UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

GABRIELA BESSA MARCONATO ANTUNES

VALIDAÇÃO DO PADRÃO DE EXPRESSÃO DE GENES DIFERENCIALMENTE EXPRESSOS ENTRE CÉLULAS MESENQUIMAIS INDIFERENCIADAS DO LIGAMENTO PERIODONTAL COM ALTO E BAIXO POTENCIAL OSTEOGÊNICO

VALIDATION OF DIFFERENTIALLY EXPRESSED GENE PATTERNS BETWEEN UNDIFFERENTIATED MESENCHYMAL CELLS FROM THE PERIODONTAL LIGAMENT WITH HIGH AND LOW OSTEOGENIC POTENTIAL

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Mestra em Clínica Odontológica, na Área de Periodontia.

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Orientadora: Profa. Dra. Catharina Marques Sacramento

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RESUMO

A regeneração periodontal em defeitos extensos permanece um grande desafio clínico, em grande parte devido à imprevisibilidade dos tratamentos disponíveis. Essa dificuldade está associada à heterogeneidade das células-tronco do ligamento periodontal (hPDLSCs), que incluem populações com alto (HOP) e baixo (LOP) potencial osteogênico. A predominância de células LOP, caracterizadas por barreiras significativas à diferenciação osteogênica, compromete a eficácia e a consistência dos resultados terapêuticos. Este estudo investigou a interação antagônica entre os genes ASPN e BMP2, fortemente relacionados ao padrão de diferenciação osteogênica e à mineralização de hPDLSCs, com o objetivo de identificar estratégias terapêuticas para otimizar a regeneração periodontal. Células HOP e LOP foram cultivadas em condições padrão (SDM) e osteogênicas (OM). A mineralização foi avaliada por coloração com Alizarina Red S (AR-S), enquanto a expressão gênica de ASPN, BMP2, OCN e RUNX2 foi quantificada por qRT-PCR, e os níveis proteicos de BMP2 foram medidos por multiplex Luminex. A análise estatística incluiu correlações de Pearson, Análise de Componentes Principais (PCA), Análise Discriminante Linear (LDA) e modelagem multivariada, realizadas no RStudio (versão 4.3.0–R 4.4.2) para explorar as associações entre os fatores analisados. Os resultados revelaram que células HOP apresentaram maior capacidade de mineralização, associada à regulação positiva do gene BMP2 ($p<0,05$) e à redução significativa da expressão de ASPN ($p<0,01$). Nas células LOP, a expressão de BMP2 também foi modulada positivamente em resposta à indução osteogênica, porém sem aumento correspondente nos níveis proteicos de BMP2. A expressão elevada de ASPN nas células LOP correlacionou-se negativamente com a expressão de BMP2 ($r=-0,565$, $p=0,004$) e com a mineralização ($r=-0,719$, $p<0,001$). Em contrapartida, os níveis proteicos de BMP2 apresentaram correlação positiva significativa com a mineralização ($r=0,422$, $p=0,041$). A matriz de correlação e a análise de rede destacaram a interação antagônica entre ASPN e BMP2, sublinhando seus papéis complementares na regulação do potencial osteogênico. O modelo multivariado confirmou ASPN como um inibidor chave da osteogênese, enquanto a proteína BMP2 foi identificada como um promotor essencial. Conclui-se que a redução na expressão de ASPN e o aumento da sinalização de BMP2 podem melhorar significativamente a regeneração periodontal. Esses achados reforçam a necessidade de intervenções moleculares direcionadas para superar as limitações associadas às células LOP, aumentando a previsibilidade e a eficácia das terapias regenerativas em defeitos periodontais complexos.

Palavras-chaves: Diferenciação Celular; Ligamento Periodontal; Proteína Morfogenética Óssea 2; Células-Tronco Mesenquimais; Regeneração Óssea; Análise Multivariada.

ABSTRACT

Periodontal regeneration in extensive defects remains a significant clinical challenge, largely due to the unpredictability of current treatments. This difficulty is linked to the heterogeneity of periodontal ligament stem cells (hPDLSCs), which include populations with high (HOP) and low (LOP) osteogenic potential. The predominance of LOP cells, characterized by significant barriers to osteogenic differentiation, undermines the efficacy and consistency of therapeutic outcomes. This study investigated the antagonistic interaction between the genes ASPN and BMP2, which are strongly associated with osteogenic differentiation patterns and mineralization in hPDLSCs, aiming to identify therapeutic strategies to optimize periodontal regeneration. HOP and LOP cells were cultured under standard (SDM) and osteogenic (OM) conditions. Mineralization was assessed using Alizarin Red S (AR-S) staining, while the gene expression of ASPN, BMP2, OCN, and RUNX2 was quantified by qRT-PCR, and BMP2 protein levels were measured using multiplex Luminex assays. Statistical analyses included Pearson correlations, Principal Component Analysis (PCA), Linear Discriminant Analysis (LDA), and multivariate modeling, performed in RStudio (version 4.3.0–R 4.4.2), to explore associations among the studied factors. The results showed that HOP cells exhibited greater mineralization capacity, associated with upregulation of BMP2 ($p<0.05$) and a significant reduction in ASPN expression ($p<0.01$). In LOP cells, BMP2 expression was also positively modulated in response to osteogenic induction; however, this was not accompanied by a corresponding increase in BMP2 protein levels. Elevated ASPN expression in LOP cells correlated negatively with BMP2 expression ($r=-0.565$, $p=0.004$) and mineralization ($r=-0.719$, $p<0.001$). Conversely, BMP2 protein levels showed a significant positive correlation with mineralization ($r=0.422$, $p=0.041$). The correlation matrix and network analysis highlighted the antagonistic interaction between ASPN and BMP2, emphasizing their complementary roles in regulating osteogenic potential. The multivariate model confirmed ASPN as a key inhibitor of osteogenesis, while BMP2 protein was identified as an essential promoter. It is concluded that reducing ASPN expression and enhancing BMP2 signaling can significantly improve periodontal regeneration. These findings underscore the need for targeted molecular interventions to overcome the limitations associated with LOP cells, thereby increasing the predictability and effectiveness of regenerative therapies in complex periodontal defects.

Keywords: Cell Differentiation; Periodontal Ligament; Bone Morphogenetic Protein 2; Mesenchymal Stem Cells; Bone Regeneration; Multivariate Analysis.

SUMÁRIO

1 INTRODUÇÃO

O periodonto corresponde ao aparato de proteção e inserção que circunda o dente, sendo composto por gengiva, cemento, ligamento periodontal e osso alveolar[1,2]. Sua função é ancorar o dente ao osso alveolar, permitindo a função mastigatória enquanto mantém a integridade dos tecidos ao redor do dente[3,4]. A periodontite é uma doença inflamatória iniciada pelo biofilme microbiano, que resulta na destruição da inserção conjuntiva e do osso alveolar, podendo levar à perda dentária[2]. O principal objetivo da terapia periodontal é controlar a inflamação induzida pelas bactérias periodontopatogênicas, restabelecendo a saúde periodontal e preservando o dente e o sistema mastigatório como um todo. Após o controle do aspecto infeccioso-inflamatório da doença, a correção de defeitos anatômicos e a regeneração dos tecidos periodontais perdidos tornam-se objetivos complementares da terapia periodontal[2,5].

A regeneração periodontal, de acordo com a definição da Academia Americana de Periodontia (AAP), consiste na formação de novo cemento, osso alveolar e ligamento periodontal[6,7]. Desde o início dos anos 1980, diversas técnicas têm sido propostas para alcançar esse objetivo, como a regeneração tecidual guiada (RTG), o uso de enxertos e substitutos ósseos, o condicionamento da superfície radicular, proteínas derivadas do esmalte e fatores de crescimento[5,8-10]. No entanto, por ser uma estrutura complexa composta por tecidos mineralizados (osso alveolar e cemento dental) e não mineralizados (ligamento periodontal e tecido gengival), a regeneração do periodonto tem se mostrado extremamente desafiadora, com ampla variabilidade nos resultados e baixa previsibilidade das técnicas regenerativas[5,11]. Além disso, tais abordagens apresentam indicação limitada a defeitos periodontais infra-ósseos e lesões de furca Grau II de Miller[5].

Neste cenário, a engenharia tecidual surge como uma alternativa promissora para a regeneração dos tecidos periodontais, fundamentando-se em conceitos de biologia celular, molecular e engenharia de biomateriais, com o objetivo de aumentar a previsibilidade dos resultados[12]. As células desempenham um papel central nesse processo, pois são responsáveis pela formação dos tecidos a serem regenerados. Desde o final da década de 1970, especula-se que o tipo celular que povoa a ferida cirúrgica periodontal durante os estágios iniciais de cicatrização é determinante para o tipo de cicatrização que ocorrerá[13]. Estudos posteriores confirmaram que o sucesso da regeneração periodontal depende da migração e proliferação de células provenientes do ligamento periodontal remanescente, acompanhadas pela diferenciação e síntese de componentes da matriz tecidual[14,15]. Contudo, sabe-se que as células do ligamento periodontal não são homogêneas, mas sim uma população heterogênea composta por subtipos

celulares com diferentes funções relacionadas à formação dos tecidos do periodonto de inserção (cemento, ligamento periodontal e osso alveolar)[16-19].

Do ponto de vista do desenvolvimento dental, o ligamento periodontal origina-se do folículo dental que circunda o dente em desenvolvimento, fornecendo ao tecido uma população heterogênea de células em diferentes estágios de diferenciação. Essas células desempenham papéis importantes na nutrição, homeostasia e reparo dos tecidos danificados[13,16]. Dependendo do estágio de diferenciação e do comprometimento com linhagens celulares específicas, as células-tronco do ligamento periodontal (hPDLSCs) podem apresentar maior capacidade para se diferenciar em fenótipo fibroblástico (formador de ligamento periodontal) ou em fenótipo mineralizante, incluindo subpopulações osteoblásticas e cementoblásticas (O/C)[16,20-23].

