The influence of extracellular matrix on human periodontal ligament stem cell properties

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อิทธิพลของโปรตีนเมทริกซ์นอกเซลล์ ที่มีต่อคุณสมบัติของเซลล์เอ็นยึดปริทันต์ในมนุษย์

น.ส.ดณีญา ไชยเขียวแก้ว

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เนื้อเยื่อเอ็นยึดปริทันต์เป็นส่วนประกอบสำคัญของฟัน ทำหน้าที่ยึดรากฟันให้ติดกับกระดูก และรองรับแรงบดเคี้ยว โปรตีนเมทริกซ์นอกเซลล์รอบๆเนื้อเยื่อเอ็นยึดปริทันต์เองก็มีส่วนสำคัญในการช่วยคงคุณสมบัติต่างๆ ของเซลล์ในเนื้อเยื่อเอ็นยึดปริทันต์รวมถึงการตอบสนองต่อแรงเชิงกลที่กระทำต่อเซลล์อีกด้วย โดยกระบวนการส่งผ่านแรงเชิงกลนั้นจะเริ่มต้นได้จากการที่เซลล์แต่ละเซลล์ทำการเปลี่ยนสัญญาณทางกายภาพ ให้เป็นสัญญาณทางชีวเคมี ซึ่งช่วยให้เซลล์สามารถรับรู้และสื่อสารระหว่างเซลล์เพื่อตอบสนองต่อแรงกระตุ้นภายนอกที่มากระทำต่อเซลล์ ดังนั้น โปรตีนเมทริกซ์นอกเซลล์จึงทำหน้าที่คล้ายกับเป็นตัวกลางในการส่งผ่านสัญญาณเชิงกลเพื่อเปลี่ยนเป็นสัญญาณทางชีวเคมีภายในเซลล์ โปรตีนเมทริกซ์อันได้แก่ เพอริออส-ทินและ ออสทีโอพอนทินนั้นเป็นส่วนประกอบหลักในโปรตีนเมทริกซ์นอกเซลล์ของเนื้อเยื่อเอ็นยึดปริทันต์ โดยทำหน้าที่เกี่ยวข้องกับการรักษาคุณสมบัติต่างๆของเนื้อเยื่อเอ็นยึดปริทันต์และกระดูก โดยเฉพาะภายใต้อิทธิพลของแรงเชิงกล เนื่องจากโปรตีนเพอริออสทินแต่เดิมนั้น ก็พบเจอได้มากในเนื้อเยื่อบริเวณที่ได้รับแรงอยู่เป็นประจำ เช่นฟัน และกระดูก ในขณะที่ออสทีโอพอนทินนั้น มีหน้าที่หลักเกี่ยวข้องกับการเปลี่ยนไปทำหน้าที่เป็นเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดในกระดูกและฟัน

โดยในวิทยานิพนธ์ฉบับนี้นั้น จะมุ่งเน้นศึกษาบทบาทของโปรตีนเมทริกซ์นอกเซลล์อันเกี่ยวข้องกับกระบวนการรักษาเสถียรภาพของเนื้อเยื่อเอ็นยึดปริทันต์ ประการแรกคือการศึกษาความสำคัญของโปรตีนออสทีโอพอนทิน ในกระบวนการเปลี่ยนไปทำหน้าที่เป็นเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดเอ็นยึดปริทันต์ ประการที่สอง คือการศึกษาบทบาทของโปรตีนเพอริออสทิน ในการทำหน้าที่เป็นตัวกลางในกระบวนการส่งผ่านแรงเชิงกลในเซลล์ต้นกำเนิดเอ็นยึดปริทันต์ในมนุษย์ และประการสุดท้าย คือการทำความเข้าใจเกี่ยวกับการเปลี่ยนแปลงของส่วนประกอบในโปรตีนเมทริกซ์นอกเซลล์ของเนื้อเยื่อเอ็นยึดปริทันต์ในภาวะพร่องแรงบดเคี้ยวในหนู

ความรู้ความเข้าใจในกระบวนการส่งผ่านแรงเชิงกลจากโปรตีนเมทริกซ์นอกเซลล์ เข้าสู่ภายในเซลล์นั้นจะช่วยส่งเสริมการคิดค้นกรมมวิธีใหม่ๆ ในการหลีกเลี่ยงการสูญเสียฟันอย่างไม่พึงประสงค์ได้ อย่างไรก็ตามกระบวนการส่งผ่านแรงเชิงกลเข้าสู่ภายในเซลล์ รวมถึงบทบาทของโปรตีนเมทริกซ์นอกเซลล์ในการสร้างและซ่อมแซมกระดูกยังต้องการการศึกษาเพิ่มเติม เพื่อนำมาซึ่งองค์ความรู้ที่เป็นประโยชน์มากขึ้นต่อไป

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Daneeya Chaikiawkeaw : The influence of extracellular matrix on human periodontal ligament stem cell properties. Advisor: Prof. Thanaphum Osathanoon, D.D.S., Ph.D Co-advisor: Nuttha Klincumhom, D.V.M., Ph.D.

The periodontal ligament (PDL) is a crucial tissue connecting teeth to surrounding alveolar bone, withstanding the mechanical forces of mastication, speech, and deglutition. The extracellular matrix (ECM) of the PDL plays a critical role in maintaining PDL cell stability and function in response to external forces. Mechanotransduction, the process by which cells convert mechanical signals into biochemical signals, is essential for PDL cells to sense and respond to mechanical stimuli. The ECM is a critical mediator of mechanotransduction, as it is responsible for transmitting mechanical forces to cells and regulating cellular responses to mechanical stress. Matricellular proteins, such as periostin (PN) and osteopontin (OPN), are two important components of the PDL ECM that play significant roles in regulating PDL function and bone remodeling under mechanical stress. PN is primarily located in areas exposed to mechanical loading, such as teeth and bone, and functions as a matrix-cell attachment protein that regulates cell adhesion and motility. OPN is involved in cell adhesion, migration, and biomineralization in the PDL and bone, and can regulate cellular responses to external stimulation through multiple pathways.

This dissertation focuses on the role of matricellular proteins in outside-in signaling from the surrounding ECM, specifically in maintaining the homeostasis of PDL tissue. Firstly, we investigated the importance of the OPN protein on the osteogenic capacity of human periodontal ligament stem cells (hPDLSCs). Additionally, we demonstrated the role of PN as a mediator of hPDLSCs mechanotransduction. Finally, we extended our understanding of the profile of PDL-ECM components under the influence of mechanical force using a rat occlusal hypofunction model.

Understanding the role of the ECM in PDL mechanotransduction is critical for developing new insights into the prevention and treatment of tooth loss. Further studies are needed to fully elucidate the mechanisms underlying ECM mechanotransduction and the roles of ECM protein in regulating osteogenic differentiation, bone formation, and remodeling.

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CHAPTER I

1. Introduction

1.1 Rationale

Cells reside within a non-cellular three-dimensional protein network, and this network secretes and shapes itself into the intercellular space known as the extracellular matrix (ECM). The ECM contains various components, such as collagens, fibronectin, elastin, laminins, proteoglycans/glycosaminoglycans, and other glycoproteins, which bind to one another and to cell surface receptors (Theocharis, Skandalis et al. 2016, Walma and Yamada 2020). These receptors receive signals from the ECM and transmit them through intracellular signaling pathways to regulate diverse cellular functions, including survival, growth, migration, differentiation, and the maintenance of normal homeostasis (Frantz, Stewart et al. 2010).

In general, ECM aligns cells and provides an environmental niche to maintain their stability and functionality (Spradling, Drummond-Barbosa et al. 2001). However, the study of ECM biology includes an important idea about how the mechanical characteristics of the ECM can impact cellular activity. In fact, the stiffness of the ECM is a crucial property that allows cells to detect external forces and react to their surroundings appropriately. This mechanism is known as mechanotransduction (DuFort, Paszek et al. 2011). The ECM mechanotransduction is composed of matrix protein components that serve as a mechanosensor in response to external forces. Those matrix proteins included collagen, glycosaminoglycans (GAGs), laminin, tenascin (Halper and Kjaer 2014), and other small molecules such as transforming growth factor-b(TGF-b), periostin (PN), and osteopontin (OPN) (Tomasek, Gabbiani et al. 2002). However, the role of each ECM protein in cellular activity is still unrevealed.

Apart from the matrix components, biochemical signaling is accomplished by the contribution of both transmitter and receiver. The periodontal ligament (PDL) is a distinctive type of soft connective tissue situated between the tooth root and alveolar bone. One of its functions is to serve as a source of stem cells that can help maintain a balance between the hard and soft tissues of the periodontium. Various types of cells have been identified within the PDL tissue, such as endothelial cells, epithelial rests of Malassez, osteoblasts, and cementoblasts (Ivanovski, Gronthos et al. 2006, Keinan and Cohen 2013). Nonetheless, the prevailing cell type in the periodontal ligament tissue is fibroblast-like cells, commonly known as periodontal ligament cells (PDLSCs). In addition to their crucial roles in the periodontal ligament, PDLSCs are also capable of enduring the mechanical stress caused by chewing and sustaining the equilibrium of hard tissue surrounding them (Tomokiyo, Wada et al. 2019). As with all other cells, PDLSCs inhabit a microenvironment that governs their actions. The primary constituent of the PDL extracellular matrix (PDL-ECM) is collagen fibers, which interact with matricellular proteins like PN and OPN (Kudo 2011, Wang, Papagerakis et al. 2018). These are the most extensively researched PDL-ECM proteins in this area of study.

Furthermore, the type and intensity of external mechanical forces appear to be crucial in maintaining the strength of the ECM and the stability of PDL. Mechanical force plays a significant role in regulating tissue homeostasis by maintaining the equilibrium between tissue formation and degradation (Vining and Mooney 2017), classified into physiological and pathological influence. In general, physiological forces generate signals that contribute to the maintenance of tissue integrity and homeostasis, such as those generated during mastication, occlusion, or speech (Lemarie, Tharaux et al. 2010, Anwar, Shalhoub et al. 2012). On the other hand, pathological forces generate destructive signals. However, in certain situations, such as occlusal hyperfunction habits, excessive or repeated occlusal forces are exerted on the tooth and its supporting structures, which surpasses the physiological limits of tissue tolerance.

Consequently, this leads to occlusal trauma and the destruction of periodontal tissues (Reddy and Vandana 2018). Not only the force intensity but also the type of force appears to affect tissue and cellular response. An excellent demonstration of this is seen in orthodontic treatment, where static compressive force on the compression side induces bone resorption while tension force on the tensile side induces bone formation, ultimately resulting in tooth repositioning (Tan, Xie et al. 2009). However, there is limited research and understanding of the effects of mechanical force intensity and type on ECM components.

Thus, the objective of this study was to examine the distinct function of ECM proteins in two different areas. Firstly, we aimed to investigate the significance of matricellular proteins as a microenvironmental niche for PDLSCs. Secondly, we aimed to explore the role of matricellular proteins as a mediator in the process of mechanotransduction, in addition to the mechanical impact on the PDL's extracellular matrix components.

1.2 Background and Significance

1.2.1 Collagen Type I

Collagen type I is a fundamental structural protein of the extracellular matrix (ECM) that shapes the inherent mechanical features of fibrous connective tissues like ligaments, tendons, heart valves, and blood vessels (Chang and Buehler 2014). The collagen fibril serves as the primary component of force transmission for all connective tissues. Simultaneously, mechanical force inclusion is vital for the development and remodeling of collagenous tissue. Studies have demonstrated the association between mechanical stress and the extracellular production of proteases. When high-intensity forces are applied to different dimensions of human fibroblasts, at least a 3- to 5-fold increase in matrix metalloproteinase-9 (MMP-9) (Prajapati, Eastwood et al. 2000) and a 13-fold increase in MMP-2 is observed (Prajapati, Chavally-Mis et al. 2000). These findings suggest that mechanical forces can potentially modify collagen turnover by changing collagen degradation. In addition to external environmental influences, internal matrix proteins can be synthesized intracellularly to control collagen fibrillogenesis, subsequently modifying the mechanical attributes of connective tissues.

1.2.2 Laminin

Laminin is an ECM protein that is commonly found in the basement membrane directly under the vascular endothelium, making it frequently exposed to fluid shear stress. This protein serves as a ligand for receptors such as integrins that are sensitive to mechanical force (Shyy and Chien 2002, Halper and Kjaer 2014). Moreover, laminin is known to facilitate the regulation of b-catenin signaling induced by shear force in colon cancer cells (Avvisato, Yang et al. 2007). Thus, it is possible that laminin plays a role in the response to mechanical force, particularly in the presence of shear force stimulation. The wealth of evidence indicates that ECM remodeling is regulated by specific

biomechanical forces to maintain the structural integrity and function of connective tissues, raising the potential to regulate PDL tissue response to fluid shear stress.

1.2.3 Periostin

Periostin (*POSTN*, PN) is a matricellular protein was established based on its expression in the PDL and periosteum. Periostin was expected to regulate tooth and bone remodeling especially under mechanical stress, since both PDL and periosteum are the sites that normally exposed to mechanical loading by mastication and physical exercise (Kudo, Amizuka et al. 2004, Bonnet, Standley et al. 2009). Moreover, periostin is one of major proteins in PDL-ECM (Litvin, Selim et al. 2004). Recently, several reports have demonstrated the importance of periostin in mechanical force-induced PDL tissue modulation, despite controversial results. The report by Rios and colleagues showed that oral tissues in *POSTN* null mice could not tolerate the mechanical force (Rios, Koushik et al. 2005). The severe resorption of jaw bones and roots of incisor teeth in these mice could be attenuated when the masticatory force was decreased when feeding mice with soft diets (Rios, Koushik et al. 2005, Rios, Ma et al. 2008). These results support the possible role of periostin in withstanding masticatory force. Previously, we have reported that PDLSCs could be activated by intermittent compressive force (ICF) stimulated the TGF-β signaling pathway and increased the expression of sclerostin (SOST) (Manokawinchoke, Pavasant et al. 2019). The evidence suggested that PDLSCs could function as mechanosensor cells, and it is possible that periostin might involve in PDL mechanotransduction to regulate tissue responses to mechanical forces.

1.2.4 Osteopontin

Osteopontin or OPN is a member of non-collagenous protein highly expressed in ECM of many tissues and participated in many biological functions including cell adhesion, migration, and biomineralization (Sodek, Ganss et al. 2000). OPN was considered to play important roles in bone formation and resorption. OPN null mice exhibited normal bone development with normal bone mass and structure, but increased fragility and decreased bone remodeling emphasizing the role of OPN in regulating bone quality instead of bone development (Liaw, Birk et al. 1998, Rittling, Matsumoto et al. 1998, Thurner, Chen et al. 2010). In the initial stages of physiologic and orthodontic tooth movements, OPN was observed in PDL space and osteocytes, respectively (Takano-Yamamoto, Takemura et al. 1994, Staines, MacRae et al. 2012). Moreover, OPN was found to localized in non-mineralized oral tissues (PDL tissues) while absent in mineralized tissues (Dentin and alveolar bone socket) during cementogenesis of mouse embryos suggesting that OPN is involved in bone remodeling, PDL remodeling and cementogenesis (Macneil, Berry et al. 1995). However, the function of OPN regarding bone biology is quite different and partly opposite. OPN has been considered as an inhibitory molecule for biomineralization regarding to its calcium binding domain which can strongly bind to extracellular Ca^{2+} (Klaning, Christensen et al. 2014), resulting in a decrease in hydroxyapatite crystals formation and an inhibition of ECM mineralization (Iline-Vul, Nanda et al. 2020). These diverse functions of OPN might be influenced by differences in forms, amounts and origins of OPN. Recently, we have shown that OPN might play a role in bone formation. Human recombinant OPN generated by the plant *N. benthamiana* induced osteogenic differentiation of PDLSCs. The plant-produced human OPN processes similar post-translational modifications compared to the mammalian OPN (Rattanapisit, Abdulheem et al. 2017). Therefore, it is essential to elucidate the effect of OPN on the osteogenic potential of PDLSCs regarding regulation, and further

explore the counteractive impact of the matricellular protein OPN on the osteogenic differentiation of MSCs.

1.3 Research Questions

1.3.1 Is the matricellular protein; OPN could provide the osteogenic inductive microenvironment to PDLSCs? How does it's affected other PDLSCs properties such as proliferation and the potential to use as a therapeutic treatment?

1.3.2 Is the matricellular protein; PN could function as the mediator, taking part in ECM mechanotransduction on PDLSCs? What is the mechanism?

1.3.3 If ECM plays an important part in the mechanotransduction process, how does PDL-ECM respond to inappropriate mechanical impact?

1.4 Objectives

Specific Aim 1. To elucidate the effect of OPN protein on PDLSCs osteogenicity and biomineralization.

Specific Aim 2. To investigate the role of periostin protein on PDLSCs response to mechanical intermittent compressive force (ICF) stimulation.

Specific Aim 3. To observe the impact of inappropriate force stimuli on PDL-ECM expression profile using rat occlusal hypofunction model.

1.5 Research boundaries

To begin with, primary cell cultures can exhibit variations in cellular characteristics and responses, even when exposed to similar substances or stimuli. The stability of the plant-produced protein, OPN produced from tobacco plants is unstable, easy to degrade, and lost of its biological effect. Proper control in the occlusal hypofunction model, it will be good if we have a sample from a normal rat with no operation and use it as the control, together with the contralateral molars. Additionally, the siRNA method has limitations in terms of its efficacy for gene knockdown, with only around 70-80% effectiveness, and its silencing effects typically only last for up to 4 days following transfection. Therefore, experiments using this method must be conducted within this time frame.

1.6 Benefits of the study

This study aims to enhance our understanding of two matricellular proteins, periosin and OPN, in PDLSCs, which will provide insight into their roles in PDL homeostasis. Investigating the role of periostin in PDLSCs will contribute to the understanding of the regulatory network between cells and ECM in response to mechanical force through mechanotransduction, which supports the evidence of PDLSCs as mechanosensory cells. Additionally, the study of OPN in PDLSCs may provide further information on its osteoinductive effect, such as the suitable conformation (soluble/matrix-bound form), the proper concentration, and the specific active domain. These findings could be beneficial in developing small osteoinductive molecules for future therapeutic applications. At last, one possible approach to maintain ECM homeostasis and prevent destruction of periodontal tissue due to occlusal hypofunction, particularly in cases of unclear cause, may involve targeting matrix proteins to strengthen the PDL-ECM.

CHAPTER II

2. Literature reviews

2.1 Periodontal ligament

Periodontal ligament is the special soft connective tissue located between alveolar bone and root of the tooth. Collectively, periodontal ligament along with alveolar bone and cementum, the hard tissue covering the root surface of the teeth, are referred to as periodontium. The function of periodontium is to anchor the tooth to alveolar bone (Türp and Alt 1998). (Figure 1)

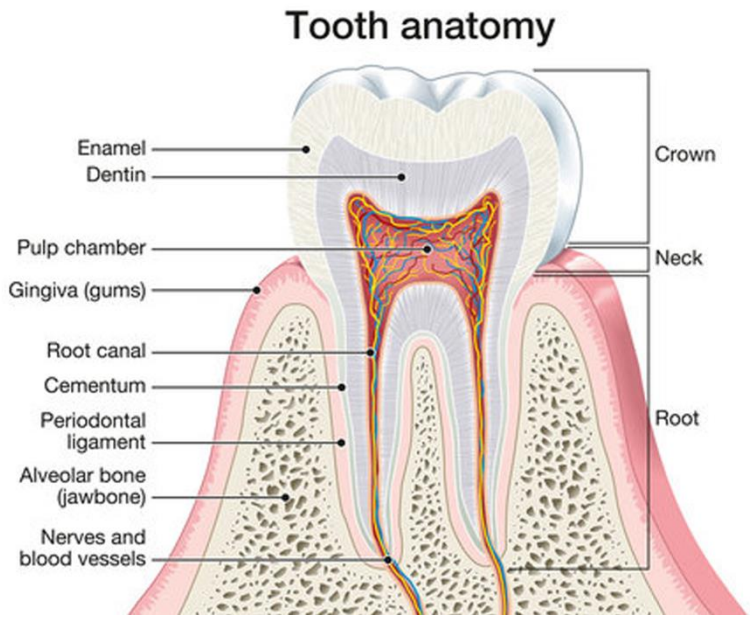

Figure 1. Diagram showing the structure of tooth.

(The illustrated is free to use, modify or reprint)

The main component of ECM in periodontal ligament tissues are collagen bundles that connect alveolar bone and cementum to hold the tooth within the bone socket (Kudo 2011, Wang, Papagerakis et al. 2018). Another important function of periodontal ligament tissues is to withstand the mechanical loading from mastication, deglutition, speech, and orthodontic treatment (Tomokiyo, Wada et al. 2019). The

influence of mechanical forces is important for the homeostasis of the tissue since improper loading stress will result in a destructive signal (Lemarie, Tharaux et al. 2010). The significant of loading forces on periodontal tissue homeostasis is to control the proper PDL space and control the tooth movement to maintain the proper occlusion (Anwar, Shalhoub et al. 2012). Regarding to the function related to mechanical loading, PDL is considered as a mechanosensory tissue.

2.2 Periodontal ligament stem cells

Human Periodontal ligament stem cells (PDLSCs) are the main cell types that resided in periodontal ligament tissue. The main function of these cells is to synthesize periodontal ligament that connect the tooth to alveolar bone. Evidence shows that PDLSCs could also withstand the mechanical forces generated from masticatory forces and help maintaining the homeostasis of periodontal tissue (Shimomoto, Chung et al. 2007, Manokawinchoke, Pavasant et al. 2019).

PDLSCs can be isolated from the periodontal ligament of extracted human third molar teeth and expanded in culture. The *In vitro* study showed that these cells possess characteristics of multipotent stem cells, including the expression of stem cells markers as well as the ability to differentiate under proper stimulation (Figure 2). PDLSCs express, like mesenchymal stem cells (MSCs), several MSC surface markers including CD105, CD90, and CD73, STRO-1 antigen, CD146, CD29, CD44, and CD106 and lack the expression of the hematopoietic and endothelial markers, CD31, CD34, and CD45 and HLA class II (Zhu and Liang 2015, Tomokiyo, Yoshida et al. 2018). Moreover, these cells have the unique capability for self-renewal as well as the ability to differentiate into osteogenic, adipogenic and neurogenic cell lineages (Huang, Pelaez et al. 2009, Grimm, Dannan et al. 2011). Under the suitable induction, PDL cells could differentiate into osteoblasts, chondrocytes, and adipocytes.

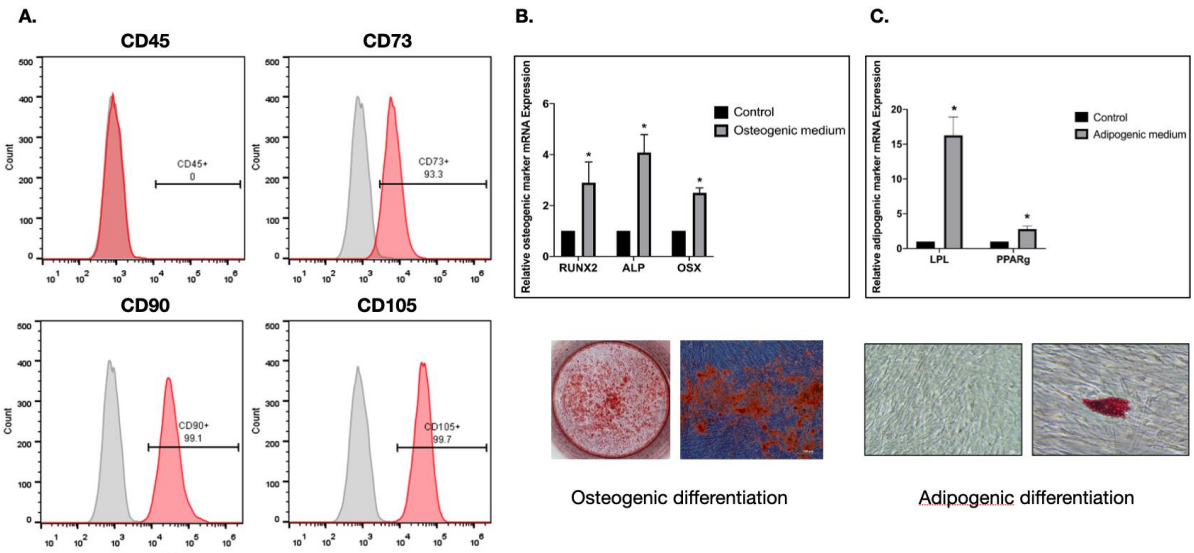

Figure 2. Stem cell characteristics of PDLSCs in culture.

