The Effects of Decellularized Extracellular Matrix Derived from Jagged1-treated Human Dental Pulp Stem Cells on Biological Responses of Stem Cells Isolated from Apical Papilla

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ผลของเมทริกซ์นอกเซลล์ที่ปราศจากเซลล์ที่ได้จากเซลล์ต้นกำเนิดจากเนื้อเยื่อในฟันที่ถูกกระตุ้นด้วยเจ็กเก็ต1 ต่อการตอบสนองทางชีววิทยาของเซลล์ต้นกำเนิดที่แยกจากเนื้อเยื่อปุ่มปลายรากฟัน

น.ส.ศุภลักษณ์ โพธิชัยเลิศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	The Effects of Decellularized Extracellular Matrix Derived from Jagged1-treated Human Dental Pulp Stem Cells on Biological Responses of Stem Cells Isolated from Apical Papilla
By	Miss Suphalak Phothichailert
Field of Study	Oral Biology
Thesis Advisor	Professor Thanaphum Osathanoon, D.D.S., PH.D.

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ศุภลักษณ์ โพธิชัยเลิศ : ผลของเมทริกซ์นอกเซลล์ที่ปราศจากเซลล์ที่ได้จากเซลล์ต้นกำเนิดจากเนื้อเยื่อในฟันที่ถูกกระตุ้นด้วยเจ็กเก็ต1 ต่อการตอบสนองทางชีววิทยาของเซลล์ต้นกำเนิดที่แยกจากเนื้อเยื่อปุ่มปลายรากฟัน. (The Effects of Decellularized Extracellular Matrix Derived from Jagged1-treated Human Dental Pulp Stem Cells on Biological Responses of Stem Cells Isolated from Apical Papilla) อ.ที่ปรึกษาหลัก : ศ.ทพ.ดร.ธนภูมิ โอสถานนท์

จุดประสงค์ : เจ็กเก็ต1 เป็นตัวสำคัญของการส่งสัญญาณของเซลล์ต้นกำเนิดจากเนื้อเยื่อในฟัน การศึกษานี้ศึกษาเกี่ยวกับลักษณะของเมทริกซ์นอกเซลล์ที่ปราศจากเซลล์ที่ได้จากเซลล์ต้นกำเนิดจากเนื้อเยื่อในฟันที่ถูกกระตุ้นด้วยเจ็กเก็ต1และการตอบสนองทางชีววิทยาของเซลล์ต้นกำเนิดที่แยกจากเนื้อเยื่อปุ่มปลายรากฟันในการเปลี่ยนเป็นเซลล์สร้างกระดูกและเนื้อฟัน ระเบียบวิธีวิจัย : ข้อมูลชีวสารสนเทศศาสตร์ของ dECM-Jagged1-hDPSCs ถูกวิเคราะห์โดยโปรแกรม NetworkAnalyst และถูกควบคุมในอาหารเลี้ยงเซลล์ชนิด N และ OM และตามด้วยกระบวนการนำเซลล์ออกจากเมทริกซ์นอกเซลล์คือ dECM-N หรือ dECM-OM หลังจากนั้นเซลล์ต้นกำเนิดที่แยกจากเนื้อเยื่อปุ่มปลายรากฟันถูกเลี้ยงบน dECM-Jagged1 ในทั้ง 2 กลุ่มและวัดการมีชีวิตของเซลล์โดยใช้การวัด MTT assay และประเมินลักษณะของและเซลล์ต้นกำเนิดที่แยกจากเนื้อเยื่อปุ่มปลายรากฟันด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดและวิเคราะห์องค์ประกอบของธาตุรวมถึงการตรวจวิเคราะห์แอนติบอดีและการสะสมแร่ธาตุถูกประเมินโดยการใช้ Multipotential differentiation assay นัยยะสำคัญทางสถิติในการทดลองนี้คือ $p<0.05$ ผลข้อมูล RNA ของ dECM-Jagged1 มีการเพิ่มขึ้นของยีนที่เกี่ยวข้องกับECM เช่น ECM organization, ECM-receptor interaction และ focal adhesion และยังพบว่ามีการเพิ่มขึ้นของ osteogenesis ในกลุ่มOM และพบว่าเมทริกซ์นอกเซลล์ที่ปราศจากเซลล์ได้แสดง fibrillar-like network structure และ ECM proteins และลดลงของแร่ธาตุและการวัดการมีชีวิตของเซลล์เป็นเวลา 7 วันไม่ได้มีการเปลี่ยนแปลงของเซลล์ต้นกำเนิดที่แยกจากเนื้อเยื่อปุ่มปลายรากฟัน การเกาะของเซลล์และ f-actin cytoskeletal organization ของ dECM-Jagged1-OM มีการแพร่เมื่อเทียบกับกลุ่มอื่นๆ และพบว่าหลังจากเลี้ยงเซลล์ต้นกำเนิดที่แยกจากเนื้อเยื่อปุ่มปลายรากฟันบน dECM-N ใน OM มีการเพิ่มขึ้นของการสะสมแร่ธาตุอย่างมีนัยยะสำคัญเมื่อเทียบกับกลุ่มอื่นๆ สรุปผลวิจัย dECM-