Diversos estudos que investigaram a multipotencialidade de clones celulares de hPDLSCs evidenciaram uma grande variabilidade no potencial dessas células para diferenciação e maturação em fenótipo O/C. No estudo de Singhatanadgit et al. (2009)[24], dentre 32 clones isolados, apenas 3 apresentaram potencial osteoblástico *in vitro*. De forma semelhante, Sununliganon & Singhatanadgit (2012)[25] isolaram 12 clones, dos quais 5 exibiram potencial osteoblástico. Em pesquisas realizadas pelo nosso grupo, de 6 clones celulares isolados de uma população de hPDLSCs, 50% demonstraram capacidade de diferenciação em fenótipo O/C[26]. Em outro estudo, envolvendo 16 clones isolados, apenas 5 apresentaram capacidade de mineralização *in vitro*[27]. Esse perfil celular heterogêneo foi corroborado por Seo et al. (2004)[18], que observaram, em um estudo *in vivo*, que 60% das colônias de hPDLSCs formaram estruturas semelhantes a tecidos periodontais, incluindo tecidos mineralizados e fibras colágenas inseridas, quando transplantadas em animais imunossuprimidos.

Essas evidências de heterogeneidade celular destacam os desafios enfrentados no contexto da regeneração periodontal. A neoformação do cemento radicular, por exemplo, é um processo essencial para a restauração do tecido conjuntivo de suporte do dente, uma vez que a superfície radicular contaminada não possui capacidade de promover a adesão e migração de células do ligamento periodontal[7,28]. Além disso, a remodelação e produção de novo osso alveolar são etapas fundamentais para a regeneração funcional do complexo periodontal[28]. Esses processos dependem de uma interação coordenada entre células-tronco, fatores de crescimento e o microambiente local do defeito periodontal[18,27]. Assim, o desenvolvimento de abordagens clínicas baseadas na engenharia tecidual, como o transplante de células progenitoras comprometidas com o fenótipo O/C ou a aplicação de moléculas sinalizadoras que estimulem a migração, proliferação e diferenciação dessas células, pode representar uma

estratégia promissora para aumentar a previsibilidade na formação do aparato cemento-ligamento[19].

Nesse contexto, compreender as diferenças intrínsecas entre as populações celulares com maior e menor potencial osteogênico torna-se essencial para aprimorar essas estratégias regenerativas. Considerando que uma parcela menor das hPDLSCs apresenta capacidade para diferenciação em osteoblastos e cementoblastos, estudos têm se concentrado na identificação de marcadores biológicos específicos (genes e proteínas) capazes de estimular a migração, proliferação e diferenciação/maturação das hPDLSCs com maior potencial O/C[25,29]. Essa heterogeneidade classifica as células-tronco do ligamento periodontal (PDLSCs) em populações com alto potencial osteogênico (HOP) e baixo potencial osteogênico (LOP)[27].

Um estudo realizado pelo nosso grupo, utilizando a técnica de sequenciamento de RNA (RNA-seq), identificou um perfil de expressão gênica distinto entre clones HOP e LOP *in vitro*[30]. Os resultados mostraram que os clones HOP apresentaram maior expressão de genes associados ao fenótipo O/C, como *RUNX2*, fosfatase alcalina (*ALP*), osteocalcina (*OC*), sialoproteína óssea (*BSP*) e osteopontina (*OPN*), em comparação aos clones LOP[30]. Além disso, foram detectadas diferenças marcantes no padrão de expressão de *ASPN* e *BMP2*. Enquanto os clones HOP exibiram níveis mais elevados de *BMP2*, um gene diretamente relacionado à diferenciação osteoblástica de células mesenquimais[36], os clones LOP apresentaram alta expressão de *ASPN*, independentemente das condições de cultura celular (meio padrão ou osteogênico)[30]. Essa alta expressão de *ASPN* parece estar associada à inibição de *BMP2* nos clones LOP, uma vez que *ASPN* atua como um regulador negativo da diferenciação O/C em hPDLSCs, suprimindo a sinalização de BMP2[50-53].

O *BMP2* é um fator de crescimento da família *TGF-β* que desempenha um papel essencial na regulação da diferenciação e proliferação de células pericondriais e osteoblastos[37,38]. Esse agente osteogênico induz a diferenciação de osteoblastos e regula a expressão de genes-chave, como *RUNX2* e *Osterix*, além de estimular a atividade de *ALP*, a síntese de proteoglicanos, colágeno tipo I, fibronectina e Osteocalcina (OCN)[39-41]. Sua ativação é amplamente mediada pela sinalização canônica *Wnt/β-catenina*[42], bem como pelas vias *Akt* e *MAPK*, que são ativadas por meio de *PI3K*[43]. Estudos indicam que *PI3K* e *Akt* são mecanismos utilizados pelo *BMP2* para promover a diferenciação osteoblástica[44-45].

Por outro lado, a expressão de *BMP2* pode ser suprimida pela inibição da via *TGF-β/Smad*, mediada pelo gene *ASPN*[45-49]. Estudos anteriores corroboram essa observação, indicando que a alta expressão de *ASPN* limita significativamente o processo de osteo/cementogênese em células do ligamento periodontal via supressão de *BMP2*[48-50]. *ASPN*

(asporina), também conhecido como PLAP-1 (Proteína Associada ao Ligamento Periodontal 1), é uma proteína da matriz extracelular associada ao ligamento periodontal e pertencente à família das pequenas proteínas de repetição ricas em leucinas[51,52]. Essa proteína desempenha um papel importante na homeostase dos tecidos periodontais[52,53] e pode estar envolvida na patogênese da periodontite[51]. Estudos demonstraram que a presença de LPS induz a expressão de *ASPN*, resultando na inibição da diferenciação osteoblástica[55.] Além disso, *ASPN* parece prevenir a anquilose em dentes submetidos a movimentação ortodôntica, inibindo a formação óssea no lado comprimido dos dentes e protegendo o ligamento periodontal contra osteogênese excessiva por meio da inibição de BMP2[47]. Há também evidências de similaridade entre o ligamento periodontal e a cartilagem articular, com a expressão de *ASPN* sendo mais elevada em indivíduos com osteoartrite, o que sugere um papel dessa proteína na formação da cartilagem articular e na regulação negativa de *TGF-β/Smad* [56].

Em um estudo anterior, demonstramos também que *ASPN* atua como um regulador negativo da diferenciação *O/C* em células *LOP* pré-tratadas com o modulador epigenético 5-aza-dC[27]. Os resultados mostraram que, apesar da hiperhidroximetilação do gene *BMP2* induzida pelo 5-aza-dC e do consequente aumento na expressão gênica de *BMP2*, as células ativaram um mecanismo de feedback inibitório. Esse mecanismo levou a um aumento na transcrição de *ASPN*, acompanhado por uma redução na expressão da proteína BMP2 e, consequentemente, a uma menor mineralização. Esses achados sugerem uma possível relação regulatória entre a expressão de *BMP2* e *ASPN* e a modulação da produção proteica de BMP2[27].

Todavia, o mecanismo de regulação antagonista entre *ASPN* e *BMP2* ainda necessita de maior elucidação, pois parece ser fundamental para a aquisição do fenótipo O/C. Uma compreensão mais detalhada dessa interação pode fornecer bases importantes para o desenvolvimento de terapias regenerativas mais eficazes. Nesse contexto, a aplicação de análises estatísticas robustas e a criação de modelos multivariados e preditivos surgem como ferramentas promissoras para identificar fatores determinantes e fornecer informações sobre os mecanismos de regulação que influenciam diretamente a diferenciação celular[59,60,61].

Entre as análises estatísticas amplamente utilizadas na biologia molecular, destaca-se o *heatmap*, uma ferramenta gráfica que permite a visualização de grandes volumes de dados organizados em uma matriz onde cores indicam variações de valores[62,63]. Essa abordagem é essencial para identificar padrões de expressão semelhantes entre grupos ou condições, facilitando a análise comparativa e a identificação de agrupamentos relevantes. A análise discriminante linear (LDA), por sua vez, é um método estatístico que separa grupos distintos com base em variáveis preditoras, otimizando a discriminação entre classes[64-68]. Essa técnica é

especialmente útil para diferenciar grupos fenotípicos ou genotípicos em estudos multivariados. Já a análise de componentes principais (PCA) reduz a dimensionalidade de conjuntos de dados complexos, destacando as variáveis que mais contribuem para a variabilidade observada[69,70,71], permitindo uma representação visual clara de agrupamentos e diferenças entre amostras.

A correlação de Pearson é outra métrica fundamental para medir associações lineares entre variáveis contínuas[72,73]. Análises mais integrativas, como matrizes de correlação e análises de rede, oferecem uma visão abrangente das interações entre genes, proteínas e outros fatores, identificando possíveis reguladores centrais em processos biológicos[74,75]. Análises multivariadas permitem explorar múltiplas variáveis simultaneamente, revelando interações complexas e padrões subjacentes nos dados[76,77]. Já os modelos preditivos baseiam-se em métricas como o *Percent Increase in Mean Squared Error* (%IncMSE) e o *Increase in Node Purity* (IncNodePurity) para avaliar a importância relativa das variáveis, fornecendo uma base quantitativa para prever comportamentos biológicos e identificar fatores determinantes em processos complexos[78,79,80].

A ampla aplicabilidade dessas ferramentas estatísticas permite integrar diferentes níveis de dados – gênicos, proteicos e fenotípicos – para explorar interações e identificar fatores críticos em processos como diferenciação celular e regulação gênica[81]. A utilização de análises multivariadas e modelos preditivos não só auxilia na interpretação de padrões e relações como também possibilita a criação de estratégias personalizadas e eficazes para o desenvolvimento de terapias inovadoras, incluindo novos biomateriais[82,83].

Nesse contexto, o objetivo deste estudo é empregar ferramentas de análise de dados e estatística para investigar os mecanismos moleculares envolvidos na regulação de *ASPN* e *BMP2*, buscando compreender como essas interações modulam o potencial O/C de hPDLSCs *in vitro*. Além disso, a investigação visa identificar alvos terapêuticos potenciais que possam contribuir para o desenvolvimento de terapias regenerativas mais eficazes e previsíveis.