(A) The expression of MSC surface markers. (B) *In vitro* differentiation capability of PDL cells toward osteogenic lineage, and (C) adipogenic lineage. (Unpublished data)

The differentiation capacity of PDLSCs was also shown in an *in vivo* study, where they generated cementum, periodontal ligament-like structures, and alveolar bone (Shi, Bartold et al. 2005). Moreover, the differentiation potential of PDLSCs has been demonstrated their different fate-committed ability which depended on their harvested location from periodontal tissue such as alveolar bone surface, root surface and capillary-associated area (Wang, Shen et al. 2011, Iwasaki, Komaki et al. 2013). These properties of PDL cells indicate an essential role of PDL cells to regulate the homeostasis of the periodontal ligament tissue as well as the periodontium.

2.3 Mechanical loading and biological response

The impact of forces generated by mechanical stress has been demonstrated to govern tissue formation, upkeep, and balance (Vining and Mooney 2017). Mechanotransduction refers to the conversion of physical cues (mechanical forces) into molecular cues (cellular signaling) which triggers cellular responses. Cellular responses to mechanical stimuli can occur rapidly, and biochemical signals are transmitted through multiple signaling pathways (Vogel and Sheetz 2006). Cells in the body have the capacity to respond to both normal and abnormal mechanical forces. Normal forces usually generate signals that sustain tissue integrity and balance, whereas abnormal forces usually cause tissue damage (Lemarie, Tharaux et al. 2010, Anwar, Shalhoub et al. 2012). While the impact of mechanotransduction on the functionality and balance of normal tissue in the human body is recognized, the precise molecular mechanisms underlying the tissue response to mechanical forces remain unclear. Three potential mechanisms of mechanotransduction have been proposed: (I) Mechanical forces trigger the activation of ion channels by altering the cell membrane potential, leading to calcium influx and intracellular cyclic adenosine monophosphate (cAMP) elevation. (II) Forces propagate signals by activating integrin-actin filaments, influencing cell-to-cell or cell-to-ECM interactions. (III) Force-induced conformational changes occur in cytoskeletal components and activate signaling pathways, such as G protein signaling (Bonnet and Ferrari 2010). The effects of mechanotransduction ultimately result in conformational changes in chromatin within the nucleus, influencing the accessibility of transcription factors or their cascades (Figure 3).

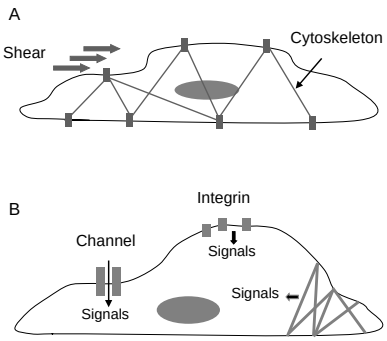

Figure 3. Diagram showed the possible pathways of mechanotransduction.

(**A**). External mechanical force causes distortion of the cell membrane, subsequently initiating signaling through the cytoskeleton.

(**B**). Force can open the ion channels on the membrane or changes the cell-matrix interaction to generate the signal via integrin receptor.

The mechanotransduction pathway can be influenced by the specific type of mechanical forces. Within our body, three types of forces can be generated: compressive force, tensile force, and shear force. It is crucial to consider not only the types of forces but also their magnitude, frequency, and duration of stimulation. However, our understanding of these factors is still incomplete.

2.4 Extracellular matrix

ECM is a highly dynamic structural network composed of non-cellular component surrounding cells, provides a physical environment, playing both structural and signaling roles (Frantz, Stewart et al. 2010, Hynes and Yamada 2011). The matrix component binds each other as well as cells surface receptor, forming the network that transduces signals from outer to inner cellular which regulate fundamental cell activities and characteristics such as adhesion, proliferation, migration, differentiation, polarity, and apoptosis (Walma and Yamada 2020). ECM structure exists in two major forms, basement membrane and interstitial ECM which are connected to cellular behaviors as shown in Figure 4. Basement membranes are sheet-like networks of ECM molecules surrounding the organs of metazoans that include collagen IV, laminins, nidogen 1 and 2, and proteoglycans (PGs) such as perlecan, agrin, collagen type XV, and collagen type XVIII (LeBleu, MacDonald et al. 2007, Pozzi, Yurchenco et al. 2017, Sekiguchi and Yamada 2018). While the interstitial matrices are characterized by a fibrous network of collagens and various non-collagenous proteins such as fibronectin, elastin, laminin, and tenascin.

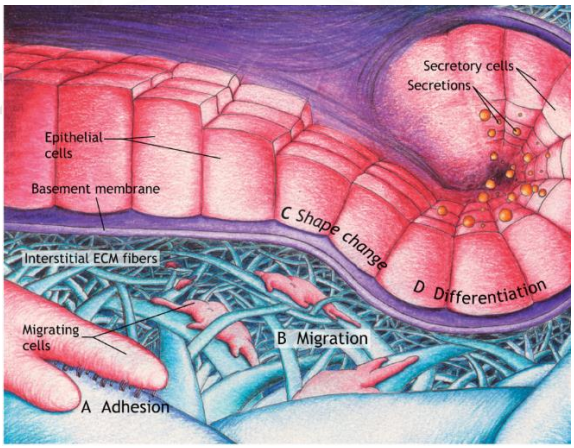

Figure 4. The structures of basement membrane and interstitial ECM fibers underneath epithelial cells that regulate cells fundamental processes.

(A) Cell adhesion, (B) Cell migration, (C) Cell shape and (D) Cell differentiation. [use under permission from (Walma and Yamada 2020)]

2.5 ECM and stem cells niche

ECM components, an integral part of the niche, play a crucial role in providing instructions within the niche (Peerani and Zandstra 2010). Through their arrangement and structural organization, these large molecules present in the extracellular space create a microenvironment where signals from interactions between cells and the ECM, as well as factors soluble or attached to the ECM, are effectively combined to support the balanced regulation of stem cell equilibrium (Watt and Fujiwara 2011). The importance of the ECM in influencing stem cell behavior is supported by in vivo evidence showing that modified or aging niches have a diminished capacity to maintain stem cell characteristics (Kurtz and Oh 2012). Furthermore, experiments conducted using decellularized tissues, which retain the ECM, offer direct evidence of the central role played by the ECM in controlling stem cell properties (Nakayama, Batchelder et al. 2010). These studies have shown that ECM scaffolds derived from decellularized tissues serve as guides for directing stem cell differentiation towards specific cell types found in the original tissue source.

2.6 ECM and its mechanotransduction

Mechanotransduction encompasses the processes through which biological systems perceive and respond to mechanical force stimuli, as well as their ability to generate force and regulate the mechanical properties of their environment field (Hoffman, Grashoff et al. 2011). These mechanotransduction effects occur at various levels of biological organization, from molecules to cells to organisms. On the tissue level, the ECM plays a central role. The ECM was initially believed to primarily serve a structural function by maintaining tissue shape under mechanical stress and providing physical support for cell adhesion and movement. Meanwhile, it also plays a crucial instructional role to provides both biochemical and biomechanical cues that influence diverse cellular activities, including migration, adhesion, changes in cell phenotype, and cell survival (Lu, Takai et al. 2011). There are three key players in ECM mechanotransduction,

2.6.1 The ECM scaffold

ECM can be classified into two primary components: the basement membrane and the interstitial matrix (Høye and Erler 2016). It consists of more than 300 proteins, 200 glycoproteins, and 30 proteoglycans (PGs). The mechanical characteristics of the ECM often rely heavily on these key constituents: elastic fibers, fibrillar collagens, and glycosaminoglycans (GAGs) along with their associated proteoglycans (PGs). Sustaining the mechanical attributes of the ECM relies on the ongoing process of synthesizing, integrating, and breaking down these essential components of the scaffold (Lu, Takai et al. 2011).

2.6.2 The mediators

Cells with high elasticity, such as fibroblasts, possess the ability to produce and release elastin, various types of collagens, glycoproteins, GAGs along with effective small molecules that form the specific composition of a particular tissue. These cells coordinate their synthetic and mechanical processes to arrange these components, contributing to the overall structural organization and mechanical properties of the

tissues (Lu, Takai et al. 2011). Additionally, they are capable of secreting proteases, particularly members of the matrix metalloproteinase family (MMPs), which facilitate the degradation of the different components (Nagase, Visse et al. 2006). When exposed to TGF-b in environments with significant tensile stress, fibroblasts have the capability to transform into myofibroblasts. This transformation enhances their capacity to produce extracellular matrix (ECM) components and increases their ability to contract (Hinz, Phan et al. 2012).

2.6.3 The receptor

The primary cellular components responsible for sensing and controlling the mechanical properties of the ECM are integrins. These integrins bind to ECM proteins and connect with cytoskeletal and signaling proteins present in focal adhesions. Another crucial group of players involved in this process are the signaling components that govern the assembly of these structures. This includes RHO-family small GTPases and their downstream effectors, such as RHO-associated protein kinase (ROCK) and myosin light chain kinases (Ciobanasu, Faivre et al. 2013).

Thus, to comprehend the mechanotransduction of ECM and tissues, it is necessary to establish a direct connection between molecular mechanisms and phenomena observed at the tissue level.

2.7 ECM small elements: Matricellular proteins

The matricellular proteins have been characterized as non-structural extracellular compartment that modulates cellular functions by facilitating the signal between ECM-cell and cell-cell interactions. Matricellular protein has been reported to promote cell adhesion and necessary for cell migration which are important parts of cell development (Bornstein and Sage 2002, Bornstein 2009). Although the structure of matricellular proteins is variable depending on their location but still contains common ECM structural motifs and similar function domains (Brekken and Sage 2000, Giachelli and Steitz 2000, Adams and Lawler 2004, Frangogiannis 2012). The relevant functions of those matricellular proteins are presented in Table 1.

Table 1. Members of matricellular proteins from different locations and its functions. [modified under permission from (Theocharis, Skandalis et al. 2016)]

Location	Matricellular proteins	Functions
Body fluids	Thrombospondin (TSP), Tenascin (TN), Secreted protein acidic and cysteine rich (SPARC), OPN, CCN family protein, cartilage oligomeric matrix protein (COMP), periostin, fibulin	**[1]** Growth factor/growth factor receptor interactions (Schiemann, Neil et al. 2003, Chen, Leask et al. 2011) **[2]** Epithelial-mesenchymal transition(Yan and Shao 2006, Du, Takeuchi et al. 2010) **[3]** Wound healing and homeostasis (Kyriakides and Bornstein 2003, Kyriakides and MacLauchlan 2009)

		[4] Innate immune function (Midwood, Sacre et al. 2009)
ECM	TSP, COMP, fibulin, periostin, TN, OPN, SPARC, hevin	**[1]** Collagen fibril/elastin/fibronectin organization (Bornstein, Agah et al. 2004) **[2]** ECM calcification (Giachelli and Steitz 2000) **[3]** Proteoglycan interactions (do Outeiro-Bernstein, Nunes et al. 2002) **[4]** Cell adhesion, migration, invasion (Murphy-Ullrich 2001, Bornstein and Sage 2002) **[5]** Stem cell niche (Chiquet-Ehrismann, Orend et al. 2014)
Inner plasma membrane	OPN	Cell adhesion and migration (Zohar, Suzuki et al. 2000)
Endoplasmic reticulum	TSP-1, TSP-4, COMP, SPARC	**[1]** ECM chaperone (Emerson, Sage et al. 2006) **[2]** ECM pre-assembly (Martinek, Shahab et al. 2008) **[3]** Calcium regulation

		(Duquette, Nadler et al. 2014) **[4]** ER function (Lynch, Maillet et al. 2012)

Matricellular proteins can bind to a large variety of surface receptors. However, most of matricellular protein members bind to and activate the signaling through multiple integrin family showing in Table 2. In addition to integrins, several matricellular proteins can bind to cell membrane heparan sulfate proteoglycans, syndecans, CD36, CD44, CD47, notch or even lipoprotein receptor-related protein (LRP) (Bedore, Leask et al. 2014, Chiquet-Ehrismann, Orend et al. 2014). The members of matricellular proteins include TSP-1 to TSP-4, TN, COMP, CCN1-2, SPARC, OPN, periostin, fibulin, hevin and R-spondins (Theocharis, Skandalis et al. 2016). Moreover, the evidence supported that cell stress from cell contractility, shear forces, or mechanical stretch affects the expression of many matricellular proteins (TSP-1, SPARC, TN-C and OPN) under fibrotic conditions (Kumei, Morita et al. 2006, Asparuhova, Ferralli et al. 2011, Chen, Leask et al. 2011).

Table 2. The matricellular proteins and specific binding integrin receptors
[modified under permission from (Murphy-Ullrich and Sage 2014)]

Matricellular proteins	Type of Integrins
TSP-1, TSP-2, TSP-4, TSP-5	αvβ3, αIIbβ3, α9β1, α6β1, α4β1, α3β1 (Calzada, Annis et al. 2004) α5β1, α5β3, α7β1, αvβ3 (Rock, Holden et al. 2010, Wang, Zheng et al. 2010)

OPN	αv (β1, β3, β5, β6) and (α8, α4, α5, α9) β1 and α4β7 (Green, Ludbrook et al. 2001, Yokosaki, Tanaka et al. 2005)
TN-C, TN-X, TN-W	α9β1, α8β1, αvβ3, αvβ6, αvβ1, α7β1, α5β1 (Yokoyama, Erickson et al. 2000, Katoh, Nagaharu et al. 2013, Tanaka, Seki et al. 2014) integrin β1 and α11β1 (Alcaraz, Exposito et al. 2014) α8β1, α4β1, αvβ1 (Scherberich, Tucker et al. 2005, Degen, Brellier et al. 2007)
CCN1, CCN2/CTGF, CCN3	αMβ2, α6β1, αvβ3, αvβ5 (Lau 2011) αMβ2, αvβ3, α4β1, α5β1 (Chen, Abraham et al. 2004) α5β1, α6β1, αvβ5 (Lin, Chen et al. 2005)
SPARC	α5β1 (Nie and Sage 2009)
Periostin	αMβ2, αvβ3, αvβ5 (Gillan, Matei et al. 2002, Johansson, Annis et al. 2013)

Fibulin-5	α5β1, α4β1 (binds, but does not activate) (Lomas, Mellody et al. 2007) αvβ3, αvβ5, α9β1 (Nakamura, Lozano et al. 2002)

2.7.1 Type I Collagen

Type I collagen (COL-I) is a widely distributed protein found abundantly in humans. It forms fibrils and fibers that vary in thickness from 50 nm to several hundred nanometers and can extend over several microns in length (Fratzl 2008). These fibers undergo crosslinking, resulting in the formation of COL-I networks. Reconstituted col-I gels are commonly utilized in three-dimensional (3D) culture studies. However, the structure and crosslinking patterns of these networks differ significantly depending on the specific tissue. The integrin receptor family, composed of heterodimers containing a and b subunits, particularly α1β1 and α2β1 integrins, play a crucial role in cell binding to col-I fibers (Frantz, Stewart et al. 2010). These COL-I networks are pivotal for facilitating numerous cell-matrix interactions.

The mechanical properties of COL-I are intricate and exhibit characteristics such as stiffness, nonlinear elasticity, viscoelasticity, and plasticity. The stiffness of individual COL-I fibrils ranges from 300 MPa to 1.2 GPa, with increased stiffness attributed to the straightening and uncoiling of the triple-helical structures of COL-I (Fratzl 2008). In reconstituted COL-I gels, microporous structures are observed, and their elastic modulus at the microscale to macroscale falls in the range of tens to hundreds of pascals. Initially, COL-I networks resist external mechanical forces or deformation, both in shear and tension, through the resistance of individual fibers to bending at low strain levels. As strain increases, the rotated and aligned fibers stretch, leading to strain stiffening (nonlinear elasticity) (Vader, Kabla et al. 2009). This strain stiffening causes a

nearly tenfold increase in stiffness with strain, which is highly dependent on the fiber length within the network. Similar to many natural extracellular matrices (ECMs), COL-I networks are susceptible to degradation by proteases, particularly MMPs (Table 3.).

Table 3. Biological activities from cleavage type I collagen mediated by MMPs (Nagase, Visse et al. 2006).

Biological effect	Responsible MMP
Keratinocyte migration and re-epithelialization	MMP-1
Osteoclast activation	MMP-13
Kidney tubulogenesis	MT1-MMP
Apoptosis	Collagenase

2.7.2 Laminin

Laminins, located in the basement membrane, are a family of large glycoproteins with multiple domains. They exist as heterotrimeric structures with molecular weights ranging from 500 to 800 kDa. In mouse and human tissues, sixteen trimeric isoforms have been identified, each with specific cell and tissue preferences (Domogatskaya, Rodin et al. 2012). Generally, a laminin isoform is composed of three chains: a, b, and g. There are genetically distinct forms of each isoform, with five a chains, four g chains, and three to six b genes typically found in most vertebrates. The variation in size among laminins is attributed to differences in chain size, with a chains being the largest (approximately 200-400 kDa) and b and g chains ranging from 120 to 200 kDa (Aumailley, Bruckner-Tuderman et al. 2005).

Proteolytic processing plays a role in the assembly of laminin molecules before they bind to their receptors. Laminins primarily engage with cells through the attachment of the G domain present in α the chains to integrins, dystroglycan, or sulfated glycolipids.

Various integrin isoforms, such as $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha V\beta 3$, are capable of binding to laminin. Moreover, specific a chains contain N-terminal globular domains that can bind to sulfatides, potentially enhancing the connection between laminin molecules and the cell surface (Domogatskaya, Rodin et al. 2012).

Laminins have significant contributions to the structure of the extracellular matrix (ECM) and exert influence on associated cells in multiple ways. These include promoting cell adhesion, affecting cell differentiation and migration, maintaining the stability of cell phenotype, and enhancing resistance against apoptosis (Aumailley, Bruckner-Tuderman et al. 2005). Laminin molecules engage in interactions not only with collagen type IV, integrins, and dystroglycan but also with additional constituents of the basement membrane matrix, thereby contributing to the overall structural integrity. Furthermore, laminins can establish connections with components present in the underlying interstitial stroma. The cellular impacts of laminins predominantly occur through the binding of these molecules to specific receptors on the cell membrane. This signaling process can lead to modifications in gene transcription levels and even influence the remodeling of gene promoters at the chromatin level. The insoluble network formed by laminin and type IV collagen plays a dual role by providing both structural support and functional capabilities within the basement membrane for the cells associated with it (Aumailley, Bruckner-Tuderman et al. 2005, Domogatskaya, Rodin et al. 2012).

Although the extent of laminin's involvement in the development of connective and soft tissue diseases remains unclear, it is evident that laminins play a significant role in the normal functioning of tendons, blood vessels, and other connective soft tissues. In studies involving mice deficient in type IV collagen, a decrease in laminin within the basement membrane covering the outermost aspect of the tendon was observed, leading to the formation of spontaneous tendon adhesions (Taylor, Al-Youha et al. 2011). Furthermore, research conducted by Molloy *et al*. (Molloy, de Bock et al. 2006) and Sato *et al*. (Sato, Nakamura et al. 1999) demonstrated that laminins are essential for the

proper healing of tendons and other connective tissues, such as the cornea. Moreover, laminin has been shown to facilitate SF regulation of β-catenin signaling in colon cancer cells (Avvisato, Yang et al. 2007). Thus, this protein is potentially associated with mechanical force responses, particularly under SF stimulation. A previous study had demonstrated that laminin can facilitate the alignment of endothelial cells in the direction of flow, thereby improving their ability to withstand and respond to shear force (Girard and Nerem 1995).

Collectively, laminins are not merely passive proteins involved in cell adhesion; rather, they actively modulate cell behavior and have an impact on processes such as differentiation, migration, and the stability of cellular phenotype. They also possess the ability against shear force. However, the specific mechanisms of laminin signaling in mechanotransduction are still predominantly unexplored, warranting further investigation.

2.7.3 Periostin

Periostin, formerly named osteoblast-specific factor OSF-2, is a matricellular protein that was first discovered in 1993 as an essential cell adhesion protein produced by mouse osteoblastic cell line (Coutu, Wu et al. 2008). In human, periostin encoded by periostin gene (*POSTN*) which is located in the long arm of chromosomal 13 (13q13.3). Periostin molecular weights are ranging from 83 to 93 kDa. These isoforms could be found in many tissues and have been found during both embryogenesis and bone development process (Hoersch and Andrade-Navarro 2010). Periostin is located in various collagen-rich connective tissues, including the periostium, periodontal ligament, skin, endocardial cushions, and cardiac valves.

Periostin can be both autocrine and paracrine factors which function as a cell attachment protein that signals through cell adhesion receptors αvβ3 and αvβ5 integrin, thereby regulating cell adhesion and mobility. Not only in the normal physiological

functions, but periostin also functions in pathophysiological conditions such as wound repair, vascular disease, osteogenesis, and tumorigenesis (Ruan, Bao et al. 2009). It has been reported that periostin-integrin activation signals through PI-3K/AKT and FAK-mediated signaling pathways, leading to the increased of cell survival, invasion, metastasis, angiogenesis, and epithelial-mesenchymal transition of carcinoma. Besides, periostin is highly expressed in tissues that subject to mechanical stress, suggesting a potential function of periostin to maintain connective tissue's structure and integrity. In connective tissue like periodontal ligament, periostin has been shown to regulates fibrillogenesis and consequently involved in biomechanical properties of fibrous tissues around the tooth (Kudo and Kii 2018). In one-point, mechanical stress increased periostin via Wnt5a expression. Wnt5a enhanced PDL-related gene expression and collagen production through TGF-β1-mediated upregulation of periostin expression, enhancing the proliferation and migration of PDL cells (Manokawinchoke, Limjeerajarus et al. 2015).

There are many other findings in roles of periostin in dental tissues as following: Due to the expression of periostin in epithelial-mesenchymal interaction during tooth development, it suggests that periostin may plays an important role in the accumulation and arrangement of ECM adhesion molecules (Rios, Koushik et al. 2005). According to Suzuki and colleagues (Suzuki, Amizuka et al. 2004), the expression of periostin in tooth and surrounding mandibular tissue suggested that periostin may involve in maintenance of the integrity of teeth at the interface site of hard-soft tissue. It functions as an adhesion between cell and matrix. It also induces the morphogenesis and assists in bearing mechanical forced.

In immunology, the immunoreactivity of periostin was found on the alveolar bone surface and incisor, as well as in surface area between the inner enamel epithelium and pre-odontoblasts. The immunoreaction to periostin also observed in PDL collagen fibrous bundles, revealed the role of periostin in the morphogenesis of the PDL and further

development. Moreover, the immunolocalization of periostin was restricted to only cell membrane of the cytoplasmic extensions of periodontal fibroblasts (Romanos, Asnani et al. 2014).

Regarding to Horiuchi et al., 1999 (Horiuchi, Amizuka et al. 1999), periostin highly expressed in PDL matrix. In addition, experiments on animals without periostin displayed in significant signs of abnormal remodeling, increase in osteoclast activity along with increased susceptibility to bacterial invasion, enamel and dentin matrix defects and unstable structure PDL leading to rapid periodontitis-liked disorder. In conclusion, periostin is critically important in maintaining integrity of PDL and is essential for remodeling of collagen matrix. This indicates that periostin is essential for controlling tooth and periodontium development.

2.7.4 Osteopontin

Osteopontin (OPN) is a major non-collagenous protein that is mainly found in the bone matrix as the bridge between the cells and hydroxyapatite (HA) in the ECM of bone and in a soluble form in body fluid. OPN, also known as secreted phosphoprotein 1 (SPP1), is a 34 kDa protein, originally identified as a highly phosphorylated and glycosylated protein that is expressed by several biomineralize related-cell types including osteocytes, osteoblasts, and odontoblasts (Sodek, Ganss et al. 2000). OPN is expressed at different stages of bone formation and has been considered as both early and late marker for osteogenic differentiation by increasing stem cells proliferation (BM-MSCs) and express in pre-mature osteoblast (Carvalho, Cabral et al. 2019). OPN belongs to the family of non-collagenous proteins known as SIBLING (small integrin-binding ligand, N-linked glycoprotein). This family consists of osteopontin , bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE) (Staines, MacRae et al. 2012).

