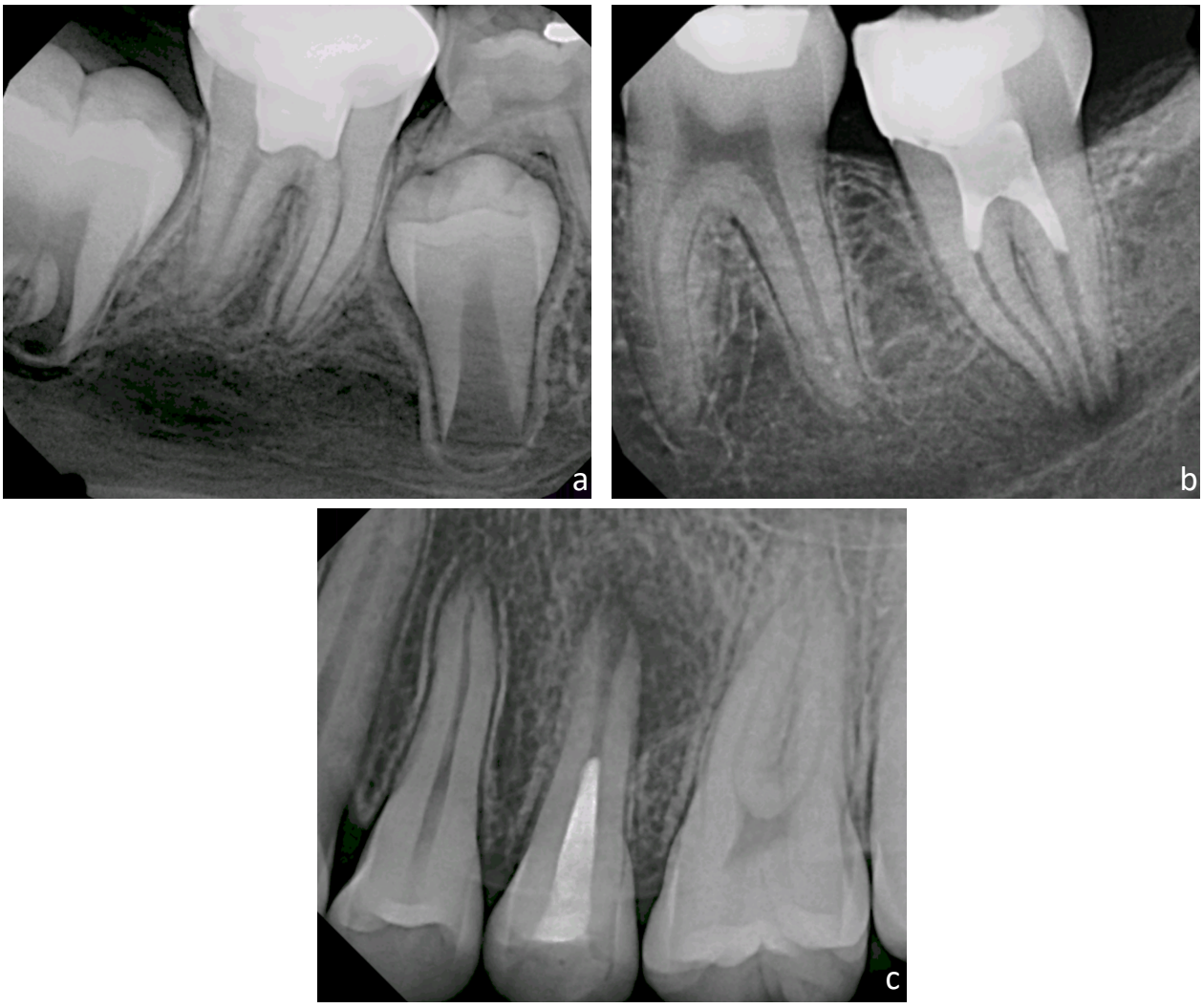

Figure 4. Clinical Cases of Utilizing VPT and RET for Young Teeth with Immature Apices. **(a)** 14-month follow-up periapical radiograph of bioceramic pulpotomy on #30, showing normal periapex; (**b**) immediate post-operative periapical radiograph of single-visit RET on #18; (**c**) immediate post-operative periapical radiograph of two-visit RET on #13.