2 ARTIGO

Modulatory Roles of Bone Morphogenetic Protein 2 and Asporin in Osteo/Cementoblast Differentiation Potential of Human Periodontal Ligament Stem Cells: A Multivariate Model Analysis

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Abstract

Objective: This study aimed to investigate the interaction between *BMP2* and *ASPN* and their relationship with the mineralization potential of human periodontal ligament stem cells (hPDLSCs). Specifically, it sought to elucidate how *BMP2/ASPN* regulation contributes to the superior mineralization capacity of high osteo/cementogenic potential cells (HOP) and to identify limitations and therapeutic targets for enhancing the osteogenic potential of low osteo/cementogenic potential cells (LOP).

Methods: Four populations of primary hPDLSCs (2 HOP and 2 LOP) were isolated and cultured under standard (SDM) and osteo/cementogenic (OM) conditions. Mineralization was assessed using Alizarin Red Staining (AR-S) and alkaline phosphatase (ALP) activity. Gene expression levels of *ASPN*, *BMP2*, *OCN*, and *RUNX2* were quantified by qRT-PCR, and *BMP2* protein levels were measured using the Luminex system. Statistical models, including Linear Discriminant Analysis (LDA), Principal Component Analysis (PCA), Pearson correlations, correlation matrices, network analysis, and multivariate modeling, were applied to explore associations between *BMP2*, *ASPN*, and mineralization, using RStudio (versions 4.3.0–4.4.2).

Results: HOP cells exhibited significantly higher mineralization potential and positive modulation of *BMP2* expression compared to LOP cells ($p<0.05$). *ASPN* was identified as a negative regulator, displaying a significant inverse correlation with *BMP2* expression and mineralization, particularly in LOP cells ($p<0.01$). The multivariate model highlighted *BMP2* as a critical positive factor for osteo/cementogenesis, whereas *ASPN* emerged as a significant inhibitory factor ($p<0.001$).

Conclusion: The upregulation of *BMP2* mRNA and protein levels, combined with the downregulation of *ASPN* expression, are key determinants of the osteo/cementogenic potential of HOP cells. Therapeutic strategies aimed at reducing *ASPN* expression and enhancing *BMP2* activity may improve the osteogenic differentiation of LOP cells, contributing to more effective periodontal regeneration

Keywords: Cell Differentiation; Periodontal Ligament; Bone Morphogenetic Protein 2; Mesenchymal Stem Cells; Bone Regeneration; Multivariate Analysis.

1. Introduction

Periodontal regeneration remains a significant challenge in clinical practice, particularly in cases involving large defects where the reconstruction of dental supporting tissues, such as bone and cementum, is essential for new anchorage of periodontal fibers and to maintain tooth stability (Stavropoulos & Sculean, 2017, Citterio et al., 2020). Despite current therapies, the predictability of outcomes remains limited, largely due to the complexity of the biological processes involved and the difficulty in promoting effective regeneration in extensive lesions (Sculean & Stavropoulos, 2017; Lin et al*.;* 2020). Proper restoration of these supporting tissues is crucial for ensuring stable attachment and prolonging the longevity of the tooth.

A key obstacle to successful periodontal regeneration lies in the limited availability of human periodontal ligament stem cells (hPDLSCs) capable of differentiating into osteo/cementogenic lineages (Silvério et al., 2010, Assis et al, 2022). HPDLSCs are a heterogeneous population that plays a crucial role in periodontal tissue regeneration; however, most of these cells exhibit low osteo/cementogenic potential (LOP), while only a minority display a high potential (HOP) for producing mineral matrix and restoring supporting tissues (Singhatanadgit, Donos & Olsen, 2009; Sununliganon L & Singhatanadgit et al., 2012; Saito et al, 2020., Sacramento et al., 2022). This discrepancy between HOP and LOP phenotypes underscores the importance of understanding the mechanisms regulating osteo/cementogenesis activation and inhibition in these cells (Sacramento et al., 2024). Identifying therapeutic targets capable of converting LOP cells to a phenotype similar to HOP could significantly enhance regenerative outcomes.

Bone morphogenetic proteins (BMPs), particularly BMP2, have emerged as key regulators in promoting osteo/cementogenesis (Silvério et al., 2012; Ru, Pan & Zheng, 2023). BMP2 is known for its ability to stimulate bone formation and is one of the most extensively studied osteo/cementogenic factors in hPDLSCs (Hayrapetyan, Jansen & van den Beucken, 2015). However, attempts to use recombinant BMP2 in periodontal treatments have yielded inconsistent results, possibly due to challenges such as inadequate dosage and limitations within the cellular microenvironment (Wikesjö et al., 2003; Takahashi et al., 2005). As a promising alternative, enhancing BMP2 expression endogenously could optimize the regenerative capacity of these cells in a more controlled and sustainable manner (Hayrapetyan, Jansen & van den Beucken, 2015; Sacramento et al., 2022).

More recently, Asporin (*ASPN*) has been identified as a potential inhibitor of *BMP2*, directly interfering with signaling pathways that promote osteo/cementogenesis (Yamada et al.,

2006; Ueda et al., 2016). *ASPN*, also known as Periodontal Ligament-Associated Protein 1 (PLAP-1), is an extracellular matrix protein that negatively regulates bone mineralization by modulating *BMP2* activity and limiting tissue formation (Ikegawa, 2008). Elevated levels of ASPN not only inhibit BMP2 by blocking its interaction with cell surface receptors but also suppress BMP2 gene expression (Tomoeda et al., 2008; Sacramento et al., 2022), further reducing its availability for osteo/cementogenic signaling. This inhibition represents a significant impediment to effective mineralization, particularly in LOP cells, where ASPN seems to act as a key barrier (Sacramento et al., 2022; Pereira et al., 2024). However, the exact regulatory mechanisms underlying the antagonistic interaction between BMP2 and ASPN in determining the mineralization capacity of hPDLSCs are still not fully understood. Gaining deeper insights into this relationship may prove instrumental in developing targeted strategies to enhance BMP2 function and improve regenerative outcomes.

To address these challenges, the application of precise data science analyses becomes highly advantageous. Predictive statistical models can help better differentiate cell populations and identify factors that directly influence regenerative capacity (Kowal et al., 2021; Zhu et al., 2021). Building a multivariate model is beneficial not only for understanding cellular interactions but also for identifying effective therapeutic targets (Tewary et al., 2018). Such an integrated approach allows us to evaluate both the individual impact of BMP2 and ASPN and their combined influence on cellular processes, helping to define strategies to improve the osteo/cementogenic capacity of hPDLSCs.

Considering that BMP2 signaling is fundamental for the differentiation of hPDLSCs into osteo/cementoblasts, promoting the expression of genes that sustain bone formation, the interaction between ASPN and BMP2 may serve as a central regulatory mechanism in osteo/cementogenesis. Specifically, in LOP cells, ASPN appears to act as a constraint, restricting BMP2 activity and consequently limiting osteo/cementogenic potential (Sacramento et al., 2022; Pereira et al., 2024).

Thus, the aim of this study was to investigate the interaction between BMP2 and ASPN and their relationship with the mineralization potential of hPDLSCs. Through an integrated statistical analysis, we aimed to understand how the regulation and modulation of these factors contribute to osteo/cementogenic differentiation, with the ultimate goal of identifying potential therapeutic interventions to enhance the osteogenic potential of LOP cells and improve the effectiveness of regenerative therapies for large periodontal defects.

2. Methods

2.1. Evaluation of mineralization potential of hPDLSCS.

This study, approved by the Ethics Committee of the Piracicaba Dental School, University of Campinas (protocol nº 63957722.0.0000.5418), utilized primary cells obtained from the Periocells biobank (B-041), which were previously isolated and characterized as human periodontal ligament stem cells (hPDLSCs) (Silvério et al, 2010). Briefly, hPDLSCs were isolated from the periodontal ligament (PDL) of extracted third molars from different donors using Magnetic Cell Sorting based on the expression of CD105+ (indicative of mesenchymal stem cells), and negative markers CD34- and CD45- to exclude hematopoietic cells. The isolated cells were further characterized by flow cytometry and immunostaining for additional markers, including CD166, CD146, and STRO-1.

Four populations of hPDLSCs (designated GB, GHP, MS, and NWB) were seeded at a density of 10^5 cells/well in 24-well plates and cultured under two distinct conditions: Standard Medium (SDM), consisting of DMEM (Gibco®) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and Osteo/cementogenic Medium (OM), comprising SDM further supplemented with 50mg/mL ascorbic acid, 10mM β-glycerophosphate, and 10^{-5}M dexamethasone. The cells were cultured for 21 days, with medium changes every other day.

To evaluate the osteo/cementogenic potential of these hPDLSCs and classify them as HOP or LOP, matrix mineralization was quantitatively assessed using Alizarin Red Staining (AR-S; Sigma-Aldrich). After fixation with absolute ethanol, cells were incubated with 40mM AR-S for 10 minutes, washed four times with phosphate-buffered saline, and subsequently solubilized in citrate-phosphate-citric acid. Absorbance was measured at 562nm using a VersaMax microplate reader (Molecular Devices).

2.2. RNA isolation and qRT-PCR.

hPDLSCs were seeded at a density of 4×10^5 cells/well in 100cm² culture plates with Standard Medium (SDM) and incubated for 24 hours. Following this, experimental treatments were applied to the designated groups, with medium changes every other day. After 14 days of culture—chosen based on the differentiation timeline of hPDLSCs into osteo/cementoblasts or cementoblasts (Choi et al., 2021)—total RNA was extracted using TRIzol reagent (Invitrogen™), followed by phenol-chloroform extraction and isopropanol precipitation. Genomic DNA contamination was removed using Turbo DNA-free treatment (Ambion Inc.). RNA quality and concentration were assessed with the NanoDrop® 2000 spectrophotometer

(Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from 2µg of total RNA using Roche Diagnostic Co. reagents.