In additional to biomineralization, OPN participates in many others biological functions including cell adhesion, migration, apoptosis, and tumor metastasis (McKee and Nanci 1996, McKee and Nanci 1996, Sodek, Ganss et al. 2000). The delay in skin wound healing is also found when lacking OPN protein in the mouse model (Liaw, Birk et al. 1998).

OPN is present and functions in two forms: a soluble and a matrix form. The soluble form of OPN has been shown to play a role in inflammation and calcification in cardio vascular diseases whereas the matrix form of this protein participates in both osteoblast and osteoclast attachment to the bone surface and regulates biomineralization (Wesson, Johnson et al. 2003). OPN molecule contains three major functional domains: integrin-binding domain, heparin-binding domain, and calcium-binding domains (Fig. 5). The integrin-binding domain sites in the middle region of the OPN molecule and the heparin-binding and calcium-binding domain are in the C-terminal region (O'Regan and Berman 2000, Kahles, Findeisen et al. 2014). However, the function of OPN regarding bone biology is still different and partly opposing.

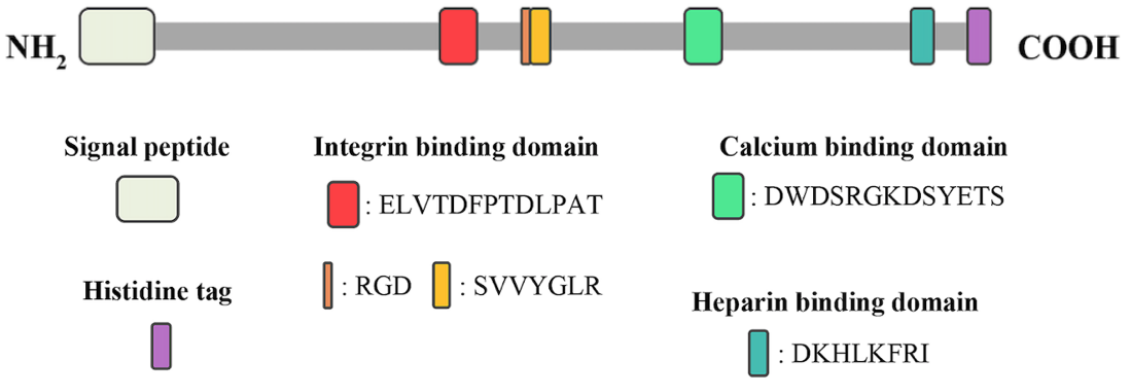

Figure 5. Structure of full length OPN and active functional domains.

Several studies have been documented the inhibitory role of OPN in HA formation and growth (Boskey, Maresca et al. 1993, Boskey, Christensen et al. 2012) regarding to its calcium binding domain which can bind directly to extracellular Ca^{2+} with high affinity (Klaning, Christensen et al. 2014) resulting in decrease of biomineralization. The effect of OPN on biomineralization depends on the acidic serine and aspartate-riches motif (ASARM) which can bind specifically to HA crystals and inhibit extracellular matrix mineralization (Iline-Vul, Nanda et al. 2020). The efficiency of OPN regulated HA crystallization depending on the number of phosphorylation sites on OPN molecules (Jono, Peinado et al. 2000, Boskey, Christensen et al. 2012). In addition to binding HA crystals, The binding between RDG motif of OPN and $\alpha V\beta 3$ integrin produced by osteoclasts modulates intracellular anion (Ca^{2+}) pump activity, leading to an increased resorption activity of mature osteoclasts (Ross, Chappel et al. 1993, Chellaiah and Hruska 2003, Tanabe, Wheal et al. 2011). This inhibitory role of OPN is confirmed by *in vivo* study in OPN null mice. Increased mineral content and size of bone were detected by Fourier transform infrared spectroscopy analysis in two different lines of *OPN*$^{-/-}$ mice at two different ages (Boskey, Spevak et al. 2002).

On the other hand, after bone resorption by osteoclasts, the adjacent bone lining cells or osteoclasts will be deposit OPN at the surface of the Howship's lacuna to induce the recruitment and differentiation of osteoblasts, suggesting the functional roles of OPN in bone remodeling (Everts, Delaisse et al. 2002, Luukkonen, Hilli et al. 2019). Interestingly, OPN null mice, exhibited the increased fragility and decreased of bone remodeling but had a normal bone development with normal bone mass and structure (Boskey, Spevak et al. 2002). These results emphasized the role of OPN in regulating bone quality, but not bone development (Liaw, Birk et al. 1998, Rittling, Matsumoto et al. 1998, Thurner, Chen et al. 2010). Although the regulatory role of OPN on biomineralization and bone remodeling is still unclear, it is possible that the key to regulate OPN functions may depend on its conformation and concentration in the initial processing.

2.8 The Role of mechanical loading in periodontal homeostasis

During normal masticatory function in the oral cavity, the periodontium is permanently subjected to various static and cyclic mechanical forces from occlusion, mastication, or chewing. Many documents support those various types of mechanical forces regulate periodontal ligament cells' behaviors to maintain their homeostasis. Early report suggested that proper cyclic mechanical stress increase PDLSCs proliferation and its DNA synthesis (Kletsas, Basdra et al. 1998) while the large magnitude of stress inhibited proliferation and induced apoptosis (Zhong, Xu et al. 2008, Hao, Xu et al. 2009, Kook and Lee 2012). However, different type and magnitude of mechanical loading seem to reveal different way of cells responses. Compressive stresses inhibit PDLSCs proliferation (Wu, Li et al. 2011) and tensile stress applied to gingival fibroblasts promotes their proliferation (Guo, Carter et al. 2011). Moreover, mechanical force seems to be involved in regulation of PDLSCs differentiation. Mechanical vibration and tensile force promote the proliferation and osteogenic differentiation of PDLSCs (Zhang, Li et al. 2012, Tantilertanant, Niyompanich et al. 2019). PDLSCs can also differentiate into keratocytes when exposed to mechanical stress (Chen, Zhang et al. 2018). Static compression could upregulate OPN and RANKL (Wongkhantee, Yongchaitrakul et al. 2007, Luckprom, Wongkhantee et al. 2010). OPN plays roles in inflammation and chemotaxis of white blood cells, while RANKL or receptor activator of nuclear factor kappa-b ligand plays an important role in the induction of osteoclastogenesis. Static compression could also induce the release of IL-1beta, one of the major inflammatory cytokines (Kanjanamekanant, Luckprom et al. 2013). Besides, compressive force could down-regulate Runx2/ALP, two osteogenic markers, indicate the inhibition of bone formation (Diercke, Sen et al. 2011). On the contrary, the intermittent compression supports the tissue homeostasis. Application of cyclic compressive force on PDLSCs increased the expression of SOST/Sclerostin expression, the antagonist of WNT

signaling pathway (Manokawinchoke, Limjeerajarus et al. 2015), suggesting the role of ICF-induced PDLSCs on the regulation of osteogenic differentiation.

Taken together, it suggested that the responses of PDLSCs to mechanical stimulation involve in both anabolic and catabolic functions of hard and soft tissues depended on different type, magnitude, duration, and frequency of forces.

2.9 Mechanical force inducible molecules:

2.9.1 Transforming growth factor beta (TGF-β)

Transforming growth factor beta (TGF-β) is a multifunctional growth factor that plays important role in proliferation, matrix synthesis and differentiation. TGF-β is a prototypic member of a large superfamily of secreted proteins that include three isoforms; TGF-β1, -β2 and -β3 (Weiss and Attisano 2013). The first discovered of TGF-β was found in cancer, cancer cells generally secrete large amount of TGF-β and its strongest involve in the most advanced stage of tumor progression (Nagaraj and Datta 2010).

However, evidence from TGF-β null mice showed the defect in the immune system indicating the important role of these growth factors in the immune modulation (Christ, McCartney-Francis et al. 1994). TGF-β has been shown to play role not only in anti-inflammatory response by regulating the function and differentiation of both T cells and dendritic cells (Strobl and Knapp 1999, Chen and Konkel 2009) but also in osteoclast formation (Quinn, Itoh et al. 2001, Itonaga, Sabokbar et al. 2004). Although the exact role of TGF-β on osteoclastogenesis is still unclear, these results indicated the involvement of TGF-β in hard tissue remodeling.

TGF-β regulate cellular activities through multiple cascades dividing to SMAD-dependent and SMAD-independent pathway (canonical and non-canonical, respectively). The intracellular mechanism was triggered via the engagement of TGF-β ligand and its specific receptors; TGF-βRI, RII and RIII. In the SMAD signaling pathway. The binding between TGF-β ligands and various of its receptor can be turned activated from TGF-βRII turn to TGF-βRI for re-activation. The TGFβ-RI-regulated SMAD2/3 phosphorylation at their C-terminal serine residues and move to form complexes with SMAD4 or co-SMAD, initiating several downstream biological processes through transcriptional regulation of target genes.

On the other hand, the non-SMAD signaling pathways. The signal from TGF-β complex transmits through other mediated factors, such as the mitogen-activated protein kinases (MAPKs), phosphatidylinositide 3-kinase (PI3K), TNF receptor-associated factor 4/6 (TRAF4/6) and Rho family of small GTPases. Activation through MAP kinases can employ regulation of transcriptional factors through direct interaction with the nuclear SMAD protein complex and via other downstream proteins. Moreover, activated JNK/p38/ERK can exert cellular apoptosis and proliferation in combination with SMAD proteins, while mediating cellular metastasis, angiogenesis, and cell growth through other transcriptional molecules, such as c-JUN and ATF. In addition, RhoA/ROCK can be activated by TGF-β to induce actin stress and fiber formation during EMT via a non-transcriptional mechanism. Moreover, TGF-β can activate interaction between the PI3K p85 subunit and the receptor complex in PI3K and AKT signaling pathway leading to translational responses via mTOR/S6kinase activation. At last, TGF-β activation also initiate nuclear factor-ϰB (NF-ϰB) from TRAF proteins signaling activity, leading to the inflammatory response among other processes (Nagaraj and Datta 2010, Weiss and Attisano 2013).

2.9.2 Alpha-smooth muscle actin (α-SMA)

Alpha-smooth muscle actin or α-SMA is one isoform of the mammalian actin gene family which is comprised of six isoforms including γ and β non-muscle actin that are well known as the cytoplasmic actin. On the contrary, α-SMA, α-cardiac, α-skeletal, and γ-smooth muscle actin are considered as tissue-specific actin isoforms (Khaitlina 2001). However, the α-SMA is also known as a specific marker for activated myofibroblasts differentiation and display increased proliferation, migration ability, cytokines production and interstitial matrix (Shirol and Shirol 2012). In connective tissue, the synergistic reaction of the α-SMA and the stress fibers provide the myofibroblast's ability with

contraction force which employ to generate forces applied to ECM adhesion receptors, these phenomena can make the whole ECM and cell unit contractile, which results in tissue contraction (Hinz and Gabbiani 2003), thereby mediating matrix remodeling. Suggested that α-SMA is a mechanosensitive protein that is strongly associated with the generation of contractile force by myofibroblast (Wang, Zohar et al. 2006).

Moreover, α-SMA was found in fibroblast that were activated by endogenously generated mechanical tension (Hinz, Mastrangelo et al. 2001) and when apply mechanical loading on connective tissue culture results in several transcriptional proteins and signaling proteins phosphorylation or even in activation of specific gene expression that may lead to enhanced the SMA expression (Khaitlina 2001, Hinz and Gabbiani 2003). Collectively, these data indicate that exogenous mechanical stress and endogenously generated force can regulate SMA expression but the mechanism of force-induced SMA expression is still unexplored. However, many reports informed that TGF-β1 is one of the most significant inducers of fibroblast and myofibroblast differentiation by increased α-SMA expression (Tomasek, Gabbiani et al. 2002, Pakyari, Farrokhi et al. 2013, Weiss and Attisano 2013), suggesting the possible related mechanism of TGF-β1 and a-SMA in response of connective tissue to mechanical force stimuli.

CHAPTER III

OSTEOPONTIN INDUCES OSTEOGENIC DIFFERENTIATION BY HUMAN PERIODONTAL LIGAMENT CELLS VIA CALCIUM BINDING DOMAIN-ALK-1 INTERACTION.

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3.1 Abstract

Background: Recently we have generated recombinant human osteopontin (rhOPN) using a plant platform (*Nicotiana benthamiana*) and demonstrated, when coated on culture plates, its osteogenic induction capacity of human periodontal ligament (PDL) cells. The aim of this study is to elucidate the molecular mechanism underlying the rhOPN-induced osteogenic differentiation of human PDL cells.

Methods: Full length rhOPN (FL-OPN) and three constructs of OPN containing integrin binding domain (N142), calcium binding domain (C122) and mutated calcium-binding domain (C122δ) were generated from *N. benthamiana*. Human PDL cells were isolated from extracted third molars and cultured on FL-OPN, N142, C122 or C122δ-coated surfaces. Real-time PCR and Western blot analyses were used to determine mRNA and protein expression. In vitro calcification was determined by Alizarin red staining. A chemical inhibitor and RNAi silencing were used to elucidate signaling pathways. In silico analyses were performed to predict the protein-protein interaction. In vivo analysis was performed using a rat calvaria defect model.

Results: Human PDL cells seeded on FL-OPN and C122-coated surfaces significantly increased both mRNA and protein expression of osterix (OSX) and enhanced in vitro calcification. Soluble FL-OPN as well as a surface coated with N142 did not affect *OSX* expression. Inhibition of activin receptor-like kinase (ALK-1) abolished the induction of osterix expression. In silico analysis suggested a possible interaction between the calcium binding domain (CaBD) of OPN and ALK-1 receptor. C122, but not C122δ coated surfaces, induced the expression of p-Smad-1 and this induction was inhibited by an ALK-1 inhibitor and RNAi against ALK-1. In vivo data showed that 3D porous scaffold containing C-122 enhanced new bone formation as compared to scaffold alone.

Conclusion: The results suggest that next to full length OPN, the CaBD of OPN, if coated to a surface, induces osteogenic differentiation via interaction with ALK-1 receptor.

Keywords: osteopontin, calcium binding domain, ALK-1, osteogenesis, periodontal ligament cells

3.2 Introduction

Osteopontin (OPN) is a major non-collagenous protein highly expressed in the bone matrix. This protein is also found in the extracellular matrix of many tissues and in a soluble form in body fluid. OPN participates in many biological functions including cell adhesion, migration, and biomineralization (McKee and Nanci 1996, Sodek, Ganss et al. 2000). Moreover, the delay in skin wound healing is also found when lacking of OPN protein in mice model (Liaw, Birk et al. 1998).

OPN is present in two forms: a soluble and a matrix-bound form. The soluble form of OPN has been shown to play role in inflammation whereas the matrix form of this protein participates in the attachment of both osteoblasts and osteoclasts to the bone surface and regulates biomineralization (Wesson, Johnson et al. 2003).

OPN contains at least three functional domains: a cell-binding, heparin-binding and the calcium-binding domains. The cell-binding domain is located in the middle region of the molecule and the heparin-binding and calcium-binding site are located in the C-terminal region (O'Regan and Berman 2000, Kahles, Findeisen et al. 2014). The function of OPN regarding bone biology is quite different and partly opposing. OPN has been considered as an inhibitory molecule for biomineralization due to its calcium binding domain that bind to extracellular Ca^{2+} with high affinity (Klaning, Christensen et al. 2014). The effect of OPN on mineralization depends on its ASARM motif (the acidic serine-and aspartate-rich), which can bind to hydroxyapatite crystals and inhibits extracellular matrix mineralization (Iline-Vul, Nanda et al. 2020). The ability to regulate hydroxyapatie crystallization depended on the phosphorylation of OPN (Jono, Peinado et al. 2000, Wesson, Johnson et al. 2003, Boskey, Christensen et al. 2012). In addition, binding of $\alpha V\beta 3$ integrin expressed by osteoclasts to bone associated OPN modulates intracellular Ca^{2+} pump activity leading to an increased resorption activity of osteoclasts (Ross, Chappel et al. 1993, Chellaiah and Hruska 2003, Tanabe, Wheal et al. 2011)

Regarding the role of OPN in bone remodeling, it has been shown that after bone resorption, bone lining cells or osteoclasts deposit OPN at the surface of the Howship's lacuna to regulate recruitment and differentiation of osteoblasts (McKee and Nanci 1996, Everts, Delaisse et al. 2002, Luukkonen, Hilli et al. 2019). Interestingly, OPN null mice showed a normal development with normal bone mass and structure but increased fragility and decreased bone remodeling. These results emphasize the role of OPN in regulating bone quality (Liaw, Birk et al. 1998, Rittling, Matsumoto et al. 1998, Thurner, Chen et al. 2010).

The role of osteopontin in bone formation is recognized by numerous previous studies (McKee and Nanci 1996, McKee and Nanci 1996, Sodek, Ganss et al. 2000). Recently, we have generated plant produced OPN and shown that this pant-produced protein could also induce osteogenic differentiation. Human recombinant OPN (rhOPN) generated by the plant *N. benthamiana* induced osteogenic differentiation (Rattanapisit, Abdulheem et al. 2017). Plant-produced rhOPN possesses post-translational modifications similar to that found in OPN expressed by mammalian cells. Our plant produced rhOPN has a MW of 60 KDa, comparable to the commercially produced rhOPN by HEK-293 cells. Plant produced rhOPN has a secondary and tertiary structure similar to that of rhOPN produced by HEK-293 cells. Coating of rhOPN to culture plates induced the expression of several osteogenic differentiation related genes such as osterix (*OSX*), dentin matrix protein-1(*DMP-1*) and *WNT3A* (Rattanapisit, Abdulheem et al. 2017, Klinthoopthamrong, Chaikiawkeaw et al. 2020). However, the molecular mechanism underlying the induction of osteogenic differentiation is still unclear. Therefore, the aim of this study was to investigate the molecular mechanism of rhOPN-induced osteogenic differentiation by human periodontal ligament cells.

3.3 Materials and Methods

3.3.1 Construction of full-length and truncated OPN fragments

Full-length OPN (FL-OPN) gene was optimized to *N. bentamiana* codon and synthesized as previously described (Rattanapisit, Abdulheem et al. 2017). The protein contained a signal peptide (SP) at N-terminus and 8His tag at C-terminus. The N-terminus half contained 142 amino acids (N142) and the C-terminus without heparin binding domain (C122) was generated and amplified by using pairs of primers: OPN-N-F + OPN-N142-R and OPN-C142-F + OPN-C122-R, respectively. The calcium binding domain within C122 was modified by site-directed mutagenesis to delete the WDSRGKDSYET (see diagram in Fig. 3) and this modified C122 or C122δ was generated by using pairs of primers; SP-F + OPN-C122δ-R and OPN-C122δ-F + 8His-R. The list of primers was shown in the supplement Table 1. After that, FL-OPN, N142, C122 and C122δ were ligated into geminiviral expression vector from bean yellow draft virus (pBY). All four recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation.

3.3.2 Plant production of full length and truncated OPN fragments

Recombinant agrobacteria were separately cultured at 28 °C for overnight. Then, cell pellets were collected and resuspended in infiltration buffer to an OD_{600} of 0.2 - 0.4. Wild type *N. bentamiana*, 6 to 8 weeks-old, was infiltrated with agrobacterium solution by vacuum condition. After that, the infiltrated leaves were harvest on 2-4 days after infiltration. Recombinant proteins were purified from the infiltrated leaves by nickel affinity chromatography.

3.3.3 OPN coated surface.

Cell culture plates * were coated with rhOPN to obtain a final concentration of 7.5, 15 and 30 ng/cm^2. Coating was performed on a shaker overnight at 4 °C and protected from light. The coated surface was air dried before seeding cells. Periodontal ligament cells were seeded at the density of 4x 10^4 cell/cm^2/ml. For examining the effect of soluble OPN, confluent cells were treated with 50, 100, 200 ng/ml of FL-OPN. OPN produced form HEK-293 † was used as a control in in vitro calcification experiments.

3.3.4 Cell culture

Human PDL cells were prepared as previously described (Rattanapisit, Abdulheem et al. 2017). A written informed consent was given by each donor and was approved by the human ethical committee of the Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2018-054). This study was conducted in accordance with the Helsinki Declaration. Human PDL cells were isolated from the periodontal ligament which was scraped from the middle-third of the root. The explants were cultured with a high glucose-Dulbecco modified eagle medium (DMEM) containing 10% fetal bovine serum, 2 mM of L-glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml) ‡ and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. After confluency, PDL cells were detached and sub-cultured at a ratio of 1:3. Cells from the third to the fifth passages were used in the study.

All the isolated cells were characterized by the expression of CD73, CD90 and CD105 by flow cytometry. The ability of osteogenic and adipogenic differentiation under appropriated induction were also examined. The characteristic results were shown in Suppl Fig. 1.1.

For the inhibition experiments, cells were pretreated for 30 min with 2 nM of the selective ALK-1 inhibitor, K02288 † before their seeding on the OPN coated surface.

3.3.5 Cell viability assay

The PDL cells were seeded in to 24 well plates at density of $6x10^4$ cells/well/ml for 24 hours prior to treatment with ALK-1 inhibitor, K02288 1, 2 and 5 nM respectively. The treated cells were incubated at 37°C for 24 hours before change the media to 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [§] solution (5 mg/ml in phenol red free DMEM) and incubate for next 20 minutes. After last incubation, the cells were dissolved in dimethyl sulfoxide solution (DMSO) [†] and measured at OD=570 nm.

3.3.6 RT-PCR

Total RNA was extracted, reversed transcribed and subjected to real time PCR analysis. Quantitation of expression was calculated based on the quantitation cycle (Cq) following normalization to expression of glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH). Primer sequence was shown in supplement Table 1.

3.3.7 Western blot

The PDL cell lysates were extracted using radio immunoprecipitation (RIPA) buffer (50 mM Tris/HCL, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate) supplemented with proteases inhibitor cocktail [†]. Total protein concentration was measured by a BCA protein assay kit [‖]. An equal amount of protein from each sample was loaded into 12% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis, transferred to nitrocellulose membrane and incubated with antibody against human OPN, OSX, Smad-1,5,8 and p-Smad-1,5,8 followed by incubating with peroxidase-labeled anti-rabbit polyclonal antibody [¶]. The signal was activated by chemiluminescence and captured using an image analyzer [#].

3.3.8 Molecular docking of calcium binding site of OPN with ALK-1

The peptide structure of the calcium binding domain (WDSRGKDSYET) of OPN was predicted by PEP-FOLD3 program (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/) (Alland, Moreews et al. 2005, Neron, Menager et al. 2009). The 3D structure of the extracellular domain of ALK-1 at the residue 30-104 (PDB code is 4FAO) (Townson, Martinez-Hackert et al. 2012) was used. The molecular docking software was iGEMDOCK (Yang and Chen 2004). The receptor was ALK-1 and the ligand was calcium binding domain of OPN.

3.3.9 Alizarin red S staining

The PDL cells cultured on FL-OPN, HEK-293-OPN, or C122 for 10-14 days were fixed with cold methanol for 10 minutes and washed with deionized water. The wells were stained with 1% Alizarin Red S solution [†] for 5 min at room temperature. The staining was analyzed by microscopy. The quantification was performed by dissolved stained sample in 10% of cetyl-pyridinium chloride and measured for absorbance at 570 nm.

3.3.10 ALK-1 knockdown attenuated the C-122-induced OSX

The predesigned small interfering RNA (siRNA) specific for *AC AVCRL1RL1* (ALK-1) gene and a non-targeting control were purchased [**]. To knockdown *ACVRL1* mRNA, human PDL cells were plated in six well plates at a density of 50% confluency and transfected with *ACVRL1* or control siRNA using transfection medium [*]. In brief, attached cells were pre-incubated with a mixture of siRNA and transfection medium in serum/antibiotic free media for 6 hours before continuing the transfection process for 24 hours. The efficacy of transfection was shown in Suppl Fig.3. Subsequently, the transfected cells were detached and seeded on C122-coated surface for 24 hours. Total RNA was harvested from treated and untreated cells and analyzed for gene expression using real time PCR.

3.3.11 Preparation of OPN-coated 3D PCL scaffolds

Double leached PCL-PEG scaffolds were fabricated as previously reported (Thadavirul, Pavasant et al. 2014). Circular scaffolds (5 mm diameter, 1 mm thickness) were prepared and treated with 1M sodium hydroxide solution † at 37˚c for 1 hour to create the hydrophilic surface. The treated scaffolds were washed thoroughly in deionized distilled water, vacuum-dried and sterilized by dipping in 70% v/v ethanol for 30 minutes and washed twice with PBS.