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Suphalak Phothichailert : The Effects of Decellularized Extracellular Matrix Derived from Jagged1-treated Human Dental Pulp Stem Cells on Biological Responses of Stem Cells Isolated from Apical Papilla. Advisor: Prof. Thanaphum Osathanoon, D.D.S., PH.D.

Objective: Indirect Jagged1 immobilization efficiently activates canonical Notch signaling in hDPSCs. This study aimed to investigate the characteristic of the Jagged1-treated hDPSCs-derived dECM and its biological activity on odonto/osteogenic differentiation SCAPs. Methods: Bioinformatic database of Jagged1-treated hDPSCs was analyzed by NetworkAnalyst. hDPSCs seeded on Jagged1 immobilized surface were maintained with N or OM followed by decellularization procedure, dECM-N or dECM-OM, respectively. SCAPs were reseeded on each dECM with either normal medium or OM. Cell viability was determined by MTT assay. Characteristics of dECMs and SCAPs were evaluated by SEM, EDX, immunofluorescent staining and alcian blue staining. A Multipotential differentiation assay. Statistical significance was considered at $p<0.05$. Results: RNA-seq database revealed upregulation of several genes involved in ECM organization, ECM-receptor interaction, and focal adhesion in Jagged1-treated hDPSCs. Immobilized Jagged1 increased the osteogenesis of hDPSCs culture with OM. dECMs showed fibrillar-like network structure and major ECM proteins, as well as glycosaminoglycans. A decrease in calcium and phosphate components was observed in dECMs after the decellularized process. Cell viability on dECMs did not alter by 7 days. Cell attachment and f-actin cytoskeletal organization of SCAPs proliferated on Jagged1-treated dECMs were comparable to those of the control

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CHAPTER I

INTRODUCTION

1.1 Importance and Rationale

Decellularization of extracellular matrix-derived human dental pulp stem cells is one of the candidate biological scaffolds for tissue regeneration. This extracellular matrix is decellularised to remove genetic materials using physical, chemical, and enzyme (Faulk, Wildemann, and Badylak 2015). In the previous reports, several organs have been successfully decellularised such as the liver, lung, kidney, heart, and tendon. These decellularised tissues are subjected to use as natural scaffolds in the tissue engineering (Agmon and Christman 2016; Faulk, Wildemann, and Badylak 2015). Jagged1, the canonical Notch ligands, enhances osteogenic differentiation in human periodontal ligament stem cells and human dental pulp cells (Osathanon, Ritprajak, et al. 2013; Manokawinchoke et al. 2017). Further, the extracellular matrix pathway is identified as the significant regulated pathway in Jagged1-treated human dental pulp cells as determined by a high throughput RNA sequencing technique. The present study investigates the characteristics of decellularised extracellular matrix derived from Jagged1-treated human dental pulp stem cells and its biological activity to stem cells isolated from apical papilla.

1.2 Objectives of Study

To investigate the characteristics of decellularised extracellular matrix derived from Jagged1-treated human dental pulp stem cells and its biological activity to stem cells isolated from apical papilla.

1.3 Research question

Could the decellularised extracellular matrix derived from Jagged1-treated human dental pulp stem cells promote the bioactivity of SCAPs?

1.4 Hypothesis

Decellularized extracellular matrix derived from Jagged1-treated human dental pulp stem cells promote proliferation and differentiation of SCAPs *in vitro.*

1.5 Advantages of study

The present study elucidates the basic knowledge of extracellular matrix component in Jagged1-treated human dental pulp cells and its effects on SCAPs responses. This can be further develop as the bioactive scaffolds in regenerative endodontics therapy.