Primers for genes 18S, ACBT, ASPN, BMP2, GAPDH, OCN, and RUNX2 were designed using Primer3web (http://primer3.ut.ee/) and verified using UCSC (https://genome.ucsc.edu/). Primer efficiency was validated, and real-time PCR was conducted on the LightCycler 480 system with the SYBR Green I Master kit (Roche Diagnostics GmbH). Each qPCR reaction used 20ng of RNA, with water as a negative control. The optimal reference gene (18S, ACBT, or GAPDH) for normalization was identified using NormFinder software (https://moma.dk/normfinder-software). Data were expressed as log2ΔCT values.

2.3. Total protein isolation and Alkaline Phosphatase enzymatic activity.

hPDLSCs were seeded and cultured as described in Section 2.2. After 14 days, cells were trypsinized, centrifuged at 3,000rpm for 5 minutes at 4°C, and washed four times with phosphate-buffered saline (PBS), centrifuging after each wash. The final pellet was resuspended in RIPA Lysis Buffer (Thermo Fisher Scientific) according to the manufacturer's guidelines. Lysates were centrifuged at 14,000rpm for 15 minutes at 4°C, and the supernatant was collected. Protein content was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

Alkaline phosphatase (ALP) activity was quantified using the 1-Step™ PNPP Substrate Solution (Thermo Fisher Scientific). A total of 100µg of each protein sample was processed in 96-well plates, and optical density was measured at 405nm using a VersaMax microplate reader (Molecular Devices). ALP activity values were normalized to total protein content.

2.4. Analysis of BMP2 protein levels via multiplex.

BMP2 protein levels were quantified from total protein samples (100µL) using the R&D Systems. The assay was conducted in 96-well plates with sensitive panels according to the manufacturer’s instructions. Microspheres conjugated with monoclonal antibodies against BMP2 (L122889; BR67; R&D Systems) were added to the wells and incubated for 2 hours at room temperature. Wells were then thoroughly washed, followed by the addition of secondary antibodies and a further 1h incubation. Detection was carried out using Streptavidin-Phycoerythrin, and fluorescence intensity was quantified with the Luminex® multiplex reader. Values were normalized to total protein content.

2.5. Statistical and data analysis.

Data analysis was conducted using *RStudio* (version 4.3.0–R 4.4.2) with specialized packages to comprehensively explore the dataset. The primary objective was to assess osteo/cementogenic potential, investigate the role of BMP2 and ASPN in mineralization, and characterize differences between HOP and LOP cells. Initially, normality was tested using the Shapiro-Wilk test.

To evaluate osteo/cementogenic potential, absolute levels of mineralization, ALP activity, gene expression, and protein expression were analyzed using the non-parametric Kruskal-Wallis test. Fold changes (log_2FC) were calculated to assess modulation between OM and SDM conditions, expressed as the ratio OM/SDM, and compared using the Mann-Whitney test. These results were used to classify cells as HOP or LOP based on markers such as mineralization, gene expressions (RUNX2, OCN), ALP activity, and BMP2/ASPN modulation.

Subsequent analyses focused on BMP2 and ASPN to understand their regulatory roles in HOP and LOP cells. Expression data (Log2ΔCt) were visualized using heatmaps (*Pheatmap*) to illustrate differences between HOP and LOP populations. Linear Discriminant Analysis (LDA; *MASS*) was used to determine if absolute gene expression (Log2ΔCt) values could differentiate HOP from LOP. Principal Component Analysis (PCA; FactoMineR) was performed to evaluate whether variations in gene and protein modulation patterns (Log2FC) could effectively differentiate HOP from LOP cells.

Pearson correlation analysis was conducted to examine linear associations between BMP2 and ASPN gene (Log2ΔCt) protein levels (Log2pg/µg), as well as mineralization. Correlations, ranging from -1 to 1, were used to identify relationships among gene/protein variables and mineralization. Positive correlations indicate direct relationships, negative correlations suggest inverse relationships.

To explore the integrated influence of multiple variables, a multivariate model was constructed to assess relationships between gene and protein expression modulation (Log2FC) and mineralization potential, accounting for group variability. The model used was as follows:

$$Mineralization \sim BMP2_{gene} + ASPN_{gene} + BMP2_{protein} + Group_{HOP/LOP}$$

A correlation matrix (*corrplot*) was generated to evaluate interactions between BMP2, ASPN, and mineralization outcomes, visualizing the strength and direction of these relationships. Network analysis (*igraph*) was also conducted to further illustrate these interactions, highlighting key nodes and connections to identify central regulators in the

osteo/cementogenic process. Metrics such as Percentage Increase in Mean Squared Error (%IncMSE) and Increase in Node Purity (IncNodePurity) were calculated using the *randomForest* algorithm to assess the relative importance of *BMP2* and *ASPN* in mineralization. %IncMSE measures the decrease in model accuracy when a variable is permuted, while IncNodePurity reflects how much a variable contributes to reducing variance within the model. Finally, the multivariate model was visualized with pairwise scatter plots in *ggplot2*, providing a comprehensive depiction of relationships among variables and illustrating interactions between *BMP2*, *ASPN*, and mineralization.

Statistical differences were considered significant at $p<0.05$. Graphical representations include symbols denoting p-values: $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

3. Results

3.1. HOP shows greater mineralization and RUNX2 modulation compared to LOP.

The AR-S staining results for HOP (GHP and NWB) and LOP (GB and MS) cells demonstrate that, even under SDM conditions, HOP cells exhibit a significantly greater mineralization capacity compared to LOP cells ($p<0.05$). Upon osteo/cementogenic induction, HOP cells displayed a significant increase in mineralization relative to both their own SDM condition ($p<0.01$) and to LOP cells ($p<0.01$), while LOP cells did not exhibit significant changes following osteo/cementogenic induction (Figure 1A, B).

For osteo/cementogenic gene modulation, RUNX2 expression did not significantly increase in either HOP or LOP cells compared to their respective SDM conditions ($p > 0.05$). However, LOP cells exhibited higher RUNX2 transcript levels compared to HOP cells in both SDM and OM conditions ($p<0.05$) (Figure 1C). In the fold change analysis (OM/SDM), both HOP and LOP showed positive modulation in response to osteo/cementogenesis, with significantly greater modulation in HOP than in LOP (Figure 1D; $p<0.01$).

For OCN, no significant differences were observed between or within groups, nor were there distinct modulation patterns during osteo/cementogenesis (Figure 1E, F; $p > 0.05$). In terms of ALP activity, LOP cells displayed higher enzymatic activity than HOP cells under both SDM and OM conditions ($p<0.05$), and both cell types showed increased ALP activity upon osteo/cementogenic induction (Figure 1G; $p<0.01$). However, there were no significant differences in fold change patterns between HOP and LOP (Figure 1H; $p > 0.05$).

These findings confirm the greater mineralization potential of HOP cells, as evidenced by their ability to produce more mineralized matrix and greater modulation of RUNX2

expression during osteo/cementogenesis. This underlines the osteo/cementogenic advantage of HOP, suggesting that LOP cells may face intrinsic regulatory challenges limiting their differentiation into osteo/cementogenic lineages.

3.2. BMP2 and ASPN gene expression, along with BMP2 protein modulation, distinguish HOP from LOP.

Under SDM conditions, BMP2 gene expression was lower in HOP compared to LOP (Figure 2A; $p<0.05$). Upon osteo/cementogenic induction, both groups increased BMP2 mRNA levels, with LOP reaching slightly higher levels ($p<0.05$). However, FC analysis (OM/SDM) revealed greater positive modulation in HOP (Figure 2B; $p<0.001$). For ASPN, HOP cells exhibited higher transcript levels under SDM conditions (Figure 2C; $p<0.05$). Upon osteo/cementogenic induction, HOP downregulated ASPN while LOP upregulated it, resulting in similar ASPN levels between the groups in OM ($p > 0.05$). FC analysis confirmed this, with HOP showing negative modulation and LOP showing positive modulation of ASPN (Figure 2D; $p<0.05$).

BMP2 protein levels were significantly higher in HOP compared to LOP under OM condition (Figure 2E; $p<0.01$). LOP cells did not exhibit significant changes, whereas HOP displayed a notable increase, indicating positive modulation ($p<0.001$). LOP remained largely unchanged (fold change ≈ 0).

The heatmap (Figure 2G) demonstrated that while overall BMP2 and ASPN levels were similar between HOP and LOP, the modulation patterns during osteo/cementogenesis differed. HOP exhibited increased BMP2 modulation and reduced ASPN expression, whereas LOP showed less pronounced BMP2 modulation and increased ASPN, indicating distinct regulatory mechanisms. LDA revealed a clear separation between HOP and LOP along the LD1 axis, which represents the variable that maximizes the difference between the two groups based on Log2ΔCT values for *ASPN* and *BMP2*. The density plot along LD1 shows distinct distributions for HOP (orange area) and LOP (blue area), reflecting consistent differences between the groups, though with some overlap. This overlap indicates intrinsic variability among the populations. (Figure 2H). PCA, based on Log2FC, effectively distinguished HOP from LOP, with *BMP2* (gene and protein) and *ASPN* contributing significantly to the separation (Figure 2I). The color gradient highlights the relative importance of each variable, with *BMP2* protein emerging as the most influential factor along Dim1 (38.6%), underscoring its critical role in osteo/cementogenic potential. In contrast, *ASPN* gene expression contributed predominantly to Dim2 (35.6%), reflecting its inhibitory effect. The size of the points represents the modulation

of individual hPDLSC populations, illustrating variability within HOP (blue) and LOP (orange).

Overall, these results indicate that HOP's superior osteo/cementogenic potential is driven by effective modulation of BMP2 and ASPN at both gene and protein levels, enhancing HOP's ability to respond to osteo/cementogenic stimuli, whereas LOP is limited by elevated ASPN expression, reducing its differentiation potential.