A 100 ml PBS solution containing 50 ng of C122 was added to each scaffold. The scaffolds were incubated in shaking incubator for 16-18 hours at room temperature and then stored at 4˚c until used.

3.3.12 Rat calvaria bone defect

The experiment was carried out with 12 six-week-old Wistar rats. The protocol was approved by the Chulalongkorn University Animal Care and Use Committee, Animal Use (Protocol No. 1773019). Two circular calvaria defects (5 mm in diameter) were created under general anesthesia using xylazine and ketamine. Rats were divided into two groups with 6 rats in each group. In group 1 the right defect was filled with a C122-coated scaffold and the other was left empty (sham). In group 2 the right defect was filled with scaffold without C122 and the other was filled with C122-coated scaffold. The wound was closed with a 4-0 nylon suture and the animals were euthanized after 4 and 8 weeks (three rats per time point).

3.3.13 Micro-computed tomography (μCT)

Bone formation in the calvaria defects was analyzed using μCT imaging. The samples were collected and fixed with 10% (v/v) formaldehyde for 24 hours, followed by

extensive washing with PBS. All specimens were scanned under 70 kVP, 114 mA, 8 W of X-ray. Total bone volume was analyzed based on hydroxyapatite (HA) at 1200 mg HA/cc using μCT scanner and μCT evaluation program [††].

3.3.14 Histological examination

Following μCT analysis the calvaria specimens were decalcified using Surgipath Decalcifier II [‡‡] and processed for paraffin embedding. Sections of 5 μm thickness were cut and stained with Masson's Trichrome. Digital images were obtained using a microscope [§§]. Four slides from middle third of the defect were randomly selected (each slide contain 3 sections) and the amount of new bone formation was measured using ImageJ Software.

3.3.15 Statistical analysis

The data was presented as mean ± SD, statistical analyses were performed by using one-way ANOVA followed by Tukey's multiple comparison test. Values of $P < 0.05$ were considered significant. The analyses were performed using GraphPad Prism9 Software.

3.4 Results

3.4.1 Recombinant human OPN stimulates osterix expression by PDL cells possibly via ALK-1 signaling pathway.

The isolated PDL cells were cultured on plant produced full length osteopontin (FL-OPN) for 24 hours. RT-PCR analysis showed that the cells cultured on FL-OPN-coated surface significantly increased the expression of *OSX,* a key transcriptional regulator of osteogenic differentiation. This was apparent at both mRNA and protein level (Fig. 6A-C). The inductive effect of surface FL-OPN on the expression of RUNX2 and ALP was also observed, but not on cell proliferation (see Suppl Fig.1.2). The inductive effect of FL-OPN proved to be a dose dependent with a maximal level at 15 ng/cm^2. Interestingly, the addition of soluble OPN to the cells did not have any effect on the expression of *OSX* (Fig. 6D-F). Fig. 6G showed that 15 ng/cm^2 of both plant and HEK293 produced OPN enhanced in vitro calcification as judged by Alizarin red S staining. The quantification analysis of the staining was shown in Fig. 6H. However, no inductive effect was observed in cells culture on 30 ng/cm^2 coated OPN surface as compared to the effect of cells cultured in osteogenic medium (OM). Therefore, the 15 ng/cm^2 FL-OPN was used in for the rest of experiments.

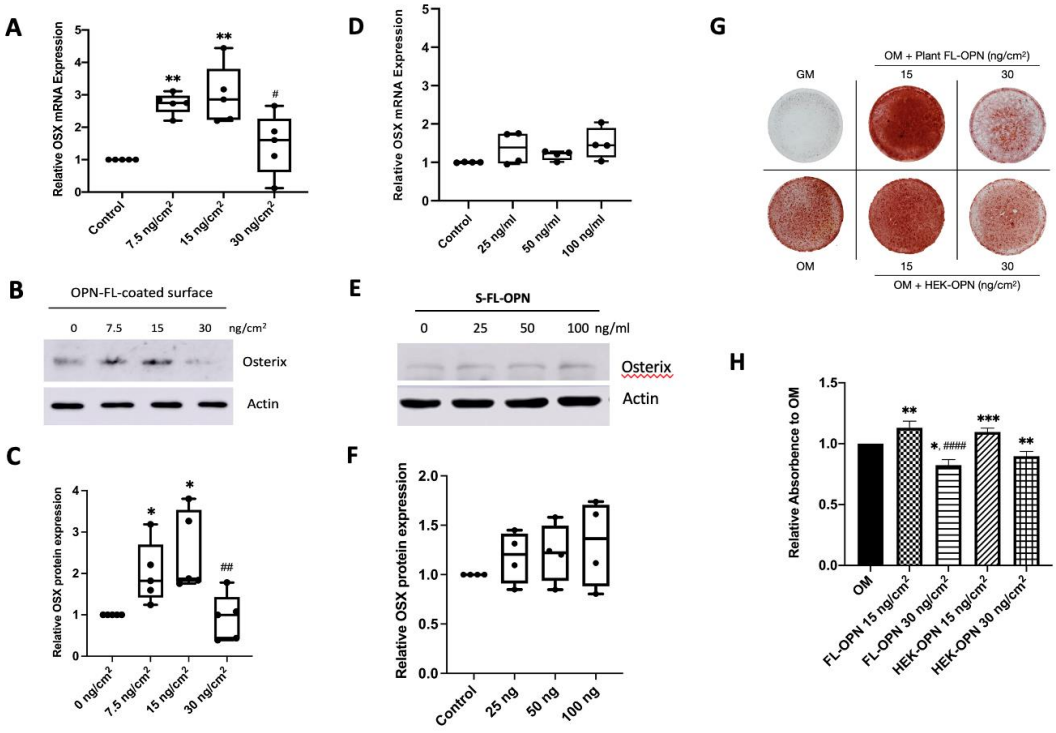

Figure 6. Plant produced osteopontin stimulates osteogenic differentiation by PDL cells.

PDL cells were seeded on FL-OPN-coated plates with a density of 150,000 cells/well/ml in 12-well plates for 24 hours. The plates were coated with the following concentrations of OPN: 0, 7.5, 15 and 30 ng/cm^2. **A)** RT-PCR analysis revealed that coated FL-OPN induced the expression of *OSX* in a dose-dependent manner. The expression of *OSX* was determined by real-time RT-PCR and normalized to the expression of *GAPDH*. **B)** Western Blot showing increased expression of OSX protein by PDL cells seeded on the OPN-coated plate for 24 hours. Actin was used as loading control. The quantitative data from Western analysis was shown in **(C). D) and E)** Soluble FL-OPN was added to the cultures and the expression of *OSX* was determined by RT-PCR and Western blot analysis, respectively. The quantitative data of Western blot analysis from Fig. **E** was shown in **(F). G)** The PDL cells were seeded on plant produced OPN or HEK293 produced OPN-coated plates (15 and 30 ng/cm^2) and cultured in osteogenic medium (OM) for 14 days. Cell cultures on uncoated surface in growth medium (GM) and OM

were used as negative and positive controls, respectively. Culture plates were stained with Alizarin Red S to detect in vitro calcification. **H)** Quantification of Alizarin Red S staining. *, ** and *** indicate significant differences from control, $p < 0.05$, 0.01 and 0.001, respectively. $^{\#}$, $^{\#\#}$ and $^{\#\#\#\#}$ indicate significant differences from OPN 15 ng/cm^2, $p < 0.05$, 0.01 and 0.0001, respectively.

Fig. 7A-C showed the effect of activin receptor-like kinase (ALK-1) inhibitor, K02288 on the inductive effect of FL-OPN. This inhibitor has been shown to bind directly to ALK-1 and inhibits BMP-9-ALK-1 signaling (Kerr, Sheldon et al. 2015). Addition of K02288 inhibited the FL-OPN-induced expression of osterix both at mRNA and protein levels without any effect on cell viability (Fig. 7D).

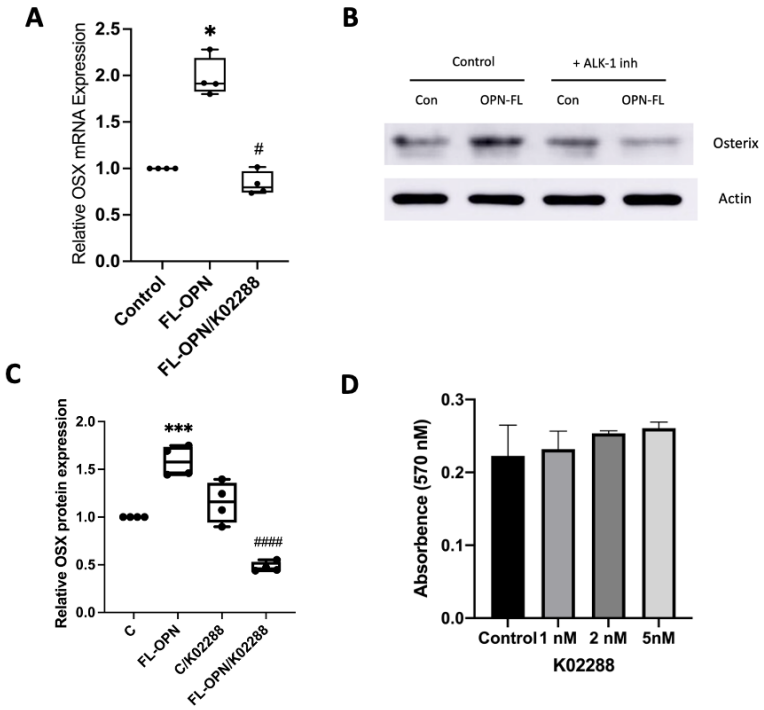

Figure 7. OPN stimulated *OSX* expression was abolished by inhibiting ALK-1

PDL cells were seeded on FL-OPN-coated plates with a density of 150,000 cells/well/ml in 12-well plates for 24 hours. The plates were coated with 15 ng/cm^2 of OPN. Addition of K02288, an ALK-1 inhibitor, abolished the OPN-induced expression of OSX as shown by RT-PCR **(A)** and Western blot analysis **(B)**. **C)** Graph showing the quantitative analysis of the Western blot. **D)** MTT cells viability assay was performed and confirmed that K02288 was not toxic. * and ** indicate significant differences from control, $p < 0.05$ and 0.01. $^{\#}$ and $^{\#\#\#\#}$ indicate significant differences from FL-OPN, $p < 0.05$ and 0.0001.

3.4.2 Prediction of OPN functional domains and ALK-1 3D structures

To confirm the interaction between the ALK-1 receptor and functional domains of OPN, a molecular docking approach was performed. The structure of three functional domains of OPN: integrin binding, calcium binding and heparin binding domains as well as the extracellular domain of ALK-1 were predicted using a computer program (Fig. 8A). The sequence of the three functional domains of OPN was derived from the study of Wai and Kuo (Wai and Kuo 2008). Ten models of each domain were generated from the input sequences. The lowest free energy model was selected for further experiments.

Fig. 8B and 8C show the three-dimensional structure of the calcium binding domain and ALK-1, respectively. From the predicted model, the overall structure of the calcium binding domain is a random coil that could interact with the extracellular domain of ALK-1 as shown in Fig. 8D and 8E. The calcium binding site of OPN binds to the extracellular domain of ALK-1 with a free energy at -138.6 kcal/mol. Most of the interactions are hydrogen bonds. The interactions of amino acids of the calcium binding domain and ALK-1 were shown in Table 4.

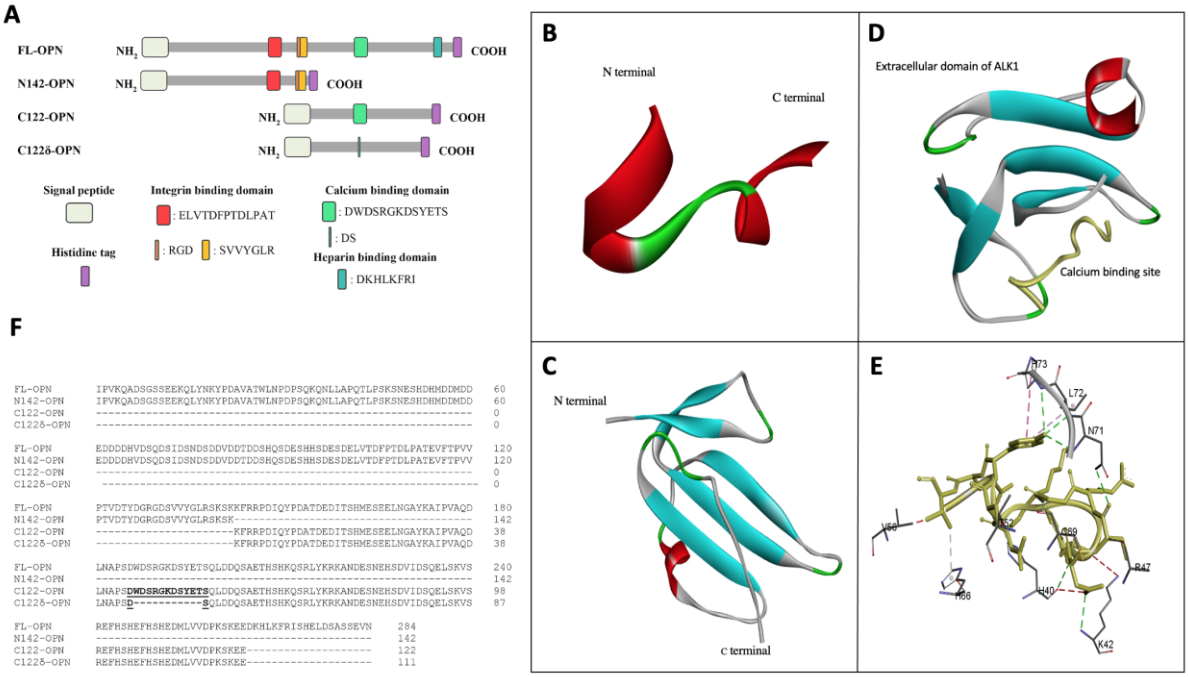

Figure 8. Molecular docking of extracellular domain of ALK-1 and calcium binding domain of OPN

A) Diagram showing the three functional domains of FL-OPN (modified form (Wai and Kuo 2008)) and three constructs that have been generated including N142 (the N-terminal part of OPN containing integrin-binding domain), C122 (the C-terminal part of OPN containing calcium-binding domain but not heparin-binding domain), and C122δ (the C122-OPN of which 11 amino acid in the middle part of calcium-binding domain were deleted). B) and C) show the predicted peptide structure of the calcium-binding site of OPN and the extracellular domain of ALK-1, respectively. D) The in silico binding of the calcium-binding domain of OPN (yellow) and extracellular domain of ALK-1. E) Interactions between the calcium-binding domain (yellow) and the extracellular domain of ALK-1. The figures of protein structures were illustrated by Discovery Studio2019 (Biovia, CA, USA). F) The amino acid alignment of these three constructs: C122, C122δ and N142.

Table 4: Interactions between calcium binding site and extracellular domain of ALK1 interface

extracellular domain of ALK1	calcium binding site of OPN
His40	Asp188, Glu196
Lys42	Asp188, Trp187
Arg47	Ser189
Thr52	Ser194
His66	Thr197
Cys69	Gly191
Asn71	Tyr195, Ser189
Leu72	Tyr195
His73	Tyr195

3.4.3 C122-OPN binds to ALK-1 and induces osteogenic differentiation.

To confirm the results from molecular docking, we generated three OPN fragments; N142, C122 and C122δ that contain an integrin binding domain, calcium binding domain (CaBD) and mutated calcium binding domain, respectively (Fig. 8A).

For functional analysis of these OPN fragments, PDL cells were seeded on the N142, C122 and C122δ coated surface and the expression of osterix was determined by RT-PCR (Fig 9A). Only C-122 coated surface showed the inductive effect on *OSX* expression. Next, PDL cells were seeded on various doses of C-122 coated surface.

The results showed that C-122 increased the expression of osterix on both mRNA (Fig. 9B) and protein levels (Fig. 9C-D) in a dose dependent manner. The optimal concentration of C122 on induction of *OSX* was found to be around 7.5-15 ng/cm^2. These two concentrations of C122 also enhance in vitro calcification when cultured in osteogenic-inductive medium (OM) as compared to those in OM alone (Fig. 9E). Addition of K02288 as well as knockdown of ALK-1 using RNAi inhibited the inductive effect of C122 on *OSX* expression (Fig. 9F-G). The efficacy of ALK-1 knockdown was about 70% (suppl Fig 1.3). Moreover, an increased level of p-Smad1 was observed in cells seeded on C122 coated surface but not on PDL cells seeded on C122d coated surface (Fig.9H-K).

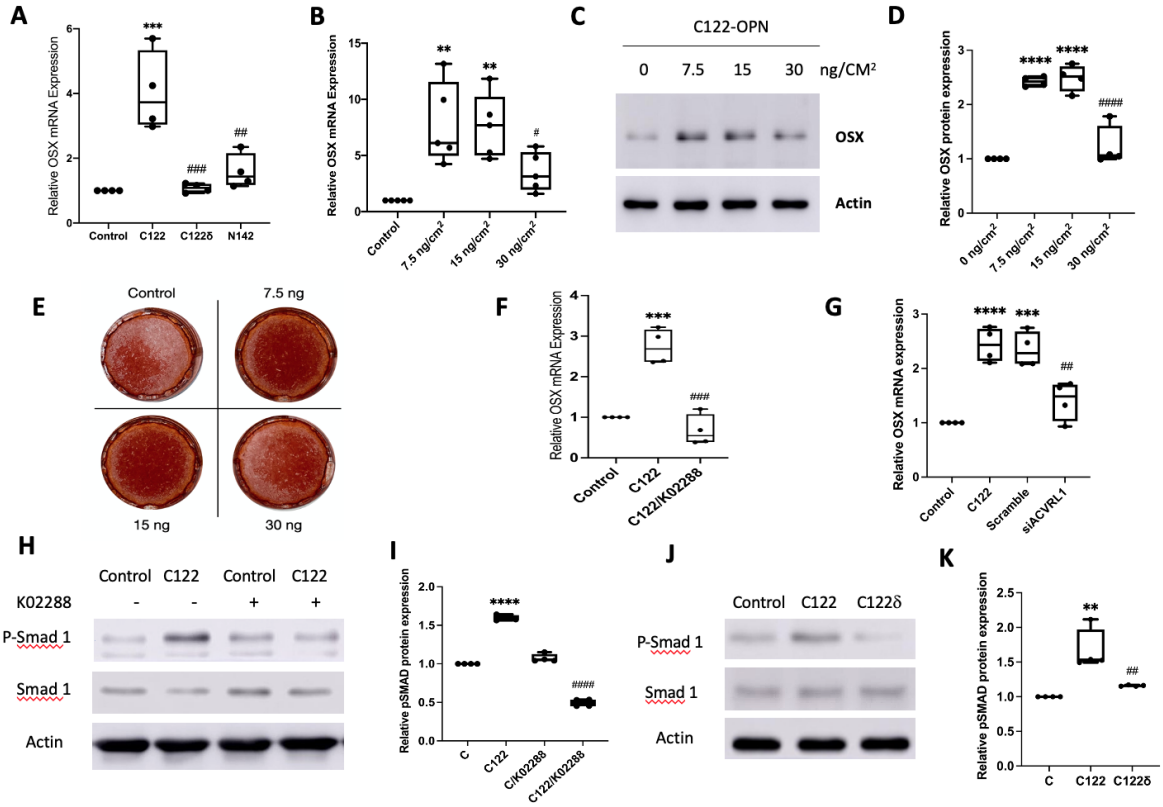

Figure 9. C122 induced the expression of *OSX* and enhanced in vitro calcification. PDL cells were seeded on surfaces coated with 15 ng/cm^2 of N-142-, C122 and C122δ. The cells were cultured for 24 hours and the expression of *OSX* was determined by real-

time PCR. The results indicated that only C122 significantly induced *OSX* mRNA expression (**A**). Next, various concentration of C-122 were examined for *OSX* expression by RT-PCR (**B**) and Western blot analysis (**C**). The quantitative data from Western blot analysis are shown in (**D**). (**E**) In vitro calcification assay showed that the C122-coated surface enhanced this parameter as judged by Alizarin Red S staining. K02288 (2 nM) inhibited the inductive effect of C122 on *OSX* expression (**F**). *ACVRL1* knockdown also abolished the inductive effect of C-122-coated surface (**G**). **H-I**) C122 increased the level of p-Smad-1 by PDL cells as judged by Western blot analysis and this induction was inhibited by the ALK-1 inhibitor. **J-K**) In contrast to p-Smad-1 induction under the influence of C122, surface coated with C122d had no effect on p-Smad-1 expression. **, *** and **** indicate significant differences from control, $p < 0.01$, 0.001 and 0.0001, respectively. $^{\#}$, $^{\#\#}$, $^{\#\#\#}$ and $^{\#\#\#\#}$ indicate significant differences from C122 7.5 and 15 ng/cm^2, $p < 0.05$, 0.01, 0.001 and 0.0001, respectively.

3.4.4 C122-OPN promotes in vivo bone formation.

The osteo-inductive effect of C122 was confirmed in a rat calvaria defect model. C-122 was coated on 3D-porous PCL scaffold and implanted in calvaria defects of Wistar rats. Fig.10A-D showed the results from μCT analysis after a healing period of 4 and 8 weeks. The 3D porous scaffold supported new bone formation when compared to the sham operated defect or blank control. Quantitative analysis from μCT micrographs revealed that the amount of new bone was significantly increased when C122-coated scaffold was used as compared to those found in uncoated scaffold (Fig.10E). Histomorphometric analysis confirmed the results from μCT analysis (Fig.10F). Sections of calvaria stained with Masson's Trichrome showed an increase of newly formed bone within the scaffold. Quantitative analysis of the histological sections support that the inductive effect was apparent at both time points of analysis, 4 and 8 weeks (Fig.10G).

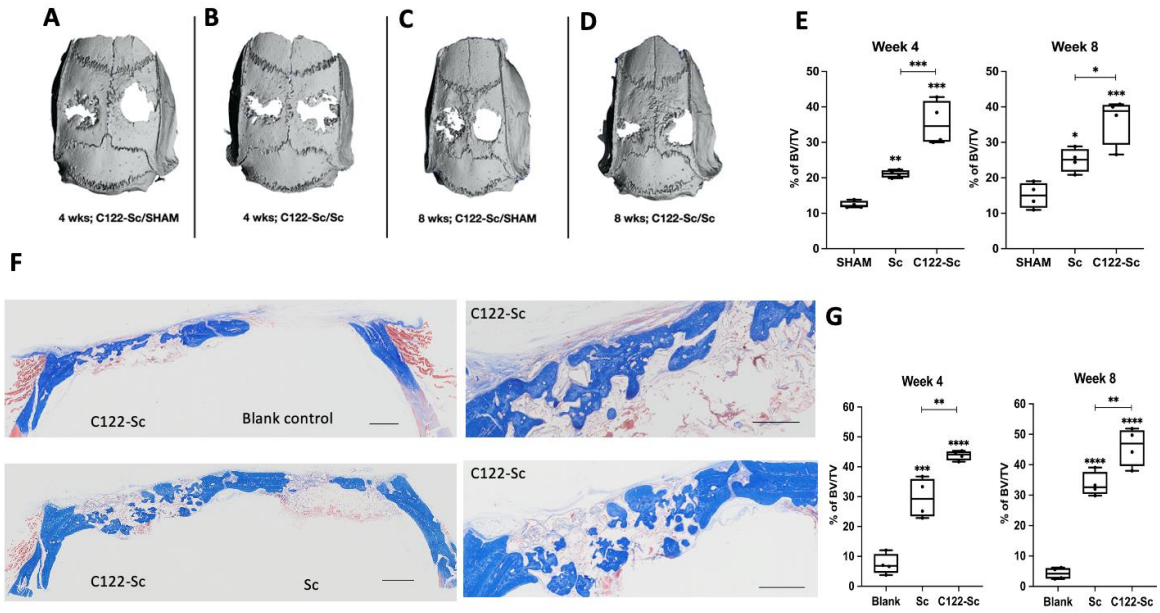

Figure 10. C122-coated PCL scaffold supported new bone formation in rat calvaria model.