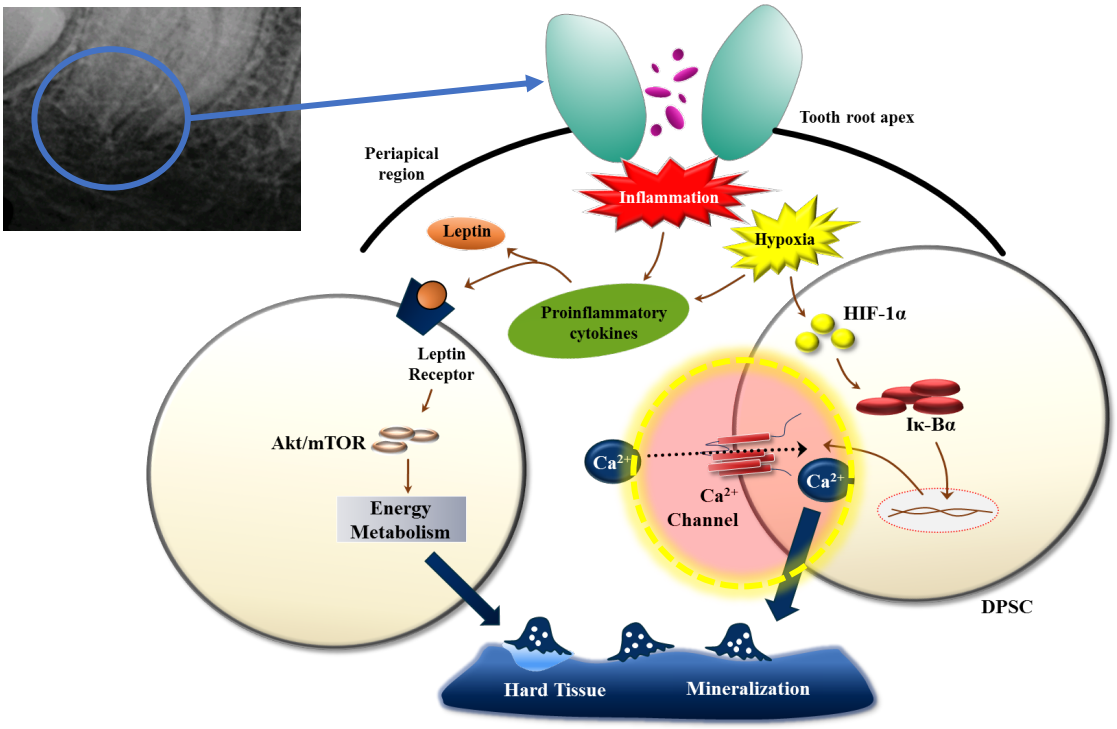

Figure 5. Proposed Model for Role of Calcium Entry Channels in DPSC. HIF-1α is translocated into the cytoplasm and leads to production of Iκ-Bα protein which inactivates the NF-κB transcription factor in the nucleus of DPSC. This leads to transcription of genes that affect calcium transport mechanisms.

6.0 CONCLUSION

The release of calcium ions from internal stores and the influx of calcium ions from the environment cause a dramatic and rapid increase in cytoplasmic calcium concentration, which has been exploited for signal transduction. In combination with power statistical analysis such as DESeq2 and Limma, key pathways can be identified that are responsible for calcium homeostasis and calcium channel signaling as key diagnostic markers for VPT and RET. Given that DPSC needs calcium mineralization to differentiate, an understanding of calcium channel signaling in DPSC will elucidate a role of calcium-dependent biomarkers that can be used as chair-side diagnosis in VPT and RET for better outcomes.

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