3.3. ASPN negatively regulates osteo/cementogenesis while BMP2 protein promotes mineralization of hPDLSCs.

Linear correlations were analyzed using absolute values (Figure 3A-C) to directly assess expression or protein levels without normalization, providing a clearer view of intrinsic relationships. A negative correlation was observed between *ASPN* transcript levels and *BMP2* protein levels ($r=-0.5652$, $p=0.004$), indicating that high *ASPN* expression negatively affects *BMP2* protein (Figure 3C). For mineralization (Figure 3D-F), *ASPN* mRNA showed a strong negative correlation ($r=-0.7186$, $p<0.001$), while *BMP2* protein levels positively correlated with mineralization ($r=0.4215$, $p=0.041$), linking higher *BMP2* to improved mineralization (Figure 3F).

These findings indicate that ASPN acts as a negative regulator of osteo/cementogenesis, reducing BMP2 protein expression and impairing mineralization, while BMP2 protein positively influences the mineralization process. The opposing roles of ASPN and BMP2 highlight their significance in regulating osteo/cementogenesis.

3.4. Integrated model highlights the influence of ASPN and BMP2 on the osteo/cementogenic potential of hPDLSCs.

To assess the relationship and impact of BMP2 and ASPN gene expression, as well as BMP2 protein levels, on mineralization, a comprehensive model was developed using FC data to reflect the relative modulation of these variables in response to osteo/cementogenic stimulation. The correlation matrix revealed significant negative correlations between ASPN gene expression and the other variables (BMP2 gene, BMP2 protein, and mineralization). The magnitude of these correlations was visually represented by dots, with larger sizes indicating stronger correlations, such as those between BMP2 protein and ASPN, and BMP2 protein and mineralization (Figure 4A). The interaction network further confirmed these findings, showing a strong positive correlation between BMP2 protein and mineralization, while ASPN was negatively correlated with all other variables (Figure 4B).

The %IncMSE metric identified ASPN expression as the most important variable distinguishing HOP from LOP, followed by BMP2 (both gene and protein), which reinforces ASPN's role as a critical negative regulator of osteo/cementogenesis in LOP (Figure 4C). Similarly, the IncNodePurity analysis highlighted ASPN as the key factor for group separation, followed by BMP2 protein, suggesting that ASPN is fundamental in defining the LOP phenotype, while BMP2 supports osteo/cementogenic differentiation (Figure 4D).

The detailed analysis of the model included correlations among ASPN gene expression, BMP2 gene and protein expression, and mineralization (Figure 4E). Overall, ASPN and BMP2 expression showed a negative correlation ($r=-0.351$). In HOP, this correlation was significant ($r=-0.640$, $p<0.05$), indicating that high ASPN levels are linked to lower BMP2 expression. Conversely, in LOP, the correlation was positive but not significant ($r=0.364$), highlighting different regulatory dynamics between these groups. ASPN gene expression also showed a significant negative correlation with BMP2 protein levels, particularly strong in HOP ($r=-0.894$, $p<0.001$), whereas in LOP, it was not significant ($r=-0.055$). BMP2 gene expression was positively correlated with its protein levels, significant only in HOP ($r=0.724$, $p<0.01$), suggesting effective translation of BMP2 in HOP. Mineralization also had a significant negative correlation with ASPN expression in HOP ($r=-0.671$, $p<0.05$). BMP2 protein expression showed a strong positive correlation with mineralization, especially in HOP ($r=0.717$, $p<0.01$).

These findings from the integrated model highlight the importance of jointly analyzing all variables to clearly demonstrate ASPN's negative regulatory role in LOP and BMP2's positive role in promoting osteo/cementogenesis, particularly in HOP.

4. Discussion

Periodontal regeneration remains a significant challenge in clinical practice, particularly in reconstructing dental supporting tissues such as bone and cementum, which are critical for providing stable anchorage to periodontal fibers and maintaining tooth stability (Stavropoulos & Sculean, 2017, Citterio et al., 2020). Achieving predictable regenerative outcomes requires a comprehensive understanding of the underlying cellular mechanisms that govern these processes, ultimately aiming to identify therapeutic targets that optimize periodontal regeneration (Galli et al., 2021).

In this context, detailed data science analyses have proven pivotal in identifying such targets, especially through predictive statistical models that allow better differentiation of cell populations and identification of key factors influencing regenerative capacity (Cahan et al., 2021; Del Sol & Jung, 2021). This study employed an integrated approach, utilizing both

univariate and multivariate analyses, to investigate the osteo/cementogenic potential of HOP and LOP cells derived from the periodontal ligament, focusing particularly on the modulation of BMP2 and ASPN genes and their protein products.

Our findings revealed significant differences in the osteo/cementogenic potential between HOP and LOP cells. HOP cells demonstrated a markedly greater intrinsic mineralization capacity than LOP cells, even before osteo/cementogenic induction. After 21 days of induction, HOP cells exhibited a significant increase in mineralization, whereas LOP cells showed no notable changes, indicating the presence of regulatory barriers that prevent their differentiation into osteo/cementogenic lineages. RUNX2 expression was more prominently modulated in HOP cells, reinforcing its role as a key transcription factor for mineralization (Liu & Lee, 2013; Vimalraj et al., 2015). However, LOP cells, despite initially higher levels of RUNX2, failed to significantly modulate this expression upon induction.

BMP2 has emerged as a critical factor for the osteo/cementogenic potential of these cells, aligning with previous findings (Zhang et al., 2017; Lee et al., 2018), which demonstrated that BMP2 is essential for osteo/cementogenesis in periodontal ligament cells, and that inhibiting BMP2 pathways results in diminished mineralization. In our data, BMP2 protein levels and their modulation were key differentiators between HOP and LOP populations. HOP cells exhibited an effective positive modulation of BMP2 protein levels, which correlated with their superior mineralization capacity, suggesting efficient post-transcriptional and translational regulatory mechanisms. On the other hand, LOP cells, despite increased BMP2 transcripts, did not translate these effectively into BMP2 protein. This finding suggests that HOP cells might possess superior mechanisms that facilitate efficient protein translation and stabilization, thereby enhancing BMP2 availability and mineralization capacity (Saito et al., 2020; Sacramento et al., 2024). In contrast, LOP cells face translational inefficiencies or post-translational issues that limit BMP2 protein levels, constraining their osteo/cementogenic potential. These might include reduced translation efficiency, improper folding, accelerated degradation, or impaired modifications essential for the pathway activation (Tintut et al., 1999; Jonason et al., 2009). Intracellular retention or competitive binding further reduces its availability (Martinez-Hackert, Sundan & Holien, 2021), reflecting a regulated environment that limits osteogenic signaling in LOP cells.

On the other hand, ASPN emerged as a pivotal factor in regulating osteo/cementogenic differentiation. Known for its inhibitory role (Yamada et al., 2006), ASPN was significantly downregulated in HOP cells during osteo/cementogenic induction, while LOP cells exhibited

increased ASPN expression. This contrasting regulation implies that reduced ASPN levels in HOP cells relieve its inhibitory influence, enabling effective BMP2 signaling and promoting mineralization. In contrast, elevated ASPN levels in LOP cells appear to act as a significant barrier, inhibiting BMP2 activity and limiting differentiation capacity. Previous studies have shown that elevated ASPN mRNA levels in hPDLSCs can interfere with BMP2 gene expression and protein synthesis (Sacramento et al., 2022; Pereira et al, 2024). ASPN protein is also believed to interact intracellularly with BMP2 (Yamada et al., 2007) and competitively prevent BMP2 from binding to the BMP receptor-IB (BMPR-IB), thereby inhibiting BMP-dependent Smad protein activation and reducing osteo/cementogenic efficiency (Tomoeda et al., 2008). Additionally, in certain LOP cells, increases in BMP2 transcript levels are accompanied by concurrent ASPN upregulation, suggesting a negative feedback mechanism that restricts differentiation synthesis (Sacramento et al., 2022; Pereira et al, 2024). In this study, the observed negative correlation between ASPN and BMP2 protein levels indicates that elevated ASPN mRNA may destabilize BMP2 or impair its modulation, ultimately limiting mineralization capacity

Indeed, studies targeting the modulation of the ASPN/BMP2 pathway through ASPN suppression have yielded promising results. Research involving vitamin D_3 (1,25(OH)2D3) demonstrated that PDLSCs treated with vitamin D_3 exhibited a significant reduction in ASPN expression, which was accompanied by an increase in BMP2 gene and protein levels in LOP cells, thereby enhancing their differentiation capacity (Pereira et al., 2024). Furthermore, 1,25(OH)2D3 was shown to reverse LPS-induced inhibition of osteoblastic differentiation in hPDLSCs by suppressing PLAP-1 expression (Zhang et al., 2020). Notably, a Vitamin D Response Element (VDRE) was identified within the PLAP-1 promoter region, demonstrating its ability to bind to the Vitamin D Receptor (Zhang et al., 2020). These findings highlight the potential of vitamin D_3, as well as other agents that may modulate ASPN expression at both gene and protein levels, to serve as effective modulators for promoting osteo/cementogenic differentiation in PDLSCs.

Building on these findings, the integrated multivariate analysis in our study provided a comprehensive perspective on the roles of BMP2 and ASPN in regulating osteo/cementogenic differentiation. The analysis emphasized the dynamic interplay between BMP2 and ASPN, with ASPN consistently acting as a strong inhibitor while BMP2 functioned as a key promoter of osteo/cementogenic potential. This underscores the importance of maintaining a balanced regulation between BMP2 and ASPN to optimize the regenerative capacity of PDLSCs (Yamada et al., 2007; Sacramento et al., 2022; Pereira et al., 2024). Focusing on achieving this

balance could lead to future regenerative strategies that enhance BMP2 activity while mitigating ASPN's inhibitory effects, ultimately improving the effectiveness of periodontal therapies.

Finally, this study has some limitations. The research was conducted with four cell populations derived from different donors. While these findings provide valuable insights, future studies should expand the sample size to validate and refine the statistical model, thereby enhancing the robustness and representativeness of the data. Moreover, although our focus was on BMP2 and ASPN gene and protein expression, further investigation into their direct protein interactions is necessary to fully elucidate the mechanism by which ASPN inhibits BMP2 activity. This could involve studying competitive binding interactions, where ASPN might directly bind to BMP2 or its receptors, blocking downstream signaling. Additionally, exploring the role of post-transcriptional and translational processes that impact these pathways could help in identifying more specific and effective therapeutic targets for periodontal regeneration. Future studies should also investigate other pharmacological agents and biomaterials capable of targeting ASPN expression or activity, potentially offering new avenues for enhancing osteo/cementogenic differentiation and promoting effective periodontal regeneration.