Two circular defects with a 5-mm diameter were created in calvariae of Wistar rats. The uncoated scaffold (Sc) or C122-coated scaffolds (C122-Sc) were embedded into the defect and kept for 4 and 8 weeks. The amount of new bone formation was monitored by μCT analysis. Fig. 5A-D are from μCT showing new bone formation within the defect at 4 (**A-B**) and 8 (**C-D**) weeks. In Fig. 10(**A**) and (**C**): The defect on the left side of the calvaria is with C122-Sc and on the right side is the sham-operated control (blank control) defect. In Fig. (**B**) and (**D**): The defect on the left side of the calvaria is with C122-Sc and on the right side is Sc. The amount of new bone formation was calculated and shown in (**E**). C122-Sc significantly induced new bone formation compared to Sc and sham operated defects ($p < 0.05$). F) Histological sections of calvariae were stained with Masson's Trichrome. The results showed the presence of fibrous tissue in blank control defects and new bone formation in defects filled with both Sc and C122-Sc. Bar at the right conner of the figure = 1 mm. The amount of new bone formation was assessed and is shown in (G). *, **, *** and **** indicated the significant compared to blank control at $p < 0.05$, 0.01, 0.001 and 0.0001, respectively.

3.5 Discussion

In this study, we demonstrated the osteogenic inductive capacity of FL-OPN. This capacity appears to occur via an interaction between the calcium-binding domain of OPN and the ALK-1 receptor. This conclusion is supported by the results obtained with a specific inhibitor of ALK-1, *ACVRL1* knockdown, the molecular docking model, and the ability of OPN-construct, C122, which contains a calcium-binding domain, to increase the expression of *OSX* and p-Smad-1, while deletion of the calcium-binding domain (C122δ) did not. Finally, in vivo study results demonstrated the ability of C122-coated scaffold to support new bone formation in rat calvaria model.

Osteopontin contains at least three functional domains (Wai and Kuo 2008). Both integrin binding domain and heparin binding domain could interact with αVβ3 integrin and heparin-like glycosaminoglycans or CD44 on the cell surface (Denhardt and Noda 1998, Giachelli and Steitz 2000, Kon, Ikesue et al. 2008) However, the interaction between CaBD and cell surface receptor was never been shown. The results of this study demonstrated for the first time the possibility that CaBD of OPN might be able to interact with ALK-1, the BMP-9 receptor and promote osteogenic differentiation by PDL cells.

ALK-1 or activin receptor-like kinase 1 is a member of transforming growth factor beta (TGF-b) receptor superfamily. It has been demonstrated that upon the engagement between ALK-1 and its ligands, BMP9 and BMP10, could induce the activation of SMAD signal transduction pathway (Townson, Martinez-Hackert et al. 2012). ALK-1 activation could lead to the phosphorylation of SMAD 1, 5, 8 that will form complex with SMAD 4 then enter translocate into the nucleus to regulate related gene expression (David, Mallet et al. 2006). In our study, we found that PDL cells seeded on OPN-coated surface could induce the phosphorylation of SMAD 1, 5, 8. Addition of ALK-1 inhibitor as well as the knockdown of ALK-1 using RNAi abolished this SMAD activation. These results support that OPN might be another ligand for ALK-1 receptor.

The OPN-ALK-1 interaction and a subsequent cellular response might depend on the concentration and the form of OPN; matrix-bound or soluble. Here we showed that soluble FL-OPN, even at a high concentration, did not induce *OSX* expression, whereas bound OPN clearly did. These findings appear to support the view that the function of matrix-bound and soluble OPN is quite different. Soluble OPN play roles in inflammatory processes (Ashkar, Weber et al. 2000, Iida, Wagatsuma et al. 2017) and is involved in cardiovascular disease (Fitzpatrick, Severson et al. 1994, Abdalrhim, Marroush et al. 2016), whereas the matrix-bound form play role in cell adhesion, biomineralization and osteogenic differentiation.

Previously, the role of matrix-bound OPN in osteogenic differentiation has been shown to require an interaction with a collagen substrate both in vitro and in vivo (Zurick, Qin et al. 2013, Carvalho, Cabral et al. 2019). This collagen-bound fraction can interact with cells of the osteogenic lineage and thus support bone formation. We now showed that the ability of FL-OPN to support osteogenic differentiation depended on its concentration. A high concentration of coated OPN did not show an inductive capacity, whereas this was apparent with a lower concentration. Our finding might explain the controversy of previous reports showing an inhibitory effect of OPN on bone formation since those studies used the soluble form or high concentrations of the matrix form (Tanabe, Wheal et al. 2011, Singh, Gill et al. 2018). A possible explanation for this discrepancy might be related to differences in protein folding of the soluble and matrix form. Moreover, low and high concentration of matrix OPN might have a different aggregation or different folding properties that will result in the exposure of the particular functional domain (Huang, Bhullar et al. 2001, van der Linden and Venema 2007).

The difference in osteogenic response related to the level of OPN present is of considerable interest. One of the mineralized tissues with a very high expression of OPN is acellular cementum of the tooth root (Bosshardt, Zalzal et al. 1998). In spite of the fact that the periodontal fibroblasts associated with this type of cementum are osteoblast-like

and have the capacity to generate bone, bone tissue is normally not deposited on this type of cementum. It may be hypothesized that the high level of OPN prevents the formation of bone.

Taken together, it is possible that the osteogenic inductive capacity of OPN depends on the availability of the CaBD domain to interact with ALK-1 on the cell surface. This availability might depend on protein concentration or specific interaction of OPN and other extracellular matrix constituents within the matrix. In addition, the C122, OPN construct that contain CaBD could induce the expression of *OSX* and increase the level of p-Smad1, the signaling target of ALK-1. Deletion of CaBD in C122δ could not induce p-Smad-1 Moreover, this activation was inhibited by inhibiting ALK-1. These results support the possible interaction of CaBD in C122 and ALK-1. Finally, our in vivo experiment further supports the role of C122 on bone formation which support the potential of C122 to be used in periodontal regeneration.

In conclusion, we have shown here that rhOPN induces the osteogenic differentiation of PDL cells. This ability proved to depend on the concentration and the way it was presented to the cells: bound to the matrix or not bound. The inductive capacity of OPN was shown to occur by an interaction between CaBD of OPN and the ALK-1 receptor. The potential of CaBD of OPN for bone tissue engineering was also demonstrated in rat calvarial model.

3.6 Footnotes

[*]; Thermo Fisher Scientific Nunc, Rochester, New York, USA.

[†]; Sigma, St. Louis, Missouri, USA.

[‡]; Gibco, Waltham, Massachusetts, USA.

[§]; Abcam, Cambridge, UK.

[‖]; Pierce Biotechnology, Rockford, Illinois, USA.

[¶]; Cell Signaling Technology, Inc., Danvers, Massachusetts, USA.

[#]; GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania, USA.

[**]; Dharmacon, Lafayette, Colorado, USA.

[††]; μCT 35 SCANCO MEDICAL, SCANCO Medical AG, Fabrikweg, Switzerland.

[‡‡]; Leica Biosystems Richmond Inc, Richmond, Illinois, USA.

[§§]; Axio Observer Z1 and ZEN pro, ZEISS International, Germany.

3.7 Acknowledgements

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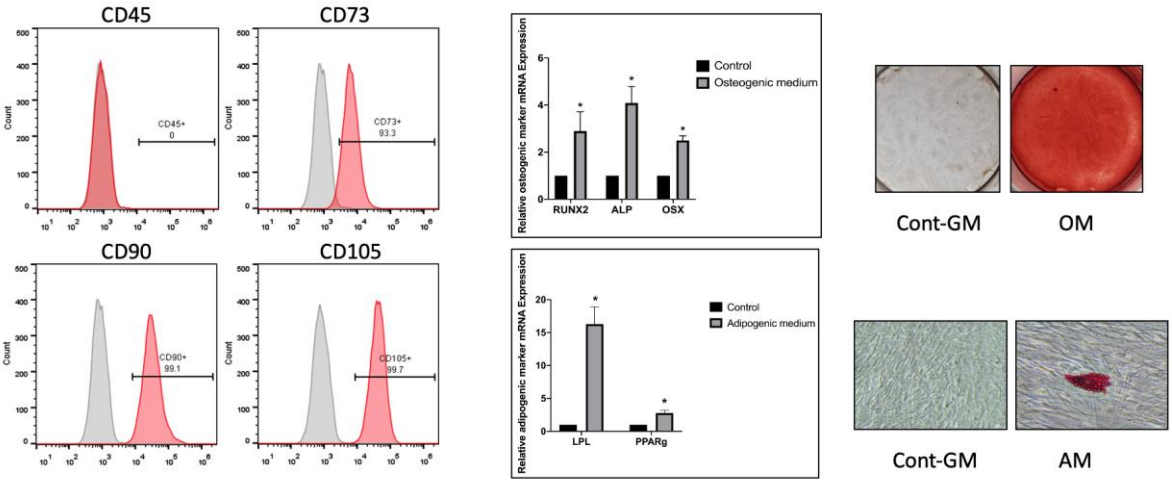
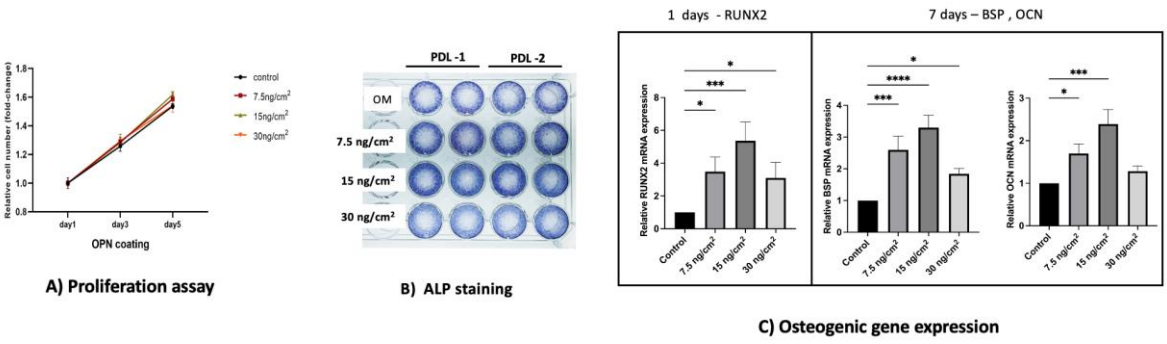
3.8 Supplementary data 1



Supplementary Fig.1.1 Characteristics of human PDL cells

PDL cells were subjected to flow cytometry analysis for the expression of mesenchymal markers. The results showed that PDLSCs highly expressed CD73, CD90 and CD105 but not CD45. PDL cells could differentiate into osteoblast-like or adipocyte-like cells when induced with osteogenic or adipogenic medium, respectively. This differentiation was judged by an increased expression of osteogenic related genes such as *RUNX2, ALP, OSX* and adipogenic related genes such as *LPL* and *PPARγ* as well as the positive staining by Alizarin red S and oil red-O. * indicates a significant difference compared to control at $p < 0.05$.

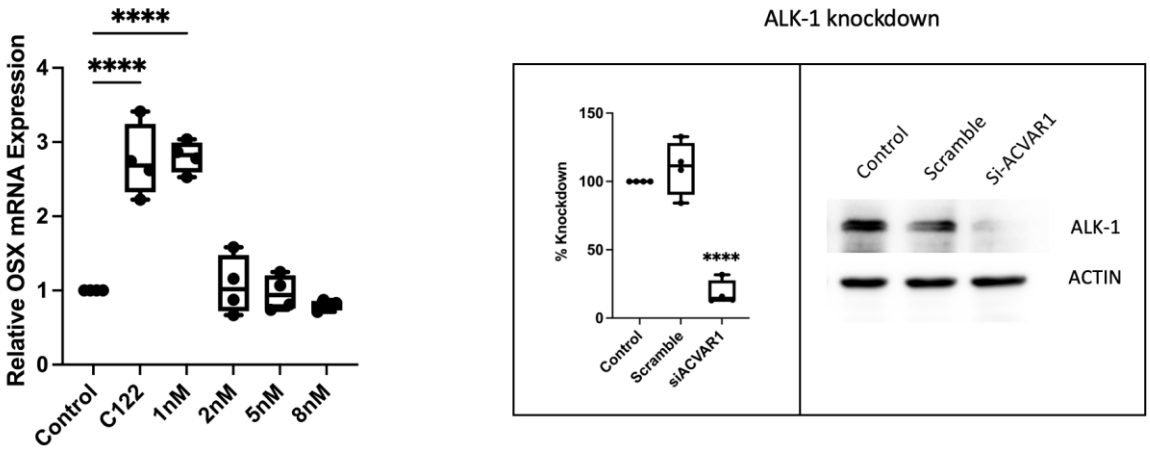


Supplementary Fig.1.2 Response of PDL to plant-produced OPN coated surface.

A) PDL cells were seeeded on the plastic surface (control) or on cultured plates coated with 7.5, 15 and 30 mg/ml at a density of $5x10^4$ cells/well/ml in 24 well plates. After 24 hours, the medium was changed to medium containing 2% serum. The cell number was determined by MTT assay after 1, 3 and 5 days. A slightly –but not significant- increased number of cells was noted when seeded on OPN at 15 and 30 ng/ml (n=3)

B) Cells were seeded as described in (A) for 24 hours. The medium was changed to osteogenic medium and ALP staining was performed using the NBT/BCIP tablets kit (Roche Diagnostics GmbH Mannheim, Germany) after 48 hours. (n=2)

C) Cells were seeded as described and the mRNA expression was determined for *Runx2, BSP* and *OCN* after 1 and 7 days cultured in osteogenic medium, respectively. *OCN* - osteocalcin, *BSP* – bone sialophosphoprotein. (n=3)



Supplementary Figure 1.3. Knockdown of ALK-1.

ALK-1 mRNA expression was knocked down using a predesigned small interfering RNA (siRNA) specific for ALK-1 (*ACVRL1* gene) and a non-targeting control. Human PDL

cells were plated in six well plates at a density of 50% confluency and transfected with *ACVRL1* or control siRNA using transfection medium. Briefly, PDL cells were pre-incubated with a mixture of siRNA and transfection medium in serum/antibiotic free media for 6 hours prior to the transfection process for another 24 hours. The efficiency of knockdown was determined by RT-PCR and Western Blot analysis.

CHAPTER IV

PERIOSTIN-INTEGRIN INTERACTION REGULATES FORCE-INDUCED TGF-β1 AND α-SMA EXPRESSION BY HPDLSCS

This study is under revision.

Publication: Oral Diseases

Manuscript number: ODI-01-23--12033.R2

4.1 Abstract

Objective Mechanical forces play essential roles in the development, tissue homeostasis, repair, and regeneration of the periodontal ligament (PDL). Periostin (PN), a major matricellular PDL protein, modulates the remodeling of the PDL and bone, especially under mechanical stress. The present study investigated the requirement of PN-integrin signaling in force-induced expression of transforming growth factor-beta 1 (TGF-β1) and alpha-smooth muscle actin (α-SMA, a myofibroblast marker) in human periodontal ligament stem cells (hPDLSCs).

Methods Cells were stimulated with intermittent compressive force using computerized controlled apparatus. Cell migration was examined using *in vitro* scratch assay. The mRNA expression was examined using real-time polymerase chain reaction. The protein expression was determined using immunofluorescent staining and western blot analysis.

Results Stimulation with an intermittent compressive force for 24 hours increased the expression of PN, TGF-β1, and α-SMA, along with increased SMAD2/3 phosphorylation. Knockdown of *POSTN* (PN gene) decreased the protein levels of TGF-β1 and pSMAD2/3 upon force stimulation. *POSTN* knockdown of hPDLSCs resulted in delayed cell migration, as determined by a scratch assay. However, migration improved after

seeding these knockdown cells on pre-PN-coated surfaces. Further, the knockdown of αVβ5 significantly attenuated the force-induced TGF-β1 expression.

Conclusion Our findings indicate the importance of PN-αVβ5 interactions in compressive force-induced TGF-β1 signaling and the expression of α-SMA. Findings support the critical role of PN in maintaining the PDL's tissue integrity and homeostasis.

Keywords: Mechanical force, Periostin, Integrin, Periodontal ligament cells.

4.2 Introduction

It is well documented that mechanical force regulates tissue growth, repair, and remodeling (LeGoff and Lecuit 2015). The importance of mechanical stimulation in periodontal tissue is also well recognized since its function is to perceive and withstand masticatory forces. Periodontal ligament (PDL) cells are mechanosensory and regulate tissue responses to mechanical forces. Mechanical force stimulation regulates hPDLs osteogenic differentiation by secretion of compounds like TGF-β1 and BMP-9 (Manokawinchoke, Pavasant et al. 2019, Tantilertanant, Niyompanich et al. 2019). Similarly to osteogenic differentiation, mechanical stimulation modulates the conversion of fibroblasts into a myofibroblast phenotype, as indicated by the expression of α-SMA (Hinz, Mastrangelo et al. 2001, Hinz and Gabbiani 2003, Yuan, Li et al. 2018). These myofibroblasts generate a tissue contractile force (Hinz and Gabbiani 2003).

Periostin (PN) is a matricellular protein that is expressed in the PDL and periosteum. PN modulates remodeling of the PDL and bone, especially under mechanical stress. This protein is located at sites generally exposed to mechanical loading exerted by mastication or physical exercise, such as teeth and bone (Kudo, Amizuka et al. 2004). A study by Rios et al (Rios, Koushik et al. 2005) showed that oral tissues in *POSTN* null mice could not tolerate mechanical forces. Severe resorption of jaw bones and roots of incisors in these mice was attenuated when masticatory-induced force was decreased by feeding mice with a soft diet. These findings support a possible role of PN in withstanding mechanical forces and suggest the significance of PN in maintaining the PDL function (Rios, Koushik et al. 2005, Rios, Ma et al. 2008).

In the context of the extracellular matrix (ECM), PN functions as a matrix-cell attachment protein that binds transmembrane heterodimer receptors, such as αvβ3 and αvβ5 integrins, thereby regulating cell adhesion and motility. The current knowledge of PN signaling mainly comes from studies in bone and cancer cells. As mentioned above, PN primarily binds to αvβ3 and αvβ5 integrin, and activation of PN-integrin was shown to,

directly and indirectly, regulate Wnt-β catenin pathway via AKT and FAK-mediated signaling pathways (Tkatchenko, Moreno-Rodriguez et al. 2009, Morra and Moch 2011). PN, a highly conserved matricellular protein in the ECM, alters collagen fibrillogenesis, fibril diameter, and collagen crosslinking (Norris, Damon et al. 2007) by enhancing the enzymatic activation of lysyl oxidase. It also changes the distribution of collagenous and non-collagenous ECM proteins such as fibronectin and tenascin C (Tabata, Hongo et al. 2014), emphasizing the importance of PN in cellular responses to external stimulation.

After mechanical forces are exerted on tissues, mechanotransduction signals are transmitted through the ECM, and these signals influence organogenesis and homeostasis (Orr, Helmke et al. 2006). When a physical force is applied to the cell surface, it is transmitted through the cell membrane and then quickly moves into the cytoplasm (Vogel and Sheetz 2006). Yet, the complex cellular mechanisms that sense those mechanotransduction signals and trigger intracellular molecular changes and differential gene expression are still unclear. We hypothesized that periostin is the linkage molecule in the ECM involved in the transition of an outside-in mechanotransduction signal through its own specific receptor.

In the present study, we aimed to investigate the involvement of PN -integrin interactions in response to intermittent compressive force (ICF) stimulation in human periodontal ligament stem cells (hPDLSCs) and to determine the potential regulatory mechanisms.

4.3 Materials and methods

4.3.1 hPDLSCs isolation and culture

The PDL tissue collection and cell isolation protocol were approved by the Human Research Ethical Committee of the Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2022-092). The third molars from healthy patients (aged 20 to 35) were surgically removed according to standard dental treatment protocol and the patient's treatment plan. PDL tissue fragments were collected by scraping them from the root of the third molars. The tissues samples were explanted and cultured in a high glucose-Dulbecco modified eagle medium (DMEM, Gibco, Waltham, MA) containing 10% fetal bovine serum, 2 mM of L-glutamine, and penicillin (100 U/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml). They were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. After reaching 100 % confluency, hPDLSCs were detached and sub-cultured at a ratio of 1:3. The isolated hPDLSCs were characterized by the expression of stem cell surface markers CD73, CD90, CD105, and negative for hematopoietic derived cell marker CD45 by flow cytometry. More than 80% of CD73, CD90, and CD 105 positive cells were considered for the experiment (see Supplementary Fig. 2.1). Cells from the third to the sixth passages were used, and cells from at least 4 different donors were employed in all experiments.

4.3.2 Flow cytometry

hPDLSCs were detached from the culture plate using 0.25% Trypsin-EDTA with less than 1 min exposure. The cells were counted and 5 $x10^5$ cells were stained with FITC-conjugated anti-human CD73, CD90, CD105 antibody and PerCP-conjugated antihuman CD45 (Abcam, Cambridge, UK). Mouse IgG2a kappa antibody was used as isotype control. The stained cells were analyzed by FACSCalibur followed by Cell Quest software (BD Bioscience, CA.).

4.3.3 Gene knockdown

Predesigned small-interfering RNA (siRNA) specific to the promoter region of POSTN gene (*POSTN*), integrin αVβ3, αVβ5 gene, and non-targeting control (scramble) were purchased (Dharmacon, CO). hPDLSCs were plated in 6-well-plates at a density of $3x10^5$ cells per well and transfected with targeted siRNA or scramble using Lipofectamine 2000 (Invitrogen, MA) following the manufacturer's instructions. Briefly, the attached cells were pre-incubated with a mixture of siRNA and transfection medium in serum- and antibiotic-free media for 48 hours before being used. The knockdown efficiency was checked by quantitative real-time PCR and western blot analysis.

4.3.4 Intermittent compressive force stimulation

An intermittent compressive force (ICF) was applied for 24 hours. A compressive force apparatus was used, as described previously (Manokawinchoke, Limjeerajarus et al. 2015, Manokawinchoke, Limraksasin et al. 2022). The compressive force was generated by applying load on the culture medium using a pestle moving up and down in a matched-size well plate. A computer-controlled apparatus was used to calculate weight and monitor the application of force by a balance placed underneath the well plate. For intermittent loading, the cycle was set as shifting between a 1-second load and 2 seconds release, yielding a loading cycle of approximately 14 rounds/minute. The force intensity was set at 1.5 g/cm^2.

4.3.5 Real-time PCR analysis

Total RNA was extracted using a total RNA isolation reagent kit (GeneAll Biotechnology, Seoul, South Korea) and converted to cDNA using the reverse transcription kit (Bio-Rad, CA). Quantitative real-time PCR was performed using a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). Cycling conditions were set at 95°C for

30s, followed by 40 cycles of 95°C for 3s and 60°C for 30s. PCR was calculated based on the quantitation cycle (Cq), followed by the normalization to the expression of the glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*). Primers had the following sequences,

GAPDH:(F)*5'CACTGCCAACGTGTCAGTGGTG3'*,(R)*5'GTAGCCCAGGATGCCCTTGAG3*

ACTA2: (F)*5'GACAATGGCTCTGGGTCGTAA3'*, (R)*5'CTGTGCTTCGTCACCCACGTA3'*

TGFB1:(F)*5'GGATACCAACTATTGCTTCAGCT3'*,(R)*5'AGGCTCCAAATGTAGGGGCAGG G3'*

and *POSTN*: (F)*5'TGTTGCCCTGGTTATATGAG3'*, (R)*5'ACTCGGTGCAAAGTAAGTGA3'*.

4.3.6 Western blot analysis

hPDLSCs lysate was extracted using RIPA buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). A BCA protein assay kit was used to measure total protein. Each sample contained an equal amount of protein and was loaded into 12% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked in 4% bovine serum albumin in tris-buffered saline with 0.1% tween-20 and incubated with primary antibodies against human PN, α-SMA, TGF-β1 (Abcam, Cambridge, UK), SMAD2/3, and pSMAD2/3 (Santa Cruz Biotechnology, Dallas, TX) (Working concentration: 100 ng/ml), paralleled with an anti-human actin antibody. β-actin was used as an internal control. After washing with tris-buffered saline, the membrane was incubated with a secondary antibody that contained HRP (20 ng/ml) (Cell Signaling Technology, Danvers, MA). The signals were activated by a substrate for enhanced chemiluminescence and captured by an image analyzer. Band density was measured using the Armersham 680 imaging software (GE HealthCare, Chicago, IL).