CHAPTER II
LITERATURE REVIEW

2.1 Human dental pulp stem cells

Dental pulp tissue is connective tissue located inside the teeth and filled in the dental pulp chamber cavity. This tissue contains arteries, veins, nerve fibre, extracellular matrix, and various cell types (Carlson 2019; Eckhard et al. 2015). Dental pulp tissues act as the inductive, formative, protective response to extrinsic stimuli such as heat, cold, and pressure. Dental pulp tissue also provides nutrition and oxygen through its vascular structures in supporting the dentin with nutrients for developing tooth and reparative activities (Takeda et al. 2008). Human dental pulp stem cells (hDPSCs) are mesenchymal stem cells that exhibit a high potential for pulp regeneration as they can regenerate a dentin/pulp-like structure *in vivo* (Tatullo et al. 2015). hDPSCs have the mesenchymal stem cell (MSCs) characteristics, for example self-renew ability and multipotential differentiation ability toward odontoblast, osteoblast, chondrocytes, adipocyte, and neural cell (Kabir et al. 2014; Gazit et al. 2013). hDPSCs express numerous MSCs surface markers, including CD29, CD44, CD59, CD73, CD90, CD146, and STRO-1, but these cells do not express surface maker of hematopoietic and endothelial stem cells such as CD14, CD34, CD45, CD133, and CD11b (Rastegar et al. 2010). The ability of hDPSCs to differentiate to osteoblast was examined by the expression of osteoblast markers such as collagen type I (Coll I), alkaline phosphatase (ALP), osteocalcin (OCL), dentin sialophosphoprotein (DSPP) and bone morphogenetic protein-2 (BMP), and osteopontin (OPN) (Mori et al. 2011; Kim et al. 2015; Tatullo et al. 2015). hDPSCs generate a dentin-like structure after seeding on hydroxyapatite/tricalcium phosphate (HA/TCP) and subsequently transplanting into immunocompromised mice (Tatullo et al. 2015). Therefore, hDPSCs are a useful cell source for regenerative dental therapies.

2.2 Human apical papilla stem cells

Stem cells isolated from apical papilla (SCAPs) are derived from the tissue at the root apex of immature permanent teeth. The apical papilla has a loose physical connection between the dental pulp and the apical papilla. This tissue has a vascular density lower than dental pulp tissue (Sonoyama et al. 2008). SCAPs exhibit the MSC marker expression similar to those observed in DPSCs. SCAPs express STRO-1, ALP, CD24, CD29, CD73, CD90, CD105, CD106, CD146, CD166 but do not express CD34, CD45, CD18, and CD150 (Sonoyama et al. 2006). CD24 is a specific marker for SCAPs which does not detect in DPSCs and BMMSCs. SCAPs are capable to differentiate into odontoblastic lineages *in vitro* (Feter et al. 2017). The previous report showed that SCAPs had a proliferation rate and tissue regeneration capacity higher than DPSCs including the ability to promote the dentin regeneration (Sonoyama et al. 2006). SCAPs derived from young adults (18-20 years old) have a high property to promote root regeneration (Sonoyama et al. 2006). Therefore, SCAPs could be a candidate cell source for cell-based therapy in the root regeneration (Sonoyama et al. 2006; Potdar and Jethmalani 2015).

SCAPs have a capacity for osteogenic differentiation potential. After osteogenic induction, SCAPs can promote calcium deposits *in vitro* (Nada and El Backly 2018). Correspondingly, the upregulation of osteogenic differentiation markers such as dentine sialophosphoprotein (DSPP), bone sialoprotein (BSP), alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP-2), runt-related transcription factor 2 (Runx2), and osteocalcin (bone gamma-carboxyglutamic acid-containing protein BGLAP) is observed (Bakopoulou et al. 2013). In contrast, SCAPs exhibit lower potency for adipogenic differentiation compared to BMMSCs as demonstrated by the intracellular lipid accumulation (Nada and El Backly 2018). In neurogenic differentiation, SCAPs have the expression of neuronal markers such as nestin and neurofilament after being cultured with a neurogenic condition (Sonoyama et al. 2008). This evidence indicates the multipotential differentiation ability of SCAPs and infers the use of these cells in regenerative therapy.

2.3 Extracellular matrix (ECM)

2.3.1 The function of Extracellular matrix (ECM)

ECM, the non-cellular structure, acts as a supporting structure and physical barrier for tissues and organs (Frantz, Stewart, and Weaver 2010). ECM composes of water, polysaccharide, mineral, ground substances, and various proteins (Frantz, Stewart, and Weaver 2010; Kendall and Feghali-Bostwick 2014) (Figure 1). ECM can regulate many biological events for example immune response, stem cell self-renewal, proliferation, and differentiation (Gattazzo, Urciuolo, and Bonaldo 2014; Ribatti 2021). ECM regulates cell response by its physiological structure and biological interaction via cell-surface receptors (Kusindarta and Wihadmadyatami 2018).