5. Conclusion

Our findings demonstrate that the superior osteo/cementogenic potential of HOP cells is driven by efficient BMP2 regulation and reduced ASPN gene expression, whereas LOP cells face inhibitory barriers due to high ASPN levels, which negatively affect BMP2 protein production and mineralization. Targeting ASPN may provide a promising therapeutic strategy to enhance BMP2 activity and improve osteo/cementogenic differentiation of hPDLSCs. Modulating these molecular targets could ultimately enhance the predictability and success of periodontal regeneration therapies.

6. Declaration of competing interest

The authors declare no conflict of interest.

7. Acknowledgments

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9. Tables

Table 1: qRT-PCR primers' sequences.

Gene	Primer (5-3 sequences)	Anneling temperature	Product Size
18S	F: CGGACAGGATTGACAGATTGATAGC R: TGCCAGAGTCTCGTTCGTTATCG	51 °C	171 bp
ACBT	F: CCAACCGCGAGAAGATGA R: CCAGAGGCGTACAGGGATAG	61 °C	538 bp
ASPN	F: CCAACCGCGAGAAGATGA R: CCAAGCAAGGTCTTCCAAAG	60 °C	223 bp
BMP-2	F: CTTCCACCCCTCTTTCTTCC R: GTCTCCCGGAAACACTTGAA	58 °C	221 bp
GAPDH	F: CCAACCGCGAGAAGATGA R: CCAGAGGCGTACAGGGATAG	51 °C	171 bp
OCN	F: AGCTCAATCCGGACTGT R: GGAAGAGGAAAGAAGGGTGC	55 °C	150 bp
RUNX2	F: CCGTCCATCCACTCTACCAC R: ATGAAATGCTTGGGAACTGC	55 °C	139 bp

18S: 18S Ribosomal RNA; ACTB: Beta-actin; ASPN: Asporin; BMP2: Bone Morphogenetic Protein 2; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; OCN: Osteocalcin; RUNX2: Runt-Related Transcription Factor 2.

10. Figures and Legends

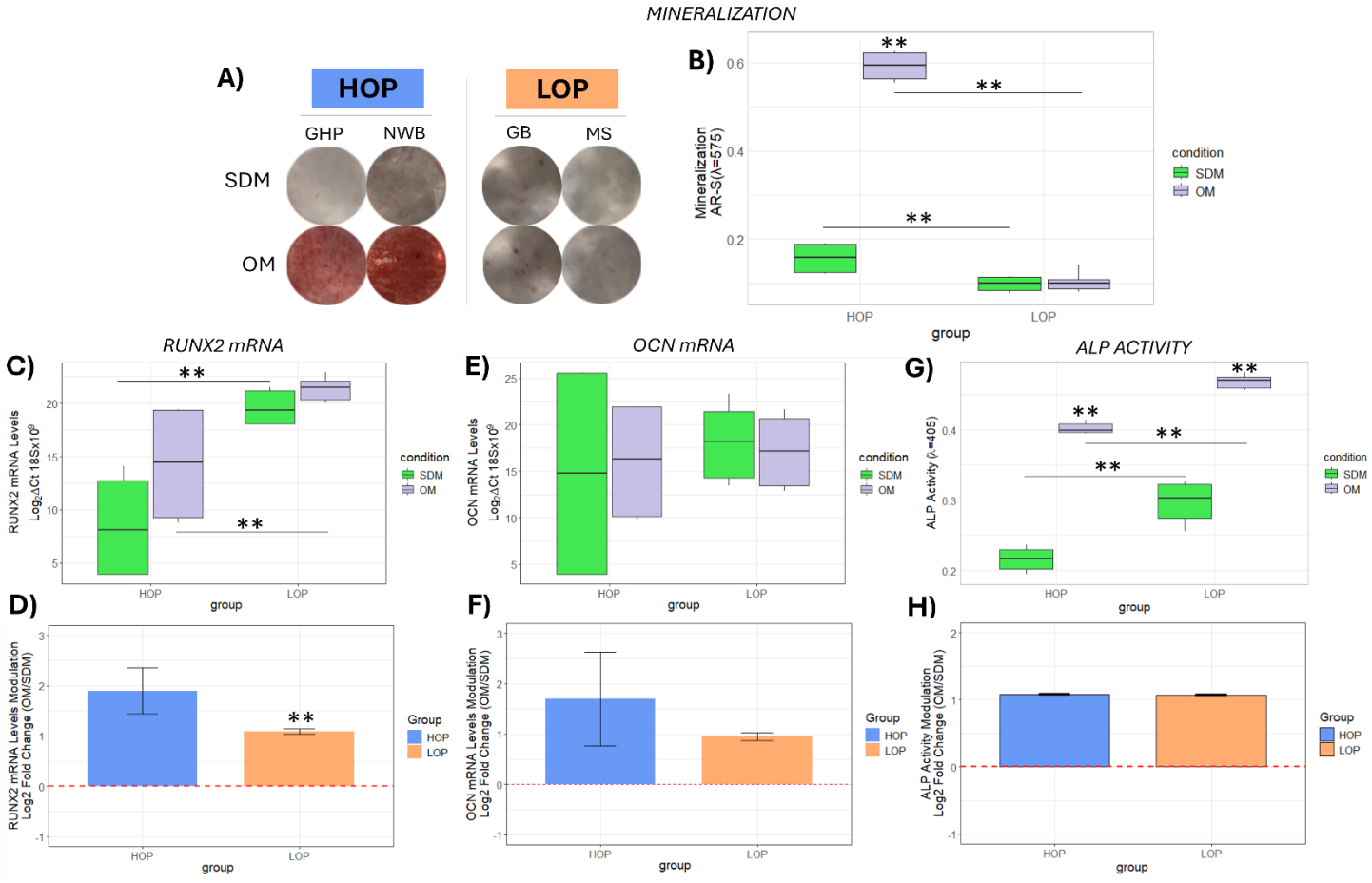

Figure 1. Mineralization capacity analysis of high and low osteo/cementoblast potential hPDLSCs. **(A)** Representative images of Alizarin Red S (AR-S) staining demonstrate that high osteo/cementogenic potential (HOP) cells cultured in osteogenic medium (OM) for 21 days exhibit markedly more intense staining than low osteo/cementogenic potential (LOP) cells, indicating superior mineralization capacity. **(B)** Quantitative analysis of AR-S staining confirms significantly higher mineralization levels in HOP cells under OM compared to LOP (**p<0.01). **(C, D)** Gene expression analysis reveals significantly greater positive modulation of *RUNX2* in HOP cells after 14 days of osteogenic induction (**p<0.01), whereas **(E, F)** *OCN* expression levels did not show significant differences between HOP and LOP under any condition. **(G, H)** Enzymatic activity analysis of alkaline phosphatase (ALP) showed no significant modulation in either group following 14 days of induction.

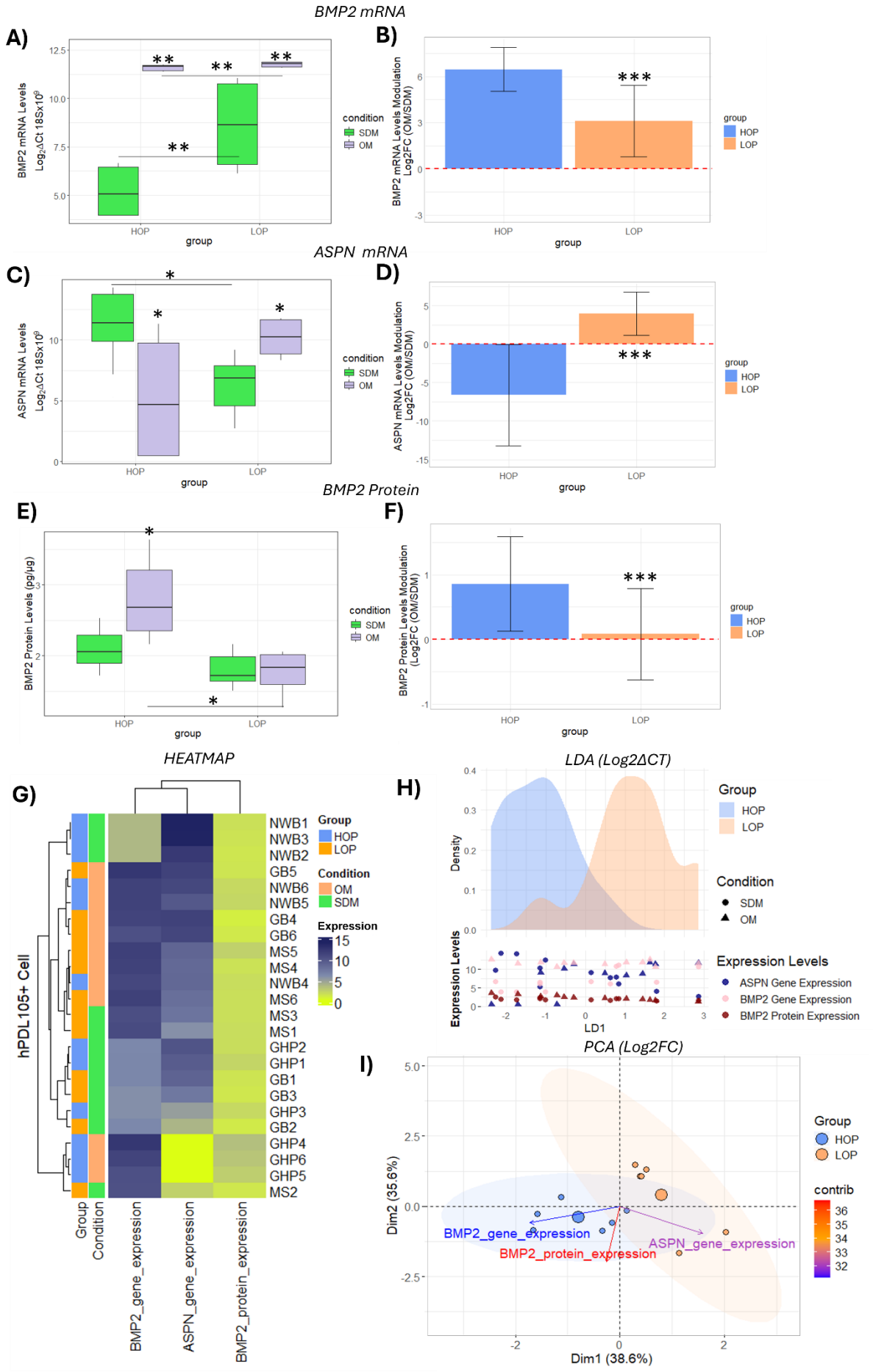
BMP2 mRNA
A)
B)
BMP2 mRNA Levels
Log2ΔCt 18Sx10^6
BMP2 mRNA Levels Modulation
Log2FC (OM/SDM)
condition
SDM
OM
group
HOP
LOP
ASPN mRNA
C)
D)
ASPN mRNA Levels
Log2ΔCt 18Sx10^6
ASPN mRNA Levels Modulation
Log2FC (OM/SDM)
BMP2 Protein
E)
F)
BMP2 Protein Levels (pg/µg)
BMP2 Protein Levels Modulation
(Log2FC (OM/SDM)
HEATMAP
G)
hPDL105+ Cell
Group
Condition
Expression
BMP2_gene_expression
ASPN_gene_expression
BMP2_protein_expression
LDA (Log2ΔCT)
H)
Density
Expression Levels
LD1
ASPN Gene Expression
BMP2 Gene Expression
BMP2 Protein Expression
PCA (Log2FC)
I)
Dim2 (35.6%)
Dim1 (38.6%)
contrib