4.3.7 Immunofluorescence staining

Cells were plated on poly-lysine pre-coated round cover glasses. Cover glasses were placed into 6-well-plates, and after 24 hours of ICF stimulation, cells were fixed with 4% paraformaldehyde. Fixed cells were incubated with anti-human α-SMA rabbit IgG antibody (1 μg/ml) (Abcam, Cambridge, UK), followed by anti-rabbit IgG Alexa Fluor 488 conjugated (50 ng/ml) (Cell Signaling Technology, Danvers). Actin filaments were stained with rhodamine-conjugated phalloidin (100 ng/ml) (Abcam, Cambridge, UK), and the cells were covered with an antifade mounting medium containing DAPI. Isotype control staining was performed in parallel with the negative control. The staining pattern was examined with an Axio Observer Z1 and ZEN pro (Zeiss International, Oberkochen, Germany).

4.3.8 Scratch assay

Culture plates were covered by 250 ng/ml of POSTN recombinant protein solution (Sigma-Aldrich, St. Louis, MO) for 16-18 hours, the plates were dried at room temperature and used immediately. Cells were seeded into 6-well-plates for 24 hours before performing the transfection process for 48 hours. Both normal and knock-down cells were scratched off using a 1000 μl pipette tip (1.2 mm diameter approximately). The medium was changed to DMEM containing 2% fetal bovine serum. A closing area was observed and measured using a microscope after 0, 24, and 48 hours of culture. The closing area was calculated using ImageJ software.

4.3.9 Cell viability assay

Cells were incubated with CCK-8 solution according to the manufacturer's instructions. To detect the proliferation signal, a conditioned medium was collected, and the optical density was measured at 460 nm wavelength. Measurements were performed at 0, 24, 48, and 72 hours.

4.3.10 Statistical analysis

Each experiment was performed with cells from four different donors. All statistical analyses were carried out using GraphPad Prism version 9 software (GraphPad Software Inc, San Diego, CA). Data were analyzed using nonparametric Mann-Whitney and Kruskal-Wallis tests, followed by Dunn's multiple comparisons. All data were presented as box and whisker plots. A $p < 0.05$ was regarded as statistically significant.

4.4 Results

4.4.1 ICF induced expression of PN, TGF-β1, and α-SMA in hPDLSCs

hPDLSCs were stimulated with intermittent compressive force (ICF) for 24 hours. Total RNA and proteins were collected and analyzed for expression of PN, TGF-β1, and α-SMA by quantitative real-time PCR and western blot, respectively. A significantly increased expression of *POSTN* (gene name of PN), *TGFB1* and *ACTA2* (gene name of α-SMA) mRNA was found in cells treated with ICF (Fig. 11A). Correspondingly, the protein levels of PN, TGF-β1, and α-SMA were markedly upregulated (Figs 11B and 11C). Moreover, the level of SMAD2/3 phosphorylation (pSMAD2/3: downstream target of TGF-β1) was increased in the ICF stimulated group compared to the control, suggesting activation of the TGF-β1 signaling pathway.

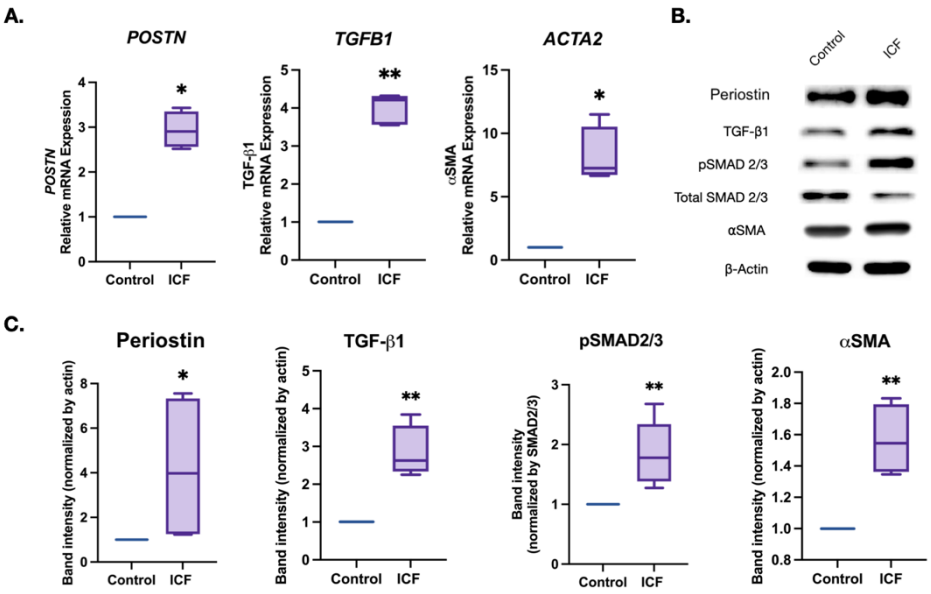


Figure 11. Intermittent compressive force (ICF) induces the upregulation of PN, TGF-β1, and α-SMA in hPDLSCs.

hPDLSCs were seeded in a 6-well-plate and subjected to ICF for 24 hours. (A) *POSTN*, *TGFB1*, and *ACTA2* mRNA levels after ICF stimulation compared to control. (B) Western

blot (WB) analysis of PN, TGF-β1, α-SMA, and SMAD2/3 phosphorylation (pSMAD2/3) expression. (C) WB band density quantification. Data are presented as box and whisker plots from four biological replicates using hPDLSCs obtained from four molars. * $p < 0.05$ and ** $p < 0.01$ compared to the control.

4.4.2 Low levels of endogenous POSTN influence hPDLSCs migration and expression of TGF-β1 and α-SMA

The importance of PN for the function of hPDLSCs was investigated following the knockdown of POSTN expression using siRNA. The decrease in expression of *POSTN* genes and protein was confirmed (Supplementary Fig. 2.1). The *POSTN* knockdown cells (*POSTN* KD) exhibited a delayed cell migration, as shown by the scratch assay (Figs 12A, C). Next, we evaluated the effect of exogenous PN on cell migration. Therefore, cells were seeded on PN-coated surfaces, and the data showed a partial rescue of cellular migration on these coated plates (Figs 12B, D). It was interesting to note that *POSTN* KD cells showed a normal viability and proliferation rate compared to control hPDLSCs (see Supplementary Fig. 2.2).

POSTN knockdown cells changed the expression profiles of genes and proteins stimulated by ICF: TGF-β1, α-SMA, and pSMAD2/3 (Figs 12E-G). *ACTA2* mRNA levels were significantly decreased while mRNA expression of *TGFB1* was not changed when compared to knockdown control (siControl) (Fig. 12E). This was not related to an increase in TGF-β1 protein expression (Figs 12F-G). No change of α-SMA protein expression was observed in POSTN knockdown cells. A decreased expression of the level of pSMAD2/3 protein was apparent in *POSTN* KD cells (Figs 12F-G).

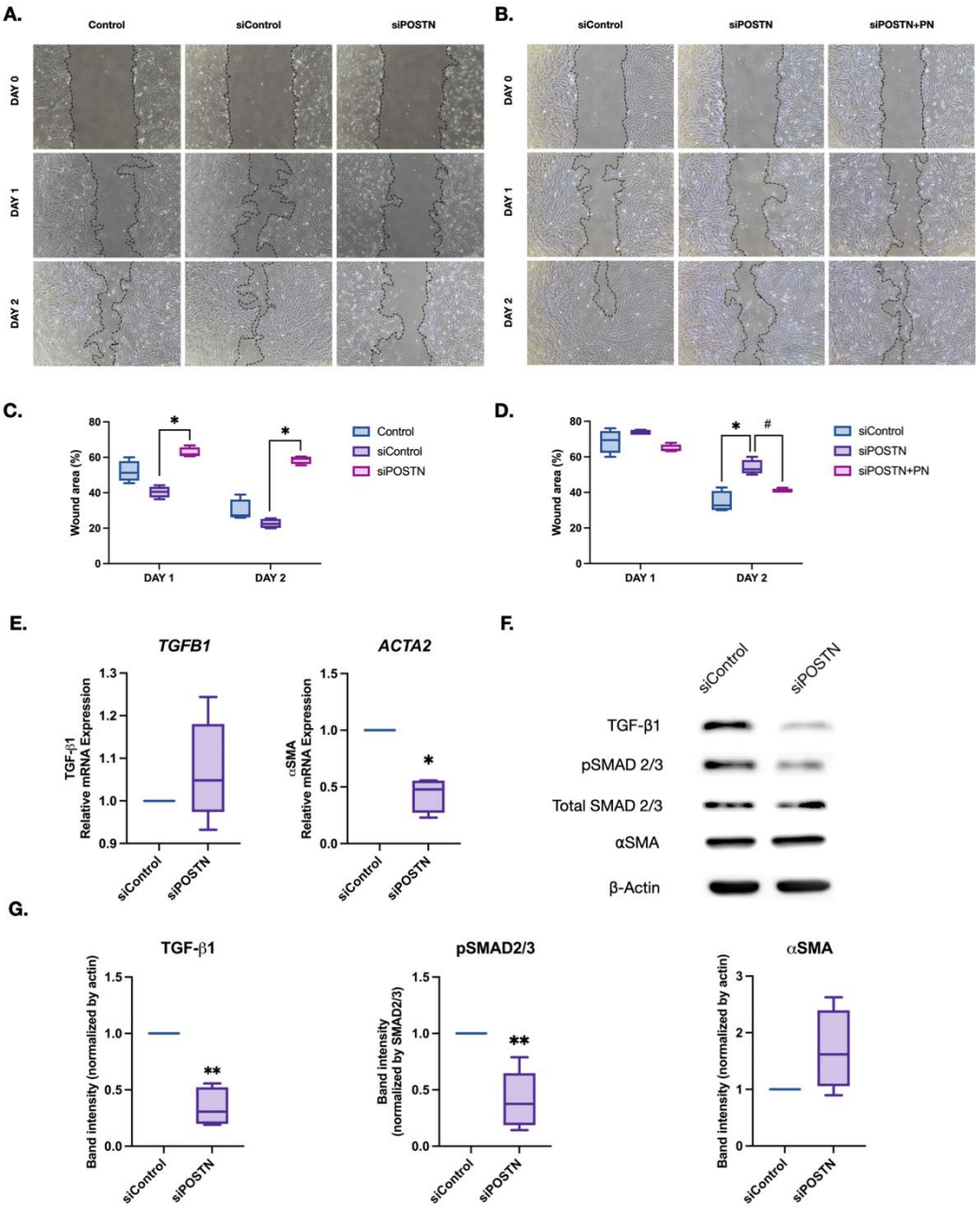


Figure 12. *POSTN* knockdown suppresses hPDLSCs migration and expression of TGF-β1 and α-SMA.

hPDLSCs were seeded in a 6-well-plate and transfected with *POSTN*-specific siRNA. (A) Scratch assay of the *POSTN* knockdown cells. (B) Scratch assay of the *POSTN* knockdown cells on pre-PN-coated surfaces. (C and D) Scratch area quantification. (E) *TGFB1*, and *ACTA2* mRNA expression of *POSTN* knockdown cells. (F) WB analysis of TGF-β1, pSMAD2/3, and α-SMA expression. (G) WB band density quantification. Data

are displayed as box and whisker plots from four biological replicates using hPDLSCs obtained from four molars. * $p < 0.05$ and ** $p < 0.01$ when compared to siControl. # $p < 0.05$ when compared to siPOSTN.

4.4.3 The absence of POSTN abrogated the expression of ICF-inducible molecules in hPDLSCs

Upon ICF stimulation, the expression of TGF-β1/SMAD2/3 and α-SMA was induced in hPDLSCs. The *POSTN* knockdown hPDLSCs exhibited a lower baseline expression of TGF-β1, α-SMA and pSMAD2/3 compared to normal hPDLSCs. Moreover, *POSTN* knockdown cells did not respond to ICF stimulation (Figs 13A-C). *POSTN* knockdown hPDLSCs were subjected to ICF for 24 hours. A decrease of α-SMA, TGF-β1, and pSMAD2/3 levels was found in *POSTN* knockdown cells under ICF stimulation compared to the siControl (Figs 13A-C). Immunofluorescence staining of α-SMA also showed lower intensity in POSTN KD cells compared to normal cells in both normal and ICF stimulation conditions (Fig. 13D).

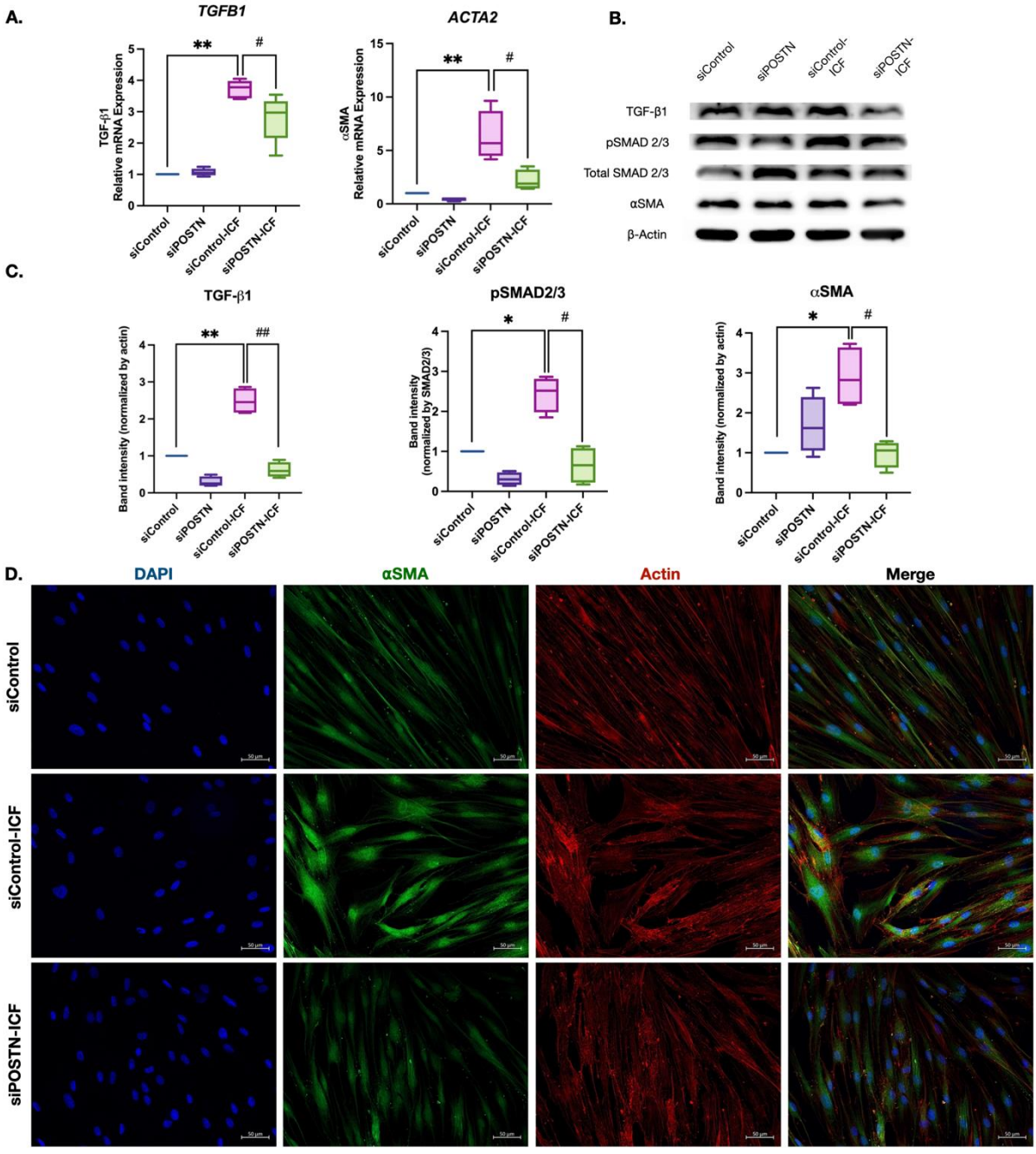


Figure 13. POSTN is essential for hPDLSCs response to ICF stimulation.

POSTN knockdown hPDLSCs were seeded and stimulated with ICF for 24 hours. (A) *TGFB1*, and *ACTA2* mRNA expression. (B) TGF-β1, pSMAD2/3, and α-SMA WB analysis. (C) WB band density quantification. (D) Immunofluorescence staining of α-SMA and Actin. Data are displayed as box and whisker plots from four biological

replicates using hPDLSCs obtained from four molars. * $p < 0.05$ and ** $p < 0.01$ when compared to siControl. # $p < 0.05$ and ## $p < 0.01$ when compared to siControl-ICF.

4.4.4 Periostin regulates ICF-induced TGF-β1 signaling via αVβ5 integrin

To evaluate how PN regulates ICF-induced expression of TGF-β1 and α-SMA, hPDLSCs were transfected with siRNA targeting integrin αVβ3 (*ITGB3*) and αVβ5 (*ITGB5*). The decrease of αVβ3 and αVβ5 protein levels was confirmed by western blot analysis (Supplementary Fig. 2.3). Interestingly, *ITGB5* knockdown cells stimulated by ICF showed a significantly decreased protein expression of TGF-β1 and pSMAD2/3 compared with those siControl cells stimulated with ICF (Fig. 14A-B). While some reduction of TGF-β1 and pSMAD2/3 was also seen in *ITGB3* knockdown cells upon ICF stimulation; this was not different from siControl cells stimulated with ICF (Fig. 14A-B). These findings suggest that ICF induced expression of TGF-β1 and activation via PN-integrin occurs via αVβ5 interaction. With respect to the expression of α-SMA, no significant changes in α-SMA levels were found when *ITGB3* and *ITGB5* knockdown cells were stimulated with ICF. These findings suggest that expression of α-SMA depends on a different regulatory pathway.

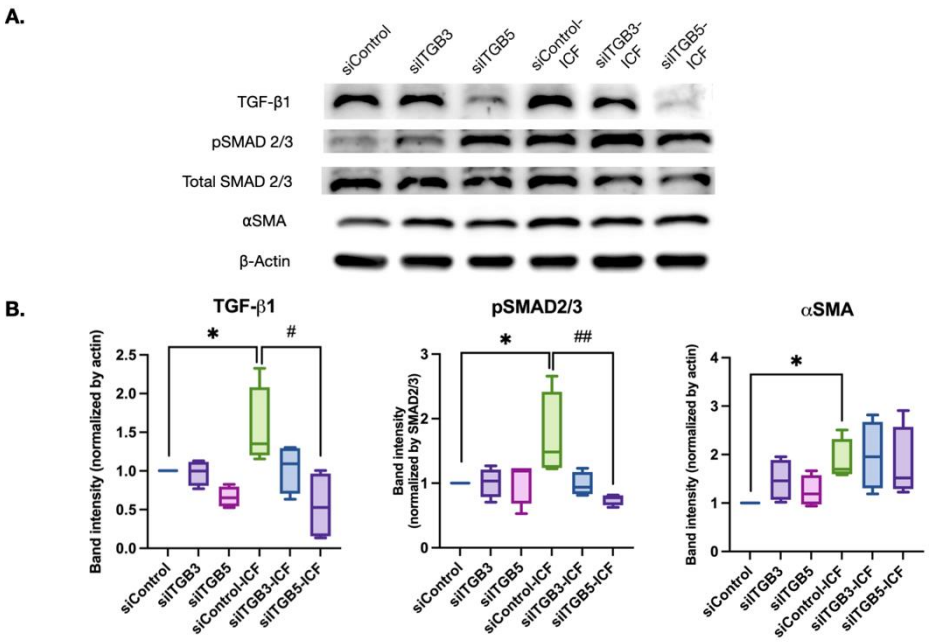


Figure 14. POSTN regulated intermittent compressive force (ICF)-induced TGF-β1 expression via integrin αVβ5.

Both *ITGB3 and ITGB5* knockdown hPDLSCs were seeded and stimulated by ICF for 24 hours. (A) WB analysis of TGF-β, pSMAD2/3, and α-SMA of both *ITGB3* and *ITGB5* knockdown cells under ICF stimulation. (B) WB band density quantification. Data are displayed as box and whisker plots from four biological replicates using hPDLSCs obtained from four molars. * $p < 0.05$ when compared to the siControl. # $p < 0.05$ and ## $p < 0.01$ when compared to siControl-ICF.

4.5 Discussion

To the best our knowledge, this study is the first to demonstrate that the matricellular protein PN modulates the response of hPDLSCs to intermittent mechanotransduction via a compressive force. ICF markedly induced the expression of TGF-β1, pSMAD2/3, and α-SMA; a process that was attenuated by the knockdown of POSTN. Moreover, ICF-induced TGF-β1/SMAD2/3 expression was also reduced upon suppressing the interaction of PN with its receptor αVβ5 integrin after knocking down this receptor. These findings suggest that ICF could promote TGF-β1/SMAD2/3 expression via PN-αVβ5 outside-in signaling. Interestingly, ICF-induced α-SMA expression seems to be independent of integrins αVβ3 and β5, indicating an alternative regulatory pathway.

It is well known that different components of the ECM play a crucial role in mechanotransduction by transmitting extracellular cues into intracellular biochemical signals (Orr, Helmke et al. 2006). As mentioned in the Introduction, matricellular proteins like PN can mediate these mechanisms. Here, we showed that the knockdown of *POSTN* attenuated the expression of TGF-β1 and α-SMA of hPDLSCs; an effect found either with or without ICF stimulation. Since its discovery, it was assumed that PN protein was somehow associated with mechanical stimuli because this protein is located at sites that are generally exposed to mechanical stress (Kudo, Amizuka et al. 2004), and, in loss-of-function POSTN mouse models, teeth and jaw bone could not tolerate mechanical forces (Rios, Ma et al. 2008). PN also plays a crucial role in the accumulation and arrangement of PDL-ECM adhesion molecules, which are directly involved in PDL integrity and energy absorption of the mechanical forces (Rios, Koushik et al. 2005). Here, we propose that the absence of PN affects the ECM structure and the responses to mechanical stresses, which could impact the induction of TGF-β1 and α-SMA in hPDLSCs upon mechanical stimulation.

It was of considerable interest to find an inductive effect of ICF on the expression of α-SMA by hPDLSCs. α-SMA is a marker for myofibroblast differentiation which is upregulated during the phenotypic transition of fibroblasts into the myofibroblasts (Shirol and Shirol 2012). Myofibroblasts play an essential role in wound healing and in the development of fibrosis after injury by altering the organization of the ECM (Gabbiani 2003). In the periodontium, α-SMA is highly expressed in PDL fibroblasts (Lekic, Rojas et al. 2001). This protein has been shown to play a potential role in PDL fibroblast differentiation along with modulating fibroblast contractility (Hinz, Celetta et al. 2001). Thus the PDL fibroblast could function like a myofibroblast in PDL. Since fibroblast-myofibroblast differentiation is one of the critical factors in regulating ligament tissue homeostasis, ICF might play a crucial role in PDL homeostasis by influencing α-SMA expression and thus steering PDL fibroblast-myofibroblast differentiation.

The data presented in this study support our previous findings that ICF induces the expression of TGF-β1 in hPDLs (Manokawinchoke, Pavasant et al. 2019, Manokawinchoke, Pavasant et al. 2021, Manokawinchoke, Chareonvit et al. 2022). TGF-β1 has been shown to be a significant inducer of fibroblast and myofibroblast differentiation by stimulating α-SMA expression (Tomasek, Gabbiani et al. 2002, Pakyari, Farrokhi et al. 2013, Weiss and Attisano 2013). Here, we propose that ICF might regulate PDL fibroblast-myofibroblast differentiation via the regulation of TGF-β1 signaling. Although our study showed an ICF-induced TGF-β1 and α-SMA expression, a possible causal relationship between TGF-β1 and α-SMA still needs to be assessed. Moreover, ICF also induced SMAD2/3 phosphorylation, suggesting that ICF-induced TGF-β1 downstream signals could activate its canonical pathway (Nagaraj and Datta 2010).

In response to external stimuli, ECM receives and sends signals to regulate cellular activity via cell surface integrins. Here, ICF-induced TGF-β1 expression was attenuated in αVβ5 knockdown cells, suggesting a relationship between this integrin and TGF-β signaling pathway upon mechanical compression forces. This observation is

in accordance with a previous report that unveiled a cross-talk between this integrin and TGF-β signaling (Nagaraj and Datta 2010). In the latter study, dynamic compression induced cartilage formation via activation of TGF-β/SMAD. This outcome was accompanied by suppression of β1 integrin in non-hypertrophic chondrocytes (Nagaraj and Datta 2010). Altogether, our findings indicate that ICF enhances TGF-β signaling through αVβ5, which may be activated via PN-integrin outside-in signaling.