2.4 Components of the extracellular matrix

2.4.1 Collagen

Collagen and elastin are structural proteins. Collagen is synthesized and secreted by fibroblasts (Theocharis et al. 2016). The structure of collagen content of repeating polypeptide chains is Gly-X-Y sequences that form into the triple-helix structure (Myllyharju and Kivirikko 2004) consisting of 3 polypeptide **α** chains. The X and Y position is essential for the function of collagen. In this regard, proline in the X position has a role in packing of the collagen structure and 4-hydroxyproline in the Y position has a role in the stability of the collagen triple helix. In vertebrates, the collagen superfamily comprises 28 types, and type I collagen is main collagen type found in the body. Type I collagen is fibril-forming collagens (Yue 2014) and a major constituent structure in tissues such as tendon, bone, and dermis (Theocharis et al. 2016). In addition, type I collagen has an essential function in tissue integrity and mechanical properties (Kular, Basu, and Sharma 2014). The important function of collagen is protecting the tissue structures. Collagen can regulate many biological responses including cell migration, cell adhesion, wound healing, differentiation, chemotaxis, and tissue remodeling (Derya 2014; Myllyharju and Kivirikko 2004).

2.4.2 Elastin

Elastin is a hydrophobic amino acid (Gly, Val, Ala) and synthesis from tropoelastin monomer (60-70 kDa) into extracellular space. (Chung et al. 2006; Wise and Weiss 2009). The cross-linking of the tropoelastin monomer to making the mature elastin (Daamen et al. 2007) leads to the important function in physiological properties like elasticity and recoil of various tissues and organs for example vessels, cartilage, bladder, skin, and heart (Theocharis et al. 2016; Daamen et al. 2007; Frantz, Stewart, and Weaver 2010). Elastin is a structural protein in vertebrates and is associated with collagen and microfibrillar proteins (Kular, Basu, and Sharma 2014; Foster 2013).

2.4.3 Fibronectin

Fibronectin (FN) is one of the adhesion glycoproteins that has an important role in the cell attachment (Frantz, Stewart, and Weaver 2010). The component of FN is two subunits of fibronectin formed by dimeric glycoprotein and connected by a disulfide bond near the C-terminal (Parisi et al. 2020; Derya 2014). FN has a binding site for contact with other molecules (Derya 2014). FN plays role in wound healing, adhesion, differentiation, migration, phagocytosis, and hemostasis (Hayashi and Yamada 1983). Moreover, FN functions in cell-matrix communication by linking cells to surrounding environment (Klecker and Nair 2017).

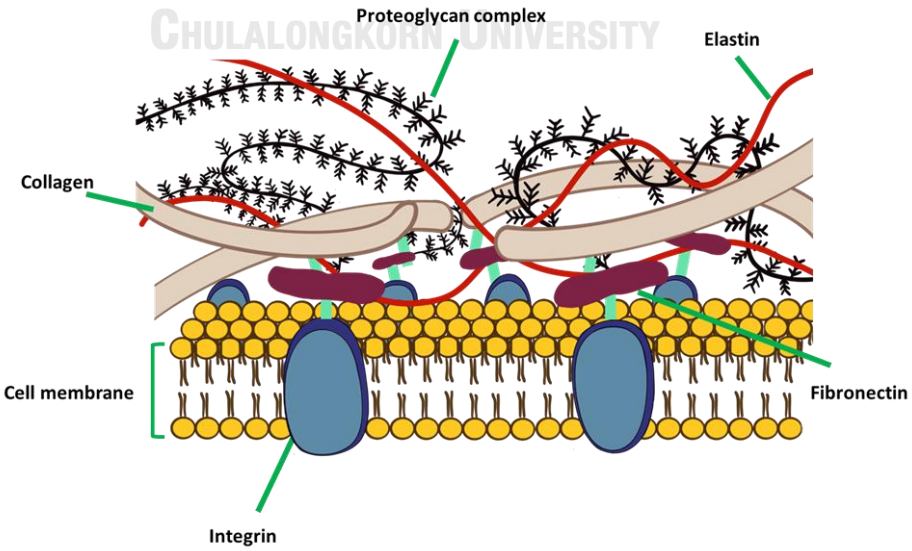

Figure 1 The component of the extracellular matrix. ECM has ground substances such as glycosaminoglycans (GAG) and proteoglycans (PG). ECM is also composed of many structural proteins, such as collagen, elastin, and fibronectin. Modified from (Kendall and Feghali-Bostwick 2014).