Figure 2. Expression and modulation of BMP2 and ASPN in hPDLSCs. **(A, B)** BMP2 gene expression analysis reveals significantly higher positive modulation (OM/SDM) in HOP cells compared to LOP (***p<0.001). **(C, D)** ASPN gene expression shows significant downregulation in HOP under OM, while LOP exhibits positive regulation following osteogenic induction (***p<0.001). **(E, F)** BMP2 protein expression demonstrates significantly elevated levels in HOP under OM (*p<0.05), with no corresponding increase observed in LOP. **(G)** Heatmap highlights the distinct expression profiles of BMP2 and ASPN across conditions, groups, and cell populations. (**H)** LDA separates HOP and LOP groups based on BMP2 and ASPN expression (Log2ΔCt). **(I)** PCA identifies BMP2 protein expression as a key contributor to the differential osteogenic potential of HOP and LOP cells.

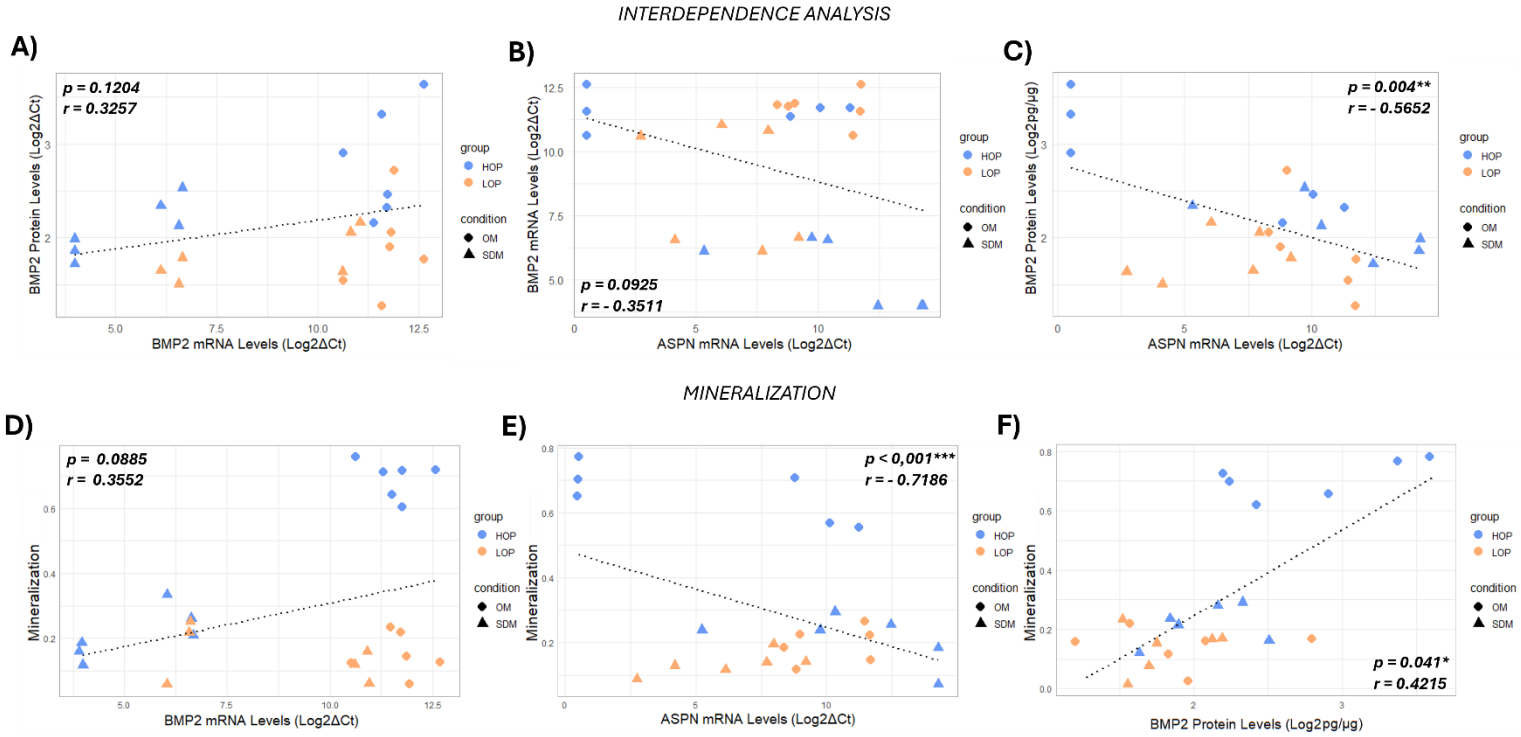

Figure 3. Linear correlation analysis between ASPN, BMP2, and mineralization in hPDLSCs. **(A)** Linear correlation between *BMP2* mRNA and BMP2 protein levels shows a positive, but non-significant association (p=0.1204; r=0.3257). **(B)** *ASPN* mRNA levels exhibit a weak negative linear correlation with *BMP2* mRNA levels, though not statistically significant (p=0.0925; r=-0.3511). **(C)** A significant negative linear correlation is observed between *ASPN* mRNA levels and BMP2 protein levels (**p=0.004; r=-0.5652). **(D)** Linear correlation between *BMP2* mRNA levels and mineralization indicates a positive trend, though not statistically significant (p=0.0885; r=0.3552). **(E)** A significant negative linear correlation is observed between *ASPN* mRNA levels and mineralization (***p<0.001; r=-0.7186). **(F)** BMP2 protein levels show a significant positive linear correlation with mineralization (*$p=0.041$; $r=0.4215$).