One of the essential functions of PN is to promote wound and tissue remodeling (Kudo 2011, Hara, Yokota et al. 2018). During these processes, cells move throughout the tissue, a process which was shown in the present study to depend on the presence of PN. Knockdown of POSTN partially inhibited hPDLSCs cell migration, a process that was precluded following the addition of exogenous PN. In line with this finding is the study by Matsuzawa and co-workers (Matsuzawa, Arai et al. 2015), who showed the migration of human PDL fibroblasts to depend on the activation of integrins. Here, PN was found to communicate with these hPDLSCs via activation of αVβ5 integrin.

Taken together, PN plays an essential role in hPDLSCs migration. It modulates the response of hPDLSCs to ICF by inducing both gene and protein expression of TGF-β1 and α-SMA. The increased levels of SMAD2/3 phosphorylation upon ICF stimulation indicated the activation of the TGF-β1 signaling pathway. Finally, the force-induced TGF-β1/SMAD2/3 expression occurred via integrin αVβ5, emphasizing the importance of PN in PDL mechanotransduction (Fig. 15).

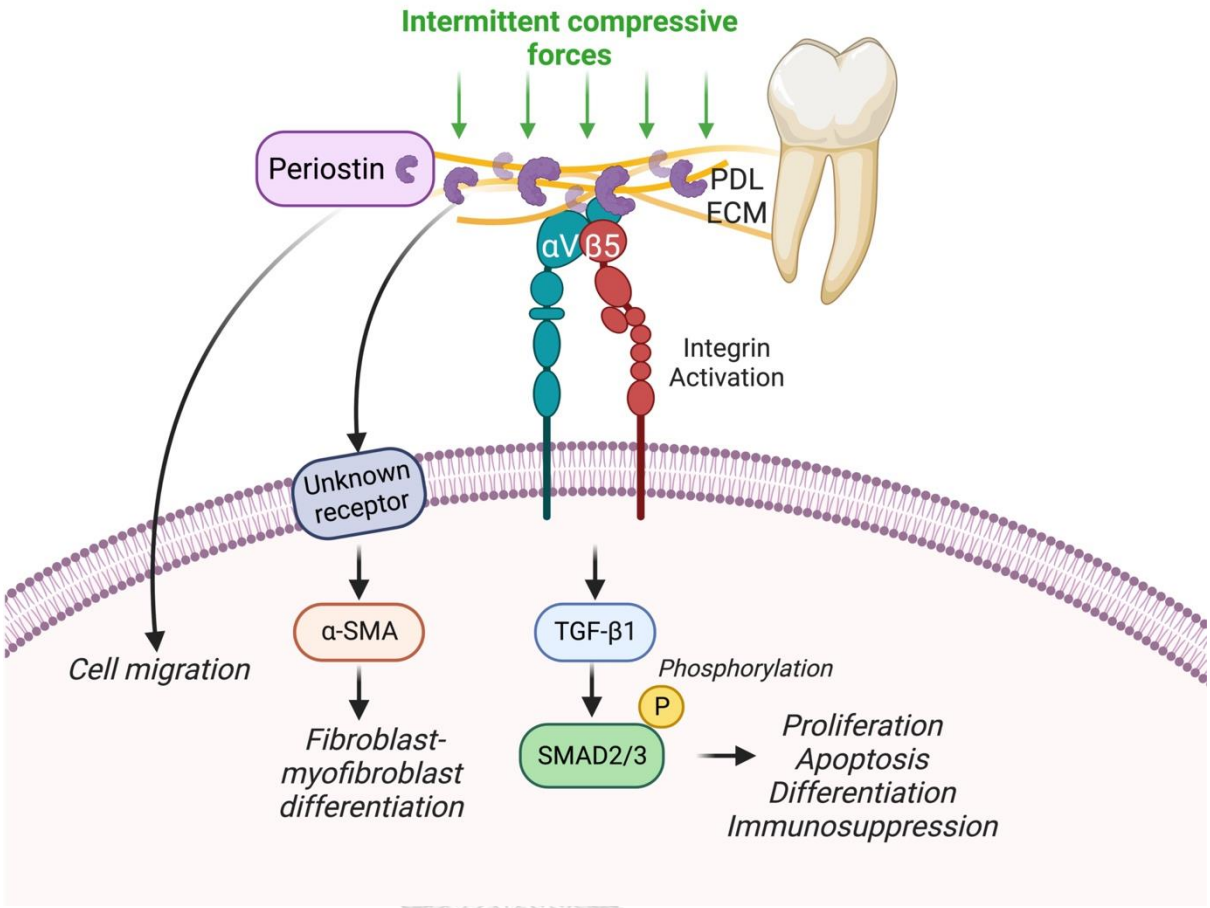


Figure 15. Schematic diagram of PN-regulated intermittent compressive force (ICF)-induced TGF-β1 expression via integrin αVβ5.

The matricellular protein, PN, takes part in hPDLSCs cell migration, and it modulates the response of hPDLSCs to ICF by inducing expression of TGF-β1, pSMAD2/3, and α-SMA. The compressive force-upregulated TGF-β1/SMAD2/3 levels occurred via integrin αVβ5 emphasizing the importance of PN in PDL mechanotransduction. Created with Biorender.com.

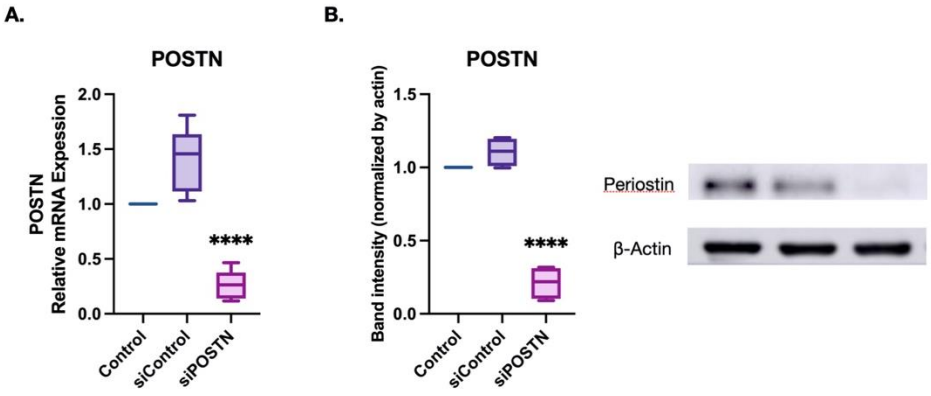
It is well known that PDL is a unique connective tissue located between the tooth and alveolar bone. The PDL functions as a mechanosensory tissue by withstanding the mechanical loading from mastication, deglutition, speech, and orthodontic treatment. Thus, the biological role of POSTN after ICF stimulation of hPDLSCs and its modulatory effects on TGF-β1 and α-SMA expression indicates that POSTN may maintain PDL

tissue homeostasis. Yet, the role of POSTN in regulating other pathways responding to mechanical forces such as shear force, tension, or vibration still requires future investigations. Additionally, the effect of POSTN in 3D models mimicking the PDL is poorly understood and this will entice future research. Ultimately, the discovery of a therapeutic agent to modulate POSTN-integrin interaction could be the key to protect against loss of PDL in patients with impaired masticatory forces. The knowledge gained herein can also assist researchers in understanding of periodontal disease progression upon specific occlusal forces.

4.6 Acknowledgments

The authors thank all staff in the Center of Excellence in Regenerative Dentistry and the Department of Oral and Maxillofacial Surgery, Chulalongkorn University, for the human specimens supported.

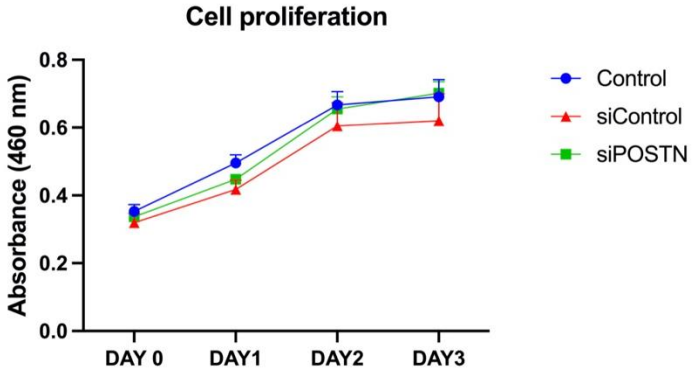
4.7 Supplementary materials 2



Supplementary Figure 2.1. The decreased POSTN level in PDL cells.

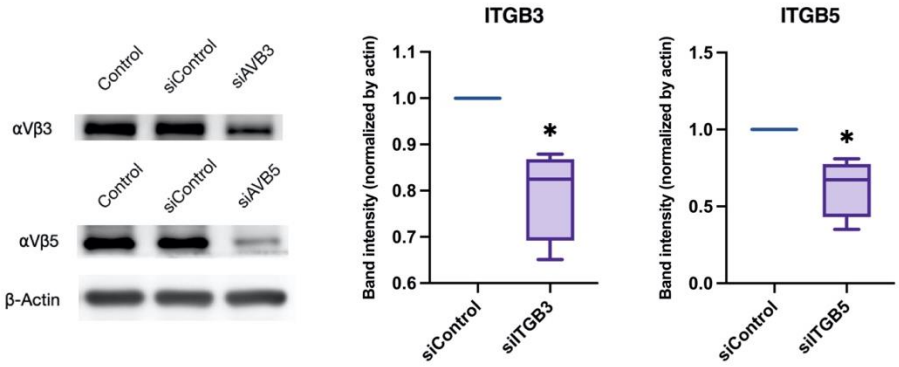
Human PDL cells were subjected to knockdown *POSTN* for at least 48 hours. Both mRNA and protein were decreased showed in A and B, respectively.

**** indicated statistically significant at $p < 0.00001$ compared to the siControl.



Supplementary Figure 2.2. Cell viability assay of *POSTN* knockdown PDL cells.

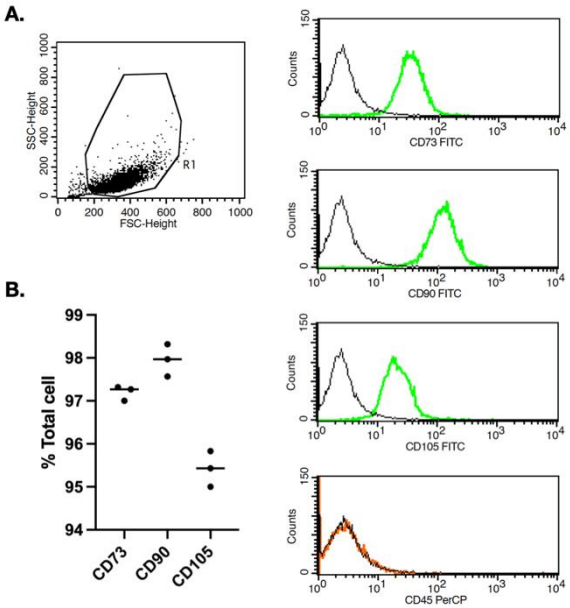
The POSTN knockdown cells showed no significantly different when compared to both Control (normal PDL cells) and siControl (Scramble).



Supplementary Figure 2.3. The decreased ITGB3 and ITGB5 levels in PDL cells.

Human PDL cells were subjected to knockdown ITGB3 and ITGB5, respectively for 48 hours. Both ITGB3 and ITGB5 protein were significantly decreased.

* indicated statistically significant at $p < 0.05$ compared to the siControl.



Supplementary Figure 2.4. PDL cell characterization.

Human PDL cells were probed with surface markers CD73, CD90 and CD105 antibodies, and CD45 was used as a negative control. **A.** Showing % Gated area and the overlayed histogram of each marker compared to isotype control. **B.** % of total positive cells compared to 100% cell count.

CHAPTER V

ALTERATION OF EXTRACELLULAR MATRIX PROTEINS IN ATROPHIC PERIODONTAL LIGAMENT OF HYPOFUNCTIONAL RAT MOLARS

This study is under revision.

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5.1 Abstract

Objectives: The aim of this study was to investigate the effect of mechanical force on possible dynamic changes of matrix protein deposition in the PDL upon in vitro mechanical stimulation and in vivo occlusal forces in a rat model under hypofunctional conditions.

Materials and methods: Intermittent compressive force (ICF) and shear force (SF) were applied to human periodontal ligament cells (PDLSCs). Protein expression of collagen I and periostin (POSTN) was analyzed by western blot technique. To establish an in vivo model, rat maxillary molars were extracted to facilitate hypofunction of the periodontal ligament (PDL) tissue of the opposing mandibular molar. The mandibles were collected after 4-, 8-, and 12-weeks post-extraction and used for μCT and immunohistochemical analysis.

Results: ICF and SF increased the synthesis of POSTN by human PDLSCs. Histological changes in the hypofunctional teeth revealed a narrowing of the PDL space, along with a decreased amount of collagen I, POSTN, and laminin in perivascular structures compared to the functional contralateral molars.

Conclusion: Our results revealed that loss of occlusal force disrupts deposition of some major matrix proteins in the PDL, underscoring the relevance of mechanical forces in maintaining periodontal tissue homeostasis by modulating ECM composition.

Keywords: Extracellular matrix, Collagen I, Periostin, Mechanical force, Hypofunction, Periodontal ligament

5.2 Introduction

The periodontal ligament (PDL) is a specialized soft tissue of the periodontium that connects the tooth to the surrounding alveolar bone. The main function of PDL is to withstand mechanical forces in the occlusal plane generated from muscular driven movements of mastication, speech, and deglutition. Proper mechanical stimulation is an important factor to maintain the integrity and function of the tooth and periodontium (Denes, Bresin et al. 2016). The lack of contact with an opposing tooth could lead to disuse-type of atrophy in the PDL, eventually resulting in tooth loss (Cohn 1965).

Atrophic changes of the PDL-surrounding hypofunctional teeth include narrowing of the periodontal space, collagen disorganization, and vascular constriction. Finally it induces dental root and alveolar bone resorption. (Sringkarnboriboon, Matsumoto et al. 2003, Hayashi, Hayashi et al. 2016). Despite the extensive literature on hypofunctional teeth and their surrounding periodontal tissues, the effect on the PDL's extracellular matrix (ECM) components remains elusive.

In vitro mechanical stimulation is known to regulate changes in the ECM protein patterns. For example, static compressive forces upregulate osteopontin, one of the bone's major non-collagenous proteins that play role in both bone formation and resorption (Wongkhantee, et al. 2007). Intermittent compressive forces (ICF) increase expression of various ECM-associated genes including insulin-like growth factor-1 (*IGF-1*), periostin (*POSTN*), sclerostin (*SOST*), and transforming growth factor-β (*TGF-β*) in PDLSCs (Manokawinchoke, Limjeerajarus et al. 2015, Pumklin, Manokawinchoke et al. 2015)

ECM is a diverse and complex structure that aligns PDL cells and provides an environmental niche to maintain their stability and functionality (Spradling, Drummond-Barbosa et al. 2001). The ECM can be regarded as a mechanotransduction system being composed of matrix protein components that serve as a mechanosensor in

response to external forces. The responsible cells then perceive the biophysical signals transmitted via the ECM, which are transduced into intracellular biochemical signals that control many biological and pathological processes such as wound healing, tissue integrity, and ECM remodeling (Gattazzo, Urciuolo et al. 2014).

Collagen type I, a primary structural protein of the ECM, shapes the intrinsic mechanical properties of fibrous connective tissues such as tendons, blood vessels, heart valves, and ligaments (Chang and Buehler 2014). Collagen fibrils are the basic units of force transmission for all connective tissues. Meanwhile, collagenous tissue development and remodeling rely on mechanical force incorporation. Studies revealed a correlation between mechanical stress and the production of extracellular proteases. High magnitude forces applied to human fibroblasts caused at least a 3- to 5-fold increase in expression of matrix metalloproteinase-9 (MMP-9) (Prajapati, Chavally-Mis et al. 2000) and a 13-fold increase in MMP-2 (Prajapati, Eastwood et al. 2000). These reports suggested that mechanical forces may modulate collagen turnover by altering collagen degradation. Apart from those external environment influences, internal matrix proteins can be generated intracellularly to govern collagen fibrillogenesis, thereby altering the mechanical characteristics of connective tissues.

Previous studies have shown that periostin (POSTN), a matricellular ECM protein and a member of the fasciclin gene family, also appears to play important roles in the collagen fibrillogenesis process (Norris, Damon et al. 2007). POSTN is a confined and secreted ECM protein that is abundant in collagen-rich fibrous connective tissues that are frequently exposed to mechanical stresses such as periosteum (Kudo 2011), cardiac valves (Norris, Kern et al. 2004), and the PDL (Horiuchi, Amizuka et al. 1999). Numerous findings report the function of POSTN in dental tissues. For instance, expression of POSTN during the interaction between epithelial and mesenchymal tissues during tooth development suggests that POSTN may be crucial for the assembling and positioning of ECM adhesion molecules (Rios, Koushik et al. 2005). The

expression of POSTN in tooth and surrounding alveolar bone, according to Suzuki and colleagues (Suzuki, Amizuka et al. 2004) indicates that POSTN may be involved in maintaining the integrity of teeth at the interface site of hard-soft tissue. POSTN acts as a point of attachment between cell and matrix, as well as triggers morphogenesis and aids in bearing occlusal biomechanical forces.

Laminin is another ECM protein that is found in the lamina basalis that covers vascular endothelium. Because of this, this protein is frequently subjected to fluid shear stress and serves as a ligand for receptors that are sensitive to mechanical force, such as integrins (Shyy and Chien 2002, Halper and Kjaer 2014). In addition, laminin has been demonstrated to facilitate the regulation of shear force (SF) induced β-catenin signaling in colon cancer cells (Avvisato, Yang et al. 2007). Hence, it is possible that this protein is involved in responses to mechanical force, particularly induced by SF stimulation. All these findings suggest that ECM remodeling is modulated by specific biomechanical force stimuli in order to maintain connective tissue structural integrity and function.

Thus, we hypothesize that occlusal hypofunction may cause dynamic changes in the periodontal tissue due to a modification of expression and structural reshaping of main ECM components in the PDL. The aim of this study was to investigate dynamic changes of expression of collagen I, POSTN, and laminin in the PDL upon a decrease in biomechanical and occlusal forces driven by hypofunction. The study outcomes will improve our understanding of how biomechanical/occlusal forces can modulate expression and structural integrity of matrix PDL proteins, unveiling the importance of these forces in the maintenance of periodontal homeostasis.

5.3 Material and methods

5.3.1 Isolation and culture of PDLSCs

The PDL tissue collection and cell isolation technique was approved by the Faculty of Dentistry's Human Research Ethics Committee at Faculty of Dentistry Chulalongkorn University (HREC-DCU 2022-092). Primary human PDLSCs were isolated from the PDL tissues obtained from three distinct healthy donors. The tissues were collected by gently scraping the periodontal ligament of extracted wisdom teeth following a standard dental treatment plan. The explanted tissue samples were cultured in a high glucose-Dulbecco modified eagle medium (DMEM, Gibco, Waltham, MA) containing 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml), and amphotericin B (5 mg/ml). They were kept in a humidified environment with 5% CO2 and 37 °C. As PDL cells reached 100% confluence, they were subcultured at a 1:3 ratio. PDLSCs characterization was shown in supplementary figure 1. Cells from the third through sixth passages and at least three separate donors were utilized in each experiment.

5.3.2 Mechanical force application

Human PDLSCs were subjected to ICF and SF for 24 hours. A compressive force device was used as described previously (Manokawinchoke, Pavasant et al. 2019). Compressive force was created by applying load to the culture media with a moving pestle in a well plate of the same size. A computer-controlled device was utilized to calculate weight and monitor the application of force through a balance positioned beneath the well plate. For intermittent loading, the cycle was adjusted to alternate between a 1-second load and a 2-second release, resulting in about 14 cycles per minute. The strength of the force was fixed at 1.5 g/cm^2. To create SF in the culture medium a revolving cone-shaped rod encased in a 34 mm-diameter stationary ring was

used. Following application, SF was distributed with a magnitude of 5 dyn/cm^2 at the bottom of the cell culture plate, analyzed by ANSYS FLUENT R16.2 software. The optimal magnitude selection was previously described (Suwittayarak, Klincumhom et al. 2022).

5.3.3 Western blot analysis

Human PDLSCs lysate was extracted using RIPA buffer including a protease inhibitor mixture. Total protein concentration was measured with a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). For electrophoresis, each sample containing an identical quantity of protein was placed onto an 8% sodium dodecyl sulfate-polyacrylamide gel. The proteins were put onto a nitrocellulose membrane after electrophoresis. The membrane was incubated with primary antibodies against human POSTN (Abcam, Cambridge, UK) and Type I collagen (Santa Cruz Biotechnology, Dallas, TX) at a working concentration of 100 ng/ml. β-Actin served as an internal control. Following Tris-buffered saline washing, the membrane was incubated with a secondary antibody containing 20 ng/ml HRP (Cell Signaling Technology, Danvers, MA). A substrate for chemiluminescence improved the signals, which were then collected by an image analyzer. The Armersham 680 imaging program was used to measure band density (GE HealthCare, Chicago, IL).

5.3.4 Animal procedure and induction of occlusal hypofunctional tooth

Nine 8-week-old Wistar male rats were included in the experiment. The animal procedure was approved by the Chulalongkorn University Animal Care and Use Committee (Animal Use Protocol No. 1773019). The sample size for this experiment was calculated using the formula from Arifin and Zahiruddin (Arifin and Zahiruddin 2017). An

occlusal hypofunction tooth model was created in mandibular right molars by extracting the first, second, and third right maxillary molars using forceps under intraperitoneal injection of xylazine and ketamine for general anesthesia (Levy and Mailland 1980, Kaneko, Ohashi et al. 2001, Xu, Wang et al. 2016). The rats and all necessary resources including facilities, equipment, and drugs were obtained from the Chulalongkorn University Laboratory Animal Center. Following the surgical procedure, the rats were individually housed and provided with a soft diet. They were granted unrestricted access to drinking water and maintained under controlled conditions of a 12-hour light-dark cycle and a constant temperature ranging from 23 to 25°C. Three rats were sacrificed for whole mandible extraction at each time point of 4-, 8- and 12-weeks post-extraction of the three maxillary molars, and their mandibles were removed. The time points represented the early, middle, and long term of losing occlusal stimulation. The set of mandibular left molars without opposing tooth extraction (contralateral tooth) was used as the control baseline.

5.3.5 Micro-computed tomography (μCT) and PDL space measurement

The whole maxillary jaw was analyzed using μCT imaging. The samples were collected and fixed with 10% (v/v) formaldehyde for 24 hours, followed by extensive washing with PBS. All specimens were scanned under 70 kVP, 114 mA, 8 W of X-ray. Total teeth and bone volume was analyzed based on hydroxyapatite (HA) at 1200 mg HA/cc using a μCT scanner. The PDL space was measured at 4 positions around the root, starting from the middle left through the apical and ending at the middle right of the root. To measure PDL space, the longest root of the first molar from each section was selected and designated as the center. The measurement was conducted on the center section and on +/-10 sections from the center using μCT evaluation program (μCT 35 SCANCO

MEDICAL, SCANCO Medical AG, Switzerland). At least 3 measurement data from 3 animals were used.

5.3.6 Histological examination and Immunohistochemistry (IHC)

Following μCT analysis, the tooth specimens were decalcified using Surgipath Decalcifier II (Leica Biosystems, Richmond, IL) and processed for paraffin embedding. Sections of 5 μm thickness were cut and stained with Masson's Trichrome and hematoxylin & eosin (H&E).

For IHC, the sections were deparaffined prior to antigen retrieval using heat-induced epitope retrieval (HIER) technique. The section was immersed in primary antibody targeting to type I collagen, POSTN and laminin (Abcam, Cambridge, UK) followed by AlexaFuor-488 labeled secondary antibody. The antibody was diluted under ratios 1:500, and 1:1000, respectively. DAPI was stained for the nuclei background. Digital and fluorescence images were obtained using an Imager.Z2 Apotome 3 microscope (ZEISS International, Germany). The positively stained ECM and blood vessels were quantified by random counting in three different samples obtained from 8-weeks post extraction group (n=3).