2.5 Extracellular matrix and a regenerative scaffold

ECM is considered as the natural biomaterial for the tissue regeneration (Zhong et al. 2019) and ECM has the capacity to control cell behaviour, adhesion, and function (Khalili and Ahmad 2015). The previous study showed that mesenchymal stem cells, osteoblasts, and osteocytes were induced from ECM derived from bone cells (Lin et al. 2020). Decellularized ECM has been used for replacing damaged kidneys, liver, heart, lung, and other diseased organs in animal studies (Gilpin and Yang 2017). The aim of the ECM scaffold for replacing damaged tissue and restorative the tissue (Scarritt, Murdock, and Badylak 2019)

ECM scaffold can be prepared by removing genetic materials (such as DNA) by various techniques for example chemical, mechanical, and enzyme-digested techniques (Faulk, Wildemann, and Badylak 2015; Gilpin and Yang 2017).

ECM derived from specific tissues inherits it ability to promote that particular tissue regeneration. In this regard, ECM derived from dental cells has potential use in dental tissue regeneration. ECM derived from DPSCs enhances odonto/osteogenic differentiation in DPSCs, PDLs, and MSCs (Ravindran, Huang, and George 2014). ECM derived from bone marrow mesenchymal stem cells (BMSCs) promotes cell adhesion and osteogenic marker gene expression, implying the candidate scaffolds for the bone regeneration (Chi et al. 2020). ECM derived from hDPSCs promotes angiogenesis as shown by the upregulation of pro-angiogenic growth factors (Ravindran et al. 2014). In addition, ECM derived from dental stem cells promotes cell survival infection and proliferation as it increased telomerase activity appropriate in tissue regeneration (Sonoyama et al. 2006). Moreover, ECM scaffold is an excellent source of bioactive proteins for example bone morphogenetic protein 2 (BMP2), transforming growth factor-beta (TGFbeta), platelet-derived growth factor (PDGF), and vascular

endothelial growth factor (VEGF) (Sonoyama et al. 2006; Ravindran et al. 2014). Taking all evidence together, ECM derived from dental cells could potentially be used as a natural biomaterial suitable scaffold for application in the clinic such as regenerative treatment.

2.6 Jagged 1

In mammalian cells, Jagged1 is a canonical Notch ligand that can interact with Notch receptors (Tien, Rajan, and Bellen 2009; Dishowitz et al. 2014) leading to activation of Notch signalling in the target cells (Dishowitz et al. 2014). Notch signalling regulates various biological processes for example embryological bone formation as well as bone healing and regeneration. Jagged1 decreases osteoprotegerin (OPG) expression and OPG/RANKL ratio, leading to an increase in the osteoclast differentiation (Guarnaccia, Pintar, and Pongor 2004; Tien, Rajan, and Bellen 2009; Dishowitz et al. 2014; Manokawinchoke et al. 2020). Canonical Notch signalling is a regulator of stem cells such as the hematopoietic stem cell (HSC), neural stem cells (NSCs), and skeletal muscle stem cells to regulate cell proliferation, differentiation, and self-renewal (Luo et al. 2019; Mancini et al. 2005; Mourikis et al. 2012). N-terminal region of Jagged1 contains a conserved DSL domain (Drosophila melanogaster and *C. elegans* ligands, delta, serrate and lag-2), 16 repeats of EGF-like (epidermal growth factor-like), DOS (Delta and OSM-11-like proteins) domain, and cysteine-rich region (Grochowski, Loomes, and Spinner 2016; Kume 2009). The activation of Notch signaling occurs after the Notch ligand from the signal sending cell interacts with the Notch receptor on the surface of the signal receiving cell. Ligands interact with receptor leads to activate the Notch intracellular domain (NICD) via-a multi-stage proteolytic by ADAM (a disintegrin and metalloprotease) protease and γ-secretase complex (Zhang et al. 2016; Youngstrom et al. 2016). After cleavage, the NICD translocated to the nucleus, and forms with CSL transcription factors and transcriptional coactivator such as mastermind-like 1 (MAML1) leading to initiating the transcription of target genes such as HES/HEY (Luo et al. 2019; Zhang et al. 2016).

Indirect affinity immobilization of Jagged1 enhanced osteogenic differentiation as demonstrated by the increase of osteogenic marker gene expression, alkaline phosphatase activity, and mineralization in human periodontal ligament stem cells (Osathanon, Ritprajak, et al. 2013), human bone-derived cells (Osathanon et al. 2019), and stem cells isolated from human exfoliated deciduous teeth (SHEDs) (Osathanon, Nowwarote, et al. 2013). Jagged1 coated polycaprolactone-based membrane enhances ALP enzymatic activity in human periodontal ligament cells (Nowwarote et al. 2018), implying the use of this Notch ligand in bone regenerative treatment.

To control cell behaviours at the biomaterials interface using Notch signalling, ligand immobilization is the crucial protocol (Beckstead, Santosa, and Giachelli 2006). The indirect affinity immobilized Jagged1 exhibits significantly higher activation of Notch signalling compared with the soluble or direct immobilized technique (Beckstead, Santosa, and Giachelli 2006).

2.7 Jagged1 and extracellular matrix

Previous work demonstrated that Jagged1 treated human dental pulp cells exhibited dysregulation in various pathways related to ECM such as extracellular matrix organization, ECM-receptor interaction and focal adhesion, elastic fibre formation, assembly of collagen fibrils, integrin cell surface interactions, laminin interaction, collagen information, ECM proteoglycans, and collagen biosynthesis (Manokawinchoke et al. 2017). In addition, Jagged1 maybe mediate the importance of the cell-matrix interaction (Nehring et al. 2005). Thus, the surface of tissue culture coated by Jagged1 could alter both the biological and mechanical properties of ECM-derived hDPSCs. This could be a benefit in dental tissue regeneration.

CHAPTER III

METERIALS AND METHODS

3.1 Cell isolation and culture

The experimental protocols were approved by the Human Research Ethics Committee, Faculty of Density, Chulalongkorn University (approval no. 106/2021). Third molars scheduled for surgical removal according to the patient's treatment plan were obtained for cell isolation. Informed consent was obtained. In brief, dental pulp and apical papilla tissues were collected and minced. Cell explantation was performed to isolate cells from both tissues. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 2mM L-glutamine, and 100 unit/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Gibco, USA) (growth medium). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C, and the culture medium was changed every 2 days. Cells were then sub-cultured, and all experiments used the cells from passages 3-5.

To identify the characteristic of mesenchymal stem cells, surface marker expression was evaluated by flow cytometry. hDPSCs and SCAPs were stained with FITC-conjugated CD44 (BD Bioscience Pharmingen, USA), FITC-conjugated CD73 (BD Bioscience Pharmingen, USA), PE-conjugated CD105 (Immuno Tools, Germany), APC-conjugated CD90 (Immuno Tools, Germany), and PerCP-conjugated CD45 antibodies (Immuno Tools, Germany). Mouse IgG isotype was used as the control. The stained cells were analysed by FACS^{Calibur} Flow Cytometer (Becton Dickinson, Worldwide Inc., San-Jose, CA, USA).

To induce osteogenic differentiation, cells ($5x10^4$) were seeded into a 24-well plate and cultured with a growth medium until confluence. Cells were maintained with an osteogenic induction medium (OM), which contained a growth medium supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich, USA), 5 mM beta-glycerophosphate (Sigma-Aldrich, USA), and 250 nM dexamethasone (Sigma-Aldrich, USA) for 14 days. For adipogenic differentiation, cells were seeded at the density of $1.25x10^4$ cells/well and maintained with an adipogenic induction medium, which

consisted of a growth medium supplemented with 1 mM IBMX (Thermo Fisher Scientific, USA), 0.1 mg/ml insulin (Sigma-Aldrich, USA), 1 μM dexamethasone (Sigma-Aldrich, USA) and 0.2 mM indomethacin (Sigma-Aldrich, USA) for 16 days. The culture medium was refreshed every 3 days.

3.2 Mineralization assay

For alizarin red s staining, cells were fixed with 4% formaldehyde in PBS for 5 min and washed with deionised water. The samples were then stained with ARS solution (pH 4.1) (Sigma-Aldrich, USA) for 5 min at room temperature and washed with deionised water. Stained mineral deposits were observed by microscope and further solubilised in 10% cetylpyridium chloride monohydrate solution for 20 min. The optical density was measured at 570 nm by a microplate reader (ELx800; BIO-TEK®). For Von Kossa staining, cells were fixed with 4% formaldehyde in PBS and stained with 5% silver nitrate in sterile deionised water under UV light for 5 min at RT.

3.3 Alkaline phosphatase assay

Cells were fixed with 4% formaldehyde in PBS and washed with deionised water. Subsequently, cells were stained with BCIP/NBT tablets (Roche, USA) in sterile deionized water for 30 min in the dark condition.

3.4 Lipid accumulation assay

The intracellular lipid accumulation was examined using oil red o staining. Cells were fixed in 4% formaldehyde in PBS for 15 min, rinse with deionized water, and stained with Oil red O solution (Sigma-Aldrich, USA) for 15 min at room temperature.

3.5 High throughput RNA sequencing data analysis

RNA sequencing data of hDPCs seeded on an indirect immobilized Jagged1 surface for 24 h were downloaded from the NCBI Sequence Read Archive and NCBI Gene Expression Omnibus (SRP100068 and GSE94989, respectively). The genes

related with ECM organization was identified and represented by the heat map. The raw expression was analyzed using NetworkAnalyst (Zhou et al. 2019; Xia, Gill, and Hancock 2015). The RNA was identified and an expression heatmap was generated using Heatmapper (Babicki et al. 2016).

3.6 Jagged1 Immobilization

Recombinant human (rh) Jagged1/FC protein (R&D Systems, Minneapolis, MN, USA) was prepared for coated on the surface of the tissue culture plate according to the previous report (Manokawinchoke et al. 2017). In short, tissue culture plates were incubated with 50 µg/ml recombinant protein G for 16h and washed with sterile phosphate buffer saline (PBS). Next, the surfaces were incubated with 10 mg/ml bovine serum albumin for 2 h, washed with PBS, and then incubated with 10 nM rhJagged1/FC for 2h. The human IgG Fc fragment (hFc) was used as the immobilization control.

3.7 Generation of decellularized extracellular matrix

Cells were seeded on rhJagged1/Fc protein-coated 24-well plates at $5x10^5$ cells and maintained with a growth medium in a humidified atmosphere with 5% CO_2 at 37 °C. To generate Jagged1-treated hDPSCs-derived ECM, the culture medium was changed to a normal medium (N medium), which is a growth medium supplemented with 50 µg/ml ascorbic acid or OM on day 7, and later cells were harvested on day 21. The culture medium was changed every 2 days.

For preparation of Jagged1-treated hDPSCs-derived decellularized ECM (dECM), samples were incubated with 0.5% Triton X-100 in 20 mM ammonium hydroxide for 5-10 min for removing all DNA components, washed by protease inhibitor in PBS. Then 0.0025% deoxyribonuclease in sterile PBS was added and incubated for 5-15 min at room temperature to break down DNA fragments and washed with a protease inhibitor in PBS. The hDPSCs-derived dECM were preserved by avoiding the dry atmosphere and kept in water at 4°C.

3.8 Scanning electron microscopy (SEM) and energy-dispersive X-ray spectrometry (EDX)

The specimens were fixed with 3% glutaraldehyde in PBS for 30 mins. The samples were dehydrated with serial graded ethanol (30-100%) and further added with hexamethyloxylane for 5 min, dried, and the gold coating was performed. The cells and dECM morphology were observed using SEM (Quanta 250, FEI, Hillsboro, OR USA). For element component detection, specimens without gold coating were examined using EDX (JSM-5410LV, JEOL, Tokyo, Japan).

3.9 Characterization of SCAPs on dECM

SCAPs were seeded at density of 2.5×10^4 cells on hDPSCs-derived dECMs for 30 min, 24h, and 7 days, incubated with a growth medium at 37 °C in a humidified atmosphere with 5% CO_2. Immunofluorescence, SEM, and EDX were performed to observe cell morphology, cell attachment, and spreading.

3.10 Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (USB Corporation). SCAPs were seeded on hDPSCs-derived dECM. On day1, 3, and 7, MTT solution (0.5 mg/ml) was added and incubated at 37°C for 15 min. The formazan crystals were dissolved with dimethylsulfoxide and glycine buffer. The absorbance at 540 nm was measured by a microplate reader (Molecular Devices, Palo Alto, CA).

3.11 Immunofluorescence staining

hDPSC-derived dECMs were assessed with fibronectin (Invitrogen, USA) and type I collagen (Abcam, UK) expression by immunofluorescence staining. The morphological appearance of the SCAPs seeded on hDPSC-derived dECMs was also evaluated by the F-actin organization using phalloidin staining (Invitrogen, USA). Briefly, dECM or cells were fixed in 4% formaldehyde in PBS, and permeabilized with 0.1% TritonX-100. Non-specific blocking was performed by incubating with 10% fetal bovine serum at 4°C overnight. The cells were stained with primary antibodies (1:200)

of type I collagen or (1:500) of fibronectin for 2 h, and further incubated with secondary antibodies. The samples were then incubated with biotinylated anti-rabbit IgG antibodies (Sigma-Aldrich, USA) at a dilution 1:2000 for 40 min and Strep-Rhodamine (Invitrogen, USA) was stained at 1:500. DAPI (Invitrogen, USA) was used for the nuclear counterstaining. Visualization using the fluorescence microscope with ApoTome system (Carl Zeiss, Germany).

3.12 Glycosaminoglycans staining

dECM were fixed with 0.1% glutaraldehyde (Sigma-Aldrich, USA) in PBS for 20 min. dECMs were stained with % Alcian Blue solution (Sigma-Aldrich, USA), incubated at RT for 24 h, and rinsed with 0.1M HCl and PBS. Stained dECM was observed by microscope.

3.13 Statistical analysis

All experiments were performed using cells from four different donors. Kruskal Wallis test followed by a pairwise comparison was employed for the more than three group comparison. Mann Whitney *U* test was used for statistical analysis of two group comparisons (GraphPad Software, CA, USA). Statistical significance was defined at $p<0.05$.

CHAPTER IV
RESULTS

4.1 Generation of decellularised extracellular matrix from human dental pulp stem cells

4.1.1 Characterization of hDPSCs

The characteristics of the human dental pulp stem cells (hDPSCs) used in this study were investigated with cell surface marker expression by flow cytometry. hDPSCs were positive for mesenchymal stem cell surface markers, CD44, CD73, and CD105, but negative for a hematopoietic surface marker, CD45 (Fig.1A). Osteogenic and adipogenic differentiation potential of hDPSCs were also examined. The mineral deposition was markedly detected by Alizarin Red S (ARS) staining after maintaining those cells with osteogenic induction medium (OM) for 14 days (Fig.2B-C). When hDPSCs were cultured with an adipogenic induction medium, the intracellular lipid droplets were observed with Oil Red O staining on day16 (Fig.2D-E). These results confirmed that isolated hDPSCs were mesenchymal stem cell populations.

4.2 Jagged1-treated hDPSCs modulated genes related to ECM organisation category

hDPSCs cultured on recombinant human Jagged1-treated surfaces (Jagged1-treated hDPSCs) significantly increased the expression of several extracellular matrix (ECM) organisation genes according to the bioinformatic analysis of the RNA sequencing database (SRP100068 and GSE94989). The upregulated ECM component genes in Jagged1-treated hDPSCs, including laminin subunit (*LAMA4, LAMB1, LAMB2, LAMC1*), elastin (*VCAN, ELN, EMILIN2*), proteoglycans (*FMOD, LUM*), glycoproteins (*FBN1, NID1, NID2, EFEMP1*), fibronectin (*TNC*), integrin (*ITG, ITGA5, ITGA11*) and glycosaminoglycans (*AGRN*) as shown in the heat map (Fig.2F). Moreover, several collagen genes were upregulated in the Jagged1-treated hDPSCs compared with the hFC-treated control such as *COL5A1, COL3A1, COL4A1, COL27A1,* and *COL5A3*. The expression profile also demonstrated an increase in the transcription level of collagen synthesis and assembly-associated genes in Jagged1-treated hDPSCs such as *P4HA3, P4HA1, ADAMTS14, ADAMTS3,* and *LOXL1* (Fig.2F).

4.3 Jagged1 promoted mineralisation in hDPSCs

The osteogenic differentiation potential of hDPSCs on indirect Jagged1-treated tissue culture surfaces was assessed after maintaining those cells in either a growth medium supplemented with 50 μg/ml ascorbic acid (a normal medium) or OM for 21 days. In normal medium conditions, the mineral deposition of hDPSCs was not observed in both Jagged1- and hFc-treated surfaces, while the ALP staining was positive (Fig.2G). ALP activity was higher in Jagged1-treated hDPSCs compared with the hFc control. When maintaining hDPSCs on Jagged1-treated surfaces with OM, significant enhancing mineralisation was observed compared to those cells maintained with a normal medium. Jagged1 immobilisation also more robustly increased the mineral deposits of hDPSCs than those of the hFc-treated control in OM condition (Fig.2G). Therefore, Jagged1 immobilised surfaces effectively enhanced an in vitro osteogenic differentiation of hDPSCs.