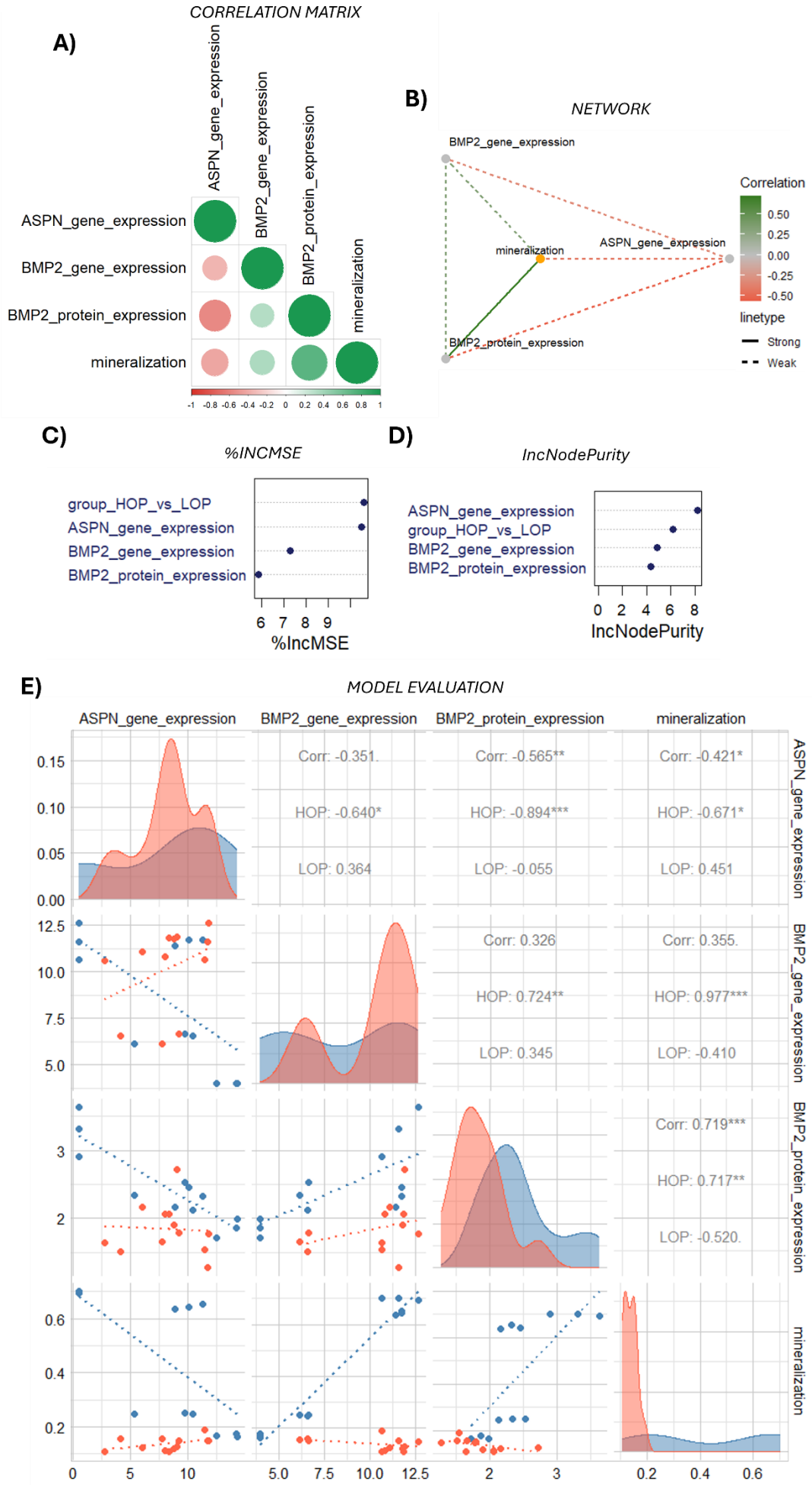
CORRELATION MATRIX
A)
ASPN_gene_expression
BMP2_gene_expression
BMP2_protein_expression
mineralization
-1 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1
B)
NETWORK
BMP2_gene_expression
mineralization
ASPN_gene_expression
BMP2_protein_expression
Correlation
0.50
0.25
0.00
-0.25
-0.50
linetype
Strong
Weak
C)
%INCMSE
group_HOP_vs_LOP
ASPN_gene_expression
BMP2_gene_expression
BMP2_protein_expression
6 7 8 9
%IncMSE
D)
IncNodePurity
ASPN_gene_expression
group_HOP_vs_LOP
BMP2_gene_expression
BMP2_protein_expression
0 2 4 6 8
IncNodePurity
E)
MODEL EVALUATION
ASPN_gene_expression
BMP2_gene_expression
BMP2_protein_expression
mineralization
Corr: -0.351.
HOP: -0.640*
LOP: 0.364
Corr: -0.565**
HOP: -0.894***
LOP: -0.055
Corr: -0.421*
HOP: -0.671*
LOP: 0.451
Corr: 0.326
HOP: 0.724**
LOP: 0.345
Corr: 0.355.
HOP: 0.977***
LOP: -0.410
Corr: 0.719***
HOP: 0.717**
LOP: -0.520.
0.15
0.10
0.05
0.00
12.5
10.0
7.5
5.0
3
2
0.6
0.4
0.2
0 5 10
5.0 7.5 10.0 12.5
2 3
0.2 0.4 0.6

Figure 4. Correlation matrix, network, and model evaluation of ASPN, BMP2, and mineralization in hPDLSCs. **(A)** Correlation matrix illustrating relationships between *ASPN* gene expression, *BMP2* gene expression, *BMP2* protein levels, and mineralization. Negative correlations are observed between *ASPN* and BMP2 or mineralization, while *BMP2* protein levels positively correlate with mineralization. **(B)** Network plot visualizing the strength and direction of correlations. Solid lines represent strong correlations, while dashed lines indicate weaker associations. %IncMSE **(C)** and IncNodePurity **(D)** feature importance plots highlight *ASPN* gene expression as the most significant predictor of group differentiation (HOP vs. LOP), followed by *BMP2* protein expression. **(E)** Pairwise scatter plots and density distributions evaluating the relationships between variables across groups. Significant negative correlations are observed between *ASPN* gene expression and mineralization (*$p<0.05$), while *BMP2* protein expression shows a strong positive correlation with mineralization (***$p < 0.001$).

3 CONCLUSÃO

O estudo destaca a importância da regulação de BMP2 e ASPN na diferenciação osteogênica de células do ligamento periodontal, fornecendo insights valiosos para a regeneração periodontal. Observou-se que a redução da expressão de ASPN em células HOP favorece um ambiente propício à mineralização, enquanto sua elevação em células LOP atua como uma barreira, limitando o potencial regenerativo. Esses achados reforçam a relevância clínica de estratégias que visem a modulação de ASPN como alvo terapêutico.

Uma das principais contribuições do estudo foi a aplicação de modelos de análise multivariada, que possibilitaram a exploração integrada das interações entre BMP2, ASPN e a mineralização. Métodos como Análise Discriminante Linear (LDA) e Análise de Componentes Principais (PCA) mostraram-se eficazes para diferenciar células HOP e LOP, evidenciando padrões regulatórios distintos. Adicionalmente, essas análises permitiram identificar correlações fundamentais, como a relação negativa entre ASPN e BMP2, bem como a associação positiva entre BMP2 e a mineralização, proporcionando uma compreensão aprofundada dos fatores que influenciam o potencial osteogênico.

Do ponto de vista clínico, a redução da expressão de *ASPN* em células LOP, visando potencializar a sinalização de *BMP2* por meio de fármacos ou outras moléculas moduladoras, destaca-se como uma estratégia promissora para aumentar a eficácia e previsibilidade das terapias regenerativas. Apoiada por análises multivariadas robustas, essa abordagem pode ter o potencial de expandir significativamente as aplicações da regeneração periodontal, especialmente em casos de defeitos extensos e complexos, historicamente associados a taxas de sucesso limitadas. Assim, a integração de moduladores moleculares inovadores pode representar um avanço substancial na prática clínica, proporcionando tratamentos mais eficazes e direcionados.

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ANEXOS

Anexo 1 - Certificado do Comitê De Ética

UNICAMP - FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNIVERSIDADE DE CAMPINAS - FOP/UNICAMP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação de vias de sinalização alternativas para potencialização osteogênica de células mesenquimais indiferenciadas do ligamento periodontal

Pesquisador: CATHARINA MARQUES SACRAMENTO

Área Temática:

Versão: 2

CAAE: 63957722.0.0000.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.756.147

Apresentação do Projeto:

O parecer inicial é elaborado com base na transcrição editada do conteúdo do registro do protocolo na Plataforma Brasil e dos arquivos anexados à Plataforma Brasil. Os pareceres de retorno, emendas e notificações são elaborados a partir do último parecer e dos dados e arquivos da última versão apresentada. A EQUIPE DE PESQUISA citada na capa do projeto de pesquisa inclui CATHARINA MARQUES SACRAMENTO (Cirurgiã-dentista, Doutoranda no PPG em Clínica Odontológica da FOP-UNICAMP, Pesquisadora responsável), KARINA GONZALES SILVÉRIO RUIZ (Cirurgiã-dentista, Docente da área de Periodontia da FOP-UNICAMP), BRUNO CAZOTTI PEREIRA (Cirurgião-dentista, Mestrando no PPG em Clínica Odontológica da FOP-UNICAMP), GABRIELA BESSA MARCONDES NONATO (Cirurgiã-dentista, Mestrando no PPG em Clínica Odontológica da FOP-UNICAMP), o que é confirmado na declaração dos pesquisadores e na PB.

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Situação do Parecer:
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Necessita Apreciação da CONEP:
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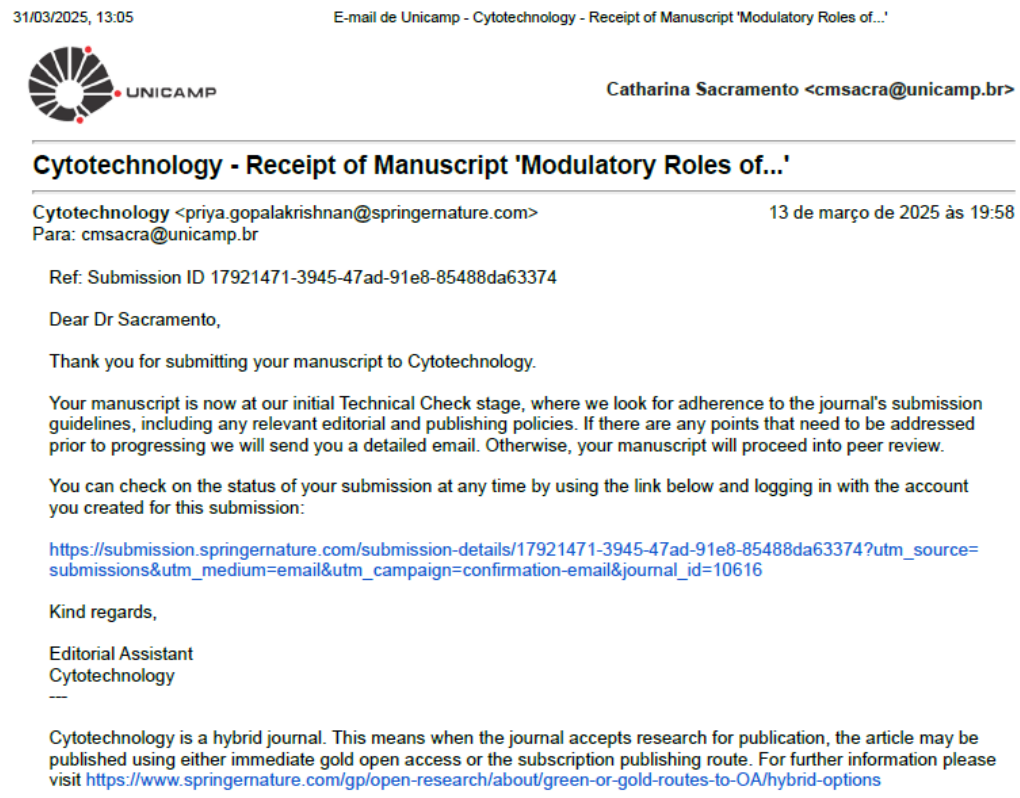
Assinado por:
jacks jorge junior
(Coordenador(a))

Anexo 2 - Comprovante de submissão de artigo científico



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Anexo 3 - Relatório de Similaridade

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