5.3.7 Statistical analysis

Data were plotted using mean and standard deviation. Statistical analysis was conducted using an unpaired Student's *t*-test and one-way ANOVA followed by Holm-Šídák's multiple comparisons test. $P < 0.05$ was considered statistically significant. GraphPad Prism9 Software was utilized to conduct the analysis.

5.4 Results

5.4.1 Mechanical forces stimulate ECM protein production by PDLSCs.

After 24 hours of stimulation, the expression of POSTN protein was significantly increased in both ICF- and SF-induced PDLSCs whereas the level of collagen I was decreased after both stimulations (Figure 16A and 16B). These findings confirm that mechanical forces can alter ECM protein expression in PDLSCs.

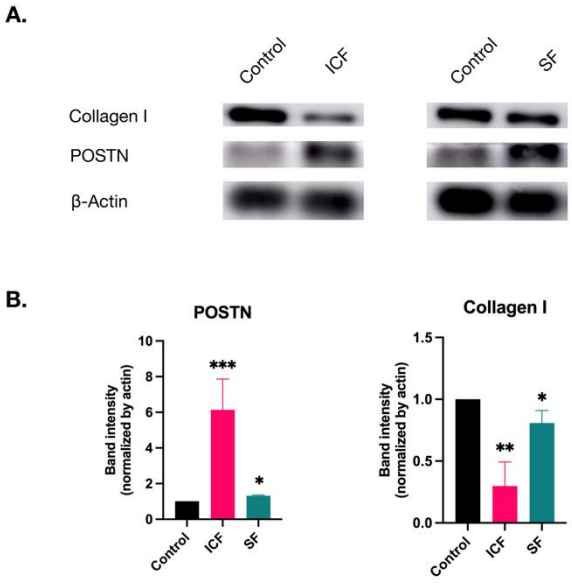

Figure 16. The mechanical forces alter the expression of collagen I and POSTN in human PDLSCs.

The PDLSCs were seeded in a 35cm culture dish and subjected to intermittent compressive force (ICF) and fluid shear force (SF). (A) Western blot analysis of collagen I and POSTN expression. (B) WB band density quantification, β-actin was used as the reference. Data are presented from three biological replicates using PDLSCs obtained from three different donors. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ compared to the control.

5.4.2 Histological features of PDL in occlusal hypofunction tooth model.

Figures 17A and 17B show the entire structure of the rat mandible, with the three molars stained with Masson's trichrome and H&E. A narrowing of the PDL space with an irregular and loose arrangement of collagen fibers was observed in the PDL of the hypofunctional tooth. This phenomenon was seen at all time points following extraction (Figures 17A and 17B). This was confirmed with micro-CT scanning analysis (Figure 17C). These findings suggest that occlusal force from the opposing tooth is required to maintain the width of the PDL.

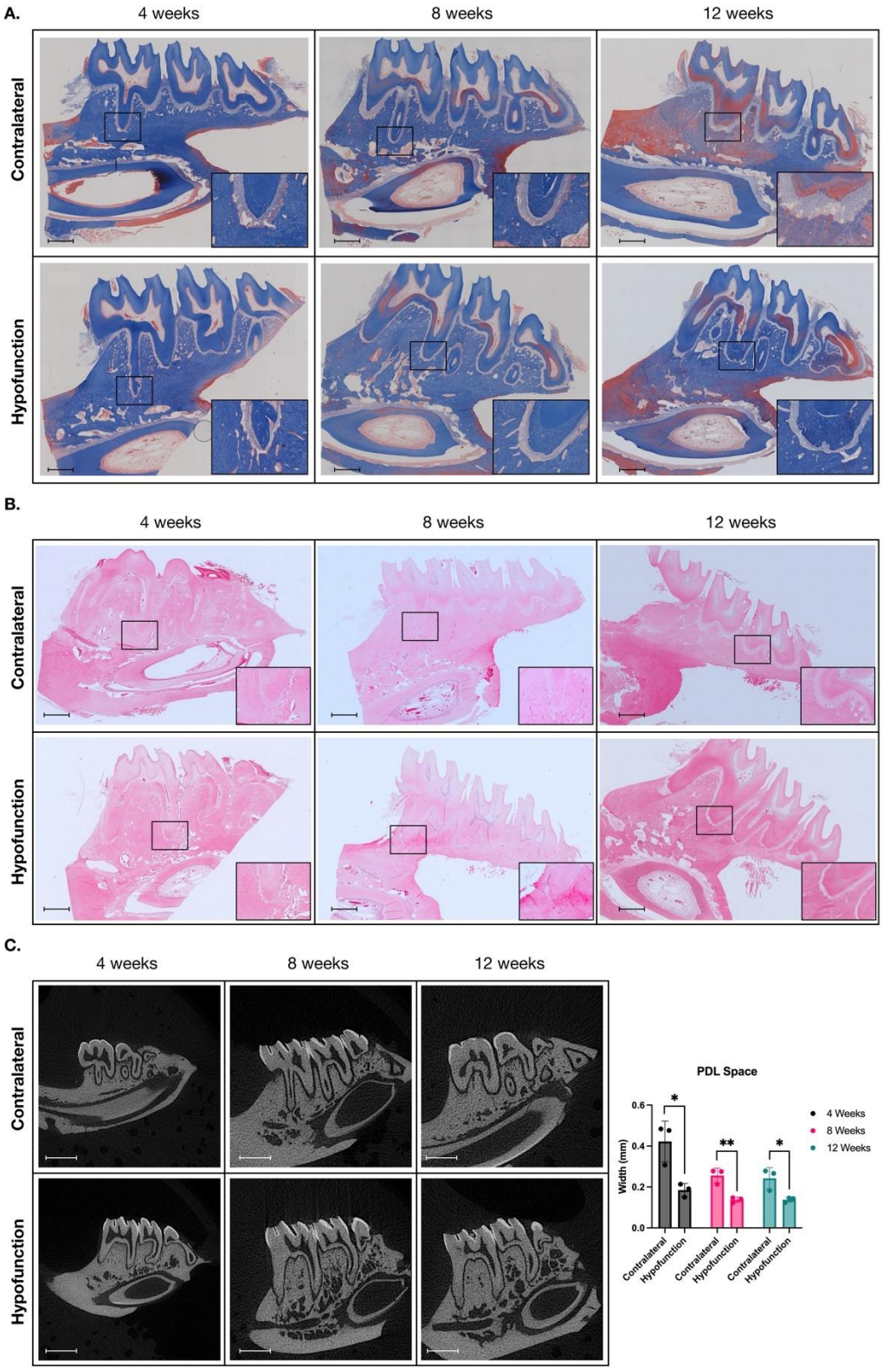

Figure 17. The Mechanical forces are essential for maintaining PDL integrity.

The paraffin sections of the mandibular right molars (hypofunction tooth) and mandibular left molars (contralateral tooth) were stained for Masson's trichrome (A) and H&E (B). (C) The micro-CT imaging showed the whole structure of the jawbone and teeth based on hydroxyapatite (HA) at 1200 mg HA/cc. The distance between cementum and alveolar bone (PDL space) is also shown in C. Data are presented from three biological replicates using samples obtained from three different rats. * $p < 0.05$ and ** $p < 0.01$ compared to the control (cotralateral). The scale bar represents 1000 μm.

5.4.3 Changes of extracellular matrix protein expression in PDL of hypofunctional teeth.

Immunolocalization of various ECM proteins revealed that collagen I, POSTN, and laminin. Both collagen I and POSTN were uniformly expressed in the PDL of the contralateral side, while the expression was strongly decreased in the hypofunctional PDL at the apical (Figures 18A and 18B) as well as middle root of mandibular molars (Figures 19A and 19B). The quantification of positive cells and blood vessels is presented in Figures 3D and 4D. In the PDL of contralateral teeth, laminin was uniformly expressed in the laminae basalis of perivascular structures. There was no difference in laminin expression between the hypofunctional and contralateral side of both apical and middle roots of molars (Figures 18C and 19C). Yet, the thickness of laminin-positive laminae basales decreased at the middle root of the hypofunctional molars (Figure 19C), but such a decrease was not found in the PDL of the apical root (Figure 18C). However, the number of laminin-positive blood vessels within the PDL space was not different (Figures 18D and 19D). Our findings suggested that deficiency of mechanical force could reduce the expression of collagen I and POSTN with less laminin-positive perivascular structures in the PDL at the middle part of the root of hypofunctional molars.

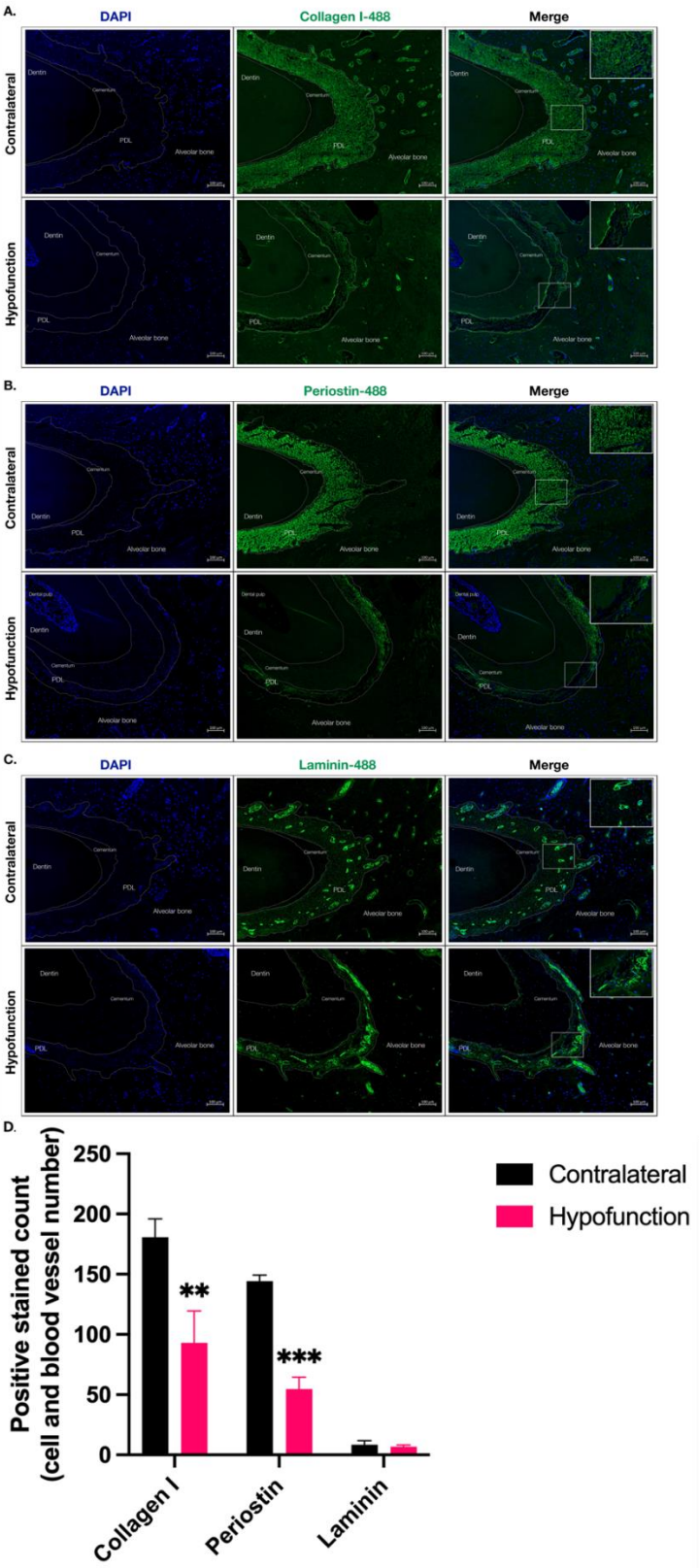

Figure 18. The effect of mechanical forces on ECM components of PDL at the apical root.

The paraffin sections of the mandibular right molars (hypofunction tooth) and mandibular left molars (contralateral tooth) were stained with the antibody specific to collagen I, POSTN, and laminin. The photomicrographs were taken at the region of the apical root. (A) collagen I (B) POSTN, and (C) laminin. All targets were labeled with green fluorescent dye; AlexaFluor-488. DAPI was used for nuclei staining. Scale bar represents 100 μm. (D) The number of collagen I, POSTN positive ECM, and laminin-positive blood vessels. ** $p < 0.01$ and *** $p < 0.001$ compared to the control (contralateral).

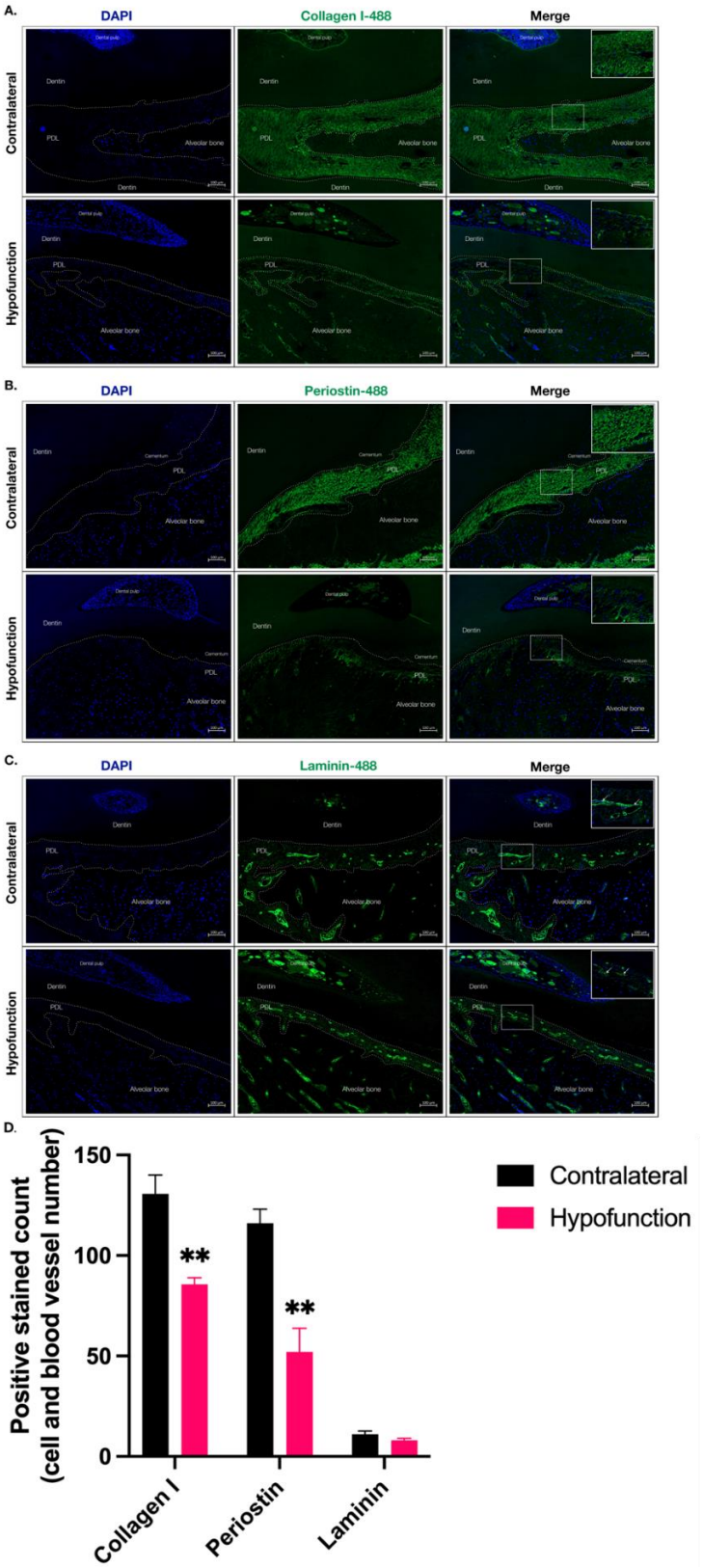

Figure 19. The effect of mechanical forces on ECM components of PDL at the middle of the root.

paraffin sections of the mandibular right molars (hypofunction tooth) and mandibular left molars (contralateral tooth) were stained with the antibody specific to collagen I, POSTN, and laminin. The micrographs were taken from the middle root. (A) collagen I (B) POSTN, and (C) laminin. All targets were labeled with green fluorescent dye; AlexaFluor-488. DAPI was used for nuclear staining. Scale bar represents 100 μm. (D) The number of collagen I, POSTN positive ECM, and laminin-positive blood vessels. ** $p < 0.01$ compared to the control (contralateral).

5.5 Discussion

The present study was conducted to investigate how biomechanical and occlusal forces may modulate expression and structural integrity of certain ECM proteins in PDL. In the *in vivo* model, the reduction of biomechanical force had a negative impact on the PDL as per the narrower PDL space with reduced collagen I and POSTN expression. This was supported by the observation that POSTN expression was increased in *in vitro* PDLSC culture in response to mechanical compressive and shear forces.

ECM is an extracellular complex structure of the tissue that primarily receives and transmits mechanical force stimuli. Matrix components usually bind to cell surface receptors, which modulate specific cellular activities through cell-ECM interaction (Frantz, Stewart et al. 2010). The most abundant component of PDL-ECM is collagen type I, as seen by the significant accumulation of collagen I in PDL space (Figure 18A). Disorientation of collagen fibers, in combination with sparse and disorganized features, was observed in hypofunctional teeth.

Collagen fibers serve as the main structural ECM component that perceives mechanical stresses and provides mechanical stability, elasticity, and structural strength (Fratzl 2008, Buehler and Yung 2009). The densely arranged collagenous tissue such as tendon or PDL responds to the mechanical force in dualistic mechanisms in tissue remodeling; by destroying an existing tissue, or by forming a new tissue (Chang and Buehler 2014). It has been shown that proper mechanical stimulation increases collagen formation, while insufficient stimulation led to collagen degradation (Langberg, Skovgaard et al. 1999). In humans, mechanical stress on the patella tendon and quadriceps muscle increased collagen synthesis. Moreover, there was a 2- to 3-fold increase in collagen production within 24 hours after exercise and this was maintained for up to 70-80 hours (Miller, Olesen et al. 2005). Alike those findings, we found an increase in expression of collagen I protein by the force-induced PDLSCs and a dense

arrangement of PDL tissue of the normal occlusal tooth of the *in vivo* model. A static mechanical loading on 3-dimensional collagen-based dermal fibroblasts resulted in 2.5 to 13-fold increased expression of MMP-2, a type IV collagenase and gelatinase A, responsible for breakdown of denatured collagen (Prajapati, Chavally-Mis et al. 2000). In our study, the presence of collagen I in the periodontal space appeared markedly reduced in hypofunctional teeth. These findings suggest that the proper balance of biomechanical forces influences collagen structure.

POSTN is a matricellular protein that is known to be highly expressed in the PDL matrix (Horiuchi, Amizuka et al. 1999). This matrix protein was proposed to regulate tooth and bone remodeling, especially under mechanical stress due to its expression on sites that are generally exposed to mechanical loading by mastication or physical exercise (Kudo, Amizuka et al. 2004, Bonnet, Standley et al. 2009). According to our *in vitro* findings, mechanical stimuli promoted production of POSTN in PDLSCs, indicating the relevance of POSTN in PDL responses to mechanical forces. In connective tissue, POSTN binds to type I collagen fibrils and control the process of collagen fibrillogenesis in mice (Norris, Damon et al. 2007). These mice had decreased collagen crosslinking and collagen fibril diameter when POSTN was absent, indicating the relationship between POSTN and the collagen fibril turnover process.

Our study revealed that collagen I expression was decreased in PDLSCs under both compression and shear forces. This expression pattern contrasts with the one of POSTN under the same conditions. In applying stress to a single cell layer without support from the ECM, direct mechanical force stimuli probably change collagen biosynthesis, which is reflected in a decrease of collagen I expression. The use of additional supplements, such as ascorbic acid, to enhance the thickness of PDLSCs-ECM layer may improve the *in vitro* study and make it more relevant to the *in vivo* investigation (Kubow, Vukmirovic et al. 2015). However, these findings may also suggest

that POSTN may maintain PDL homeostasis by modulating collagen turnover during mechanical force stimulation.

Laminin is another important component of the ECM, generally found in all laminae basalis (Timpl, Rohde et al. 1979, Halper and Kjaer 2014). In this study, laminin was highly expressed by laminae basalis of blood vessels in the PDL. This expression was slightly decreased at the middle root of hypofunctional teeth. This suggests a negative impact of low-intensity occlusal forces on the vascular structure of the PDL. Apoptosis of vascular endothelial cells with subsequent atrophic changes of vascular structure has been reported in PDL of hypofunctional tooth (Usumi-Fujita, Hosomichi et al. 2013). Laminin is an ECM protein that due to its localization adjacent to vascular endothelium is often exposed to fluid shear force and acts as a ligand for mechanical force-sensitive receptors such as integrins (Shyy and Chien 2002, Halper and Kjaer 2014). Moreover, laminin has been shown to facilitate SF regulation of β-catenin signaling in colon cancer cells (Avvisato, Yang et al. 2007). Thus, this protein is potentially associated in mechanical force responses, particularly under SF stimulation. A previous study had demonstrated that laminin can facilitate the alignment of endothelial cells in the direction of flow, thereby improving their ability to withstand and respond to shear force (Girard and Nerem 1995). In our investigation, no significant changes in the laminin expression pattern were found. It has been noted, however, that once the occlusal force is applied to a tooth, a range of forces and magnitudes are created at various root locations. Under all loading situations, the largest root biomechanical stress appear to occur in the cervical third of the root surface and not in the apical one (Wan, Chung et al. 2022). It is possible that variations in the laminin expression pattern are associated with the shear force-dominant location at the level of the root surface. Though unveiling this association will require further investigations.

Furthermore, it is interesting that no visible structural changes occurred in the PDL adjacent to the contralateral molars, the teeth that were presumably

hyperfunctional. As opposed to hypofunctional teeth, hyperfunction or parafunctional periodontium typically manifest as larger ligament space but decreased alveolar bone mass compared to normal function teeth (Johnson 1990).In hyperfunctional situations, repeated or excessive occlusal force is applied on tooth and supporting tissue which exceeds the physiologic limits of the tissue tolerance and subsequently leads to occlusal trauma and destruction of periodontal tissue (Reddy and Vandana 2018). An occlusal trauma can be generated by an excessive occlusal force which is classified as either primary or secondary depending on the condition of the connective tissue attachment and bone levels. The primary stage includes normal connective tissue attachment and normal bone levels, which are demolished in secondary stage (Fan and Caton 2018). In our study, there were no signs of alveolar bone and loss of PDL structure in the contralateral mandible during 12 weeks of the study which may imply as the primary stage of occlusal trauma with no loss of connective tissue occurs.

In summary, it is possible that the interaction between POSTN and collagen I promoted PDL-ECM homeostasis during biomechanical force stimulation. The reduction of POSTN and collagen I subsequently interfered with ECM structural integrity, reflected as narrowing PDL space under low-intensity of occlusal forces. Our findings reveal that both the type and magnitude of a biomechanical/occlusal force are essential to preserving ECM composition in the PDL via the modulation of two essential ECM proteins, collagen I and POSTN. The healthy PDL-ECM may provide an immobilized niche environment for PDLSCs to maintain their stemness and provide PDL tissue integrity. Studies have indicated that occlusal hypofunction is one of the major factors leading to dental root and bone resorption (Sringkarnboriboon, Matsumoto et al. 2003, Hayashi, Hayashi et al. 2016). It is possible that strengthening the PDL-ECM by targeting matrix proteins might be an alternative approach for maintaining ECM homeostasis and preventing the destruction of periodontal tissue caused by occlusal hypofunction, especially in cases of unknown etiology. Future studies are necessary to

further investigate the mechanisms of ECM proteins that regulate the PDL structural integrity.

5.6 Conclusions

Our findings revealed that a deficiency in occlusal force interferes with the deposition of collagen I, POSTN, and laminin and highlight the importance of mechanical forces in modulating ECM composition to preserve periodontal tissue homeostasis.

5.7 Ethics approval

Human PDL specimens were approved by the Human Research Ethical Committee of the Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2022-092). Informed consent was obtained. The animal procedure in this study was approved by the Chulalongkorn University Animal Care and Use Committee (Animal Use Protocol No. 1773019). This study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013.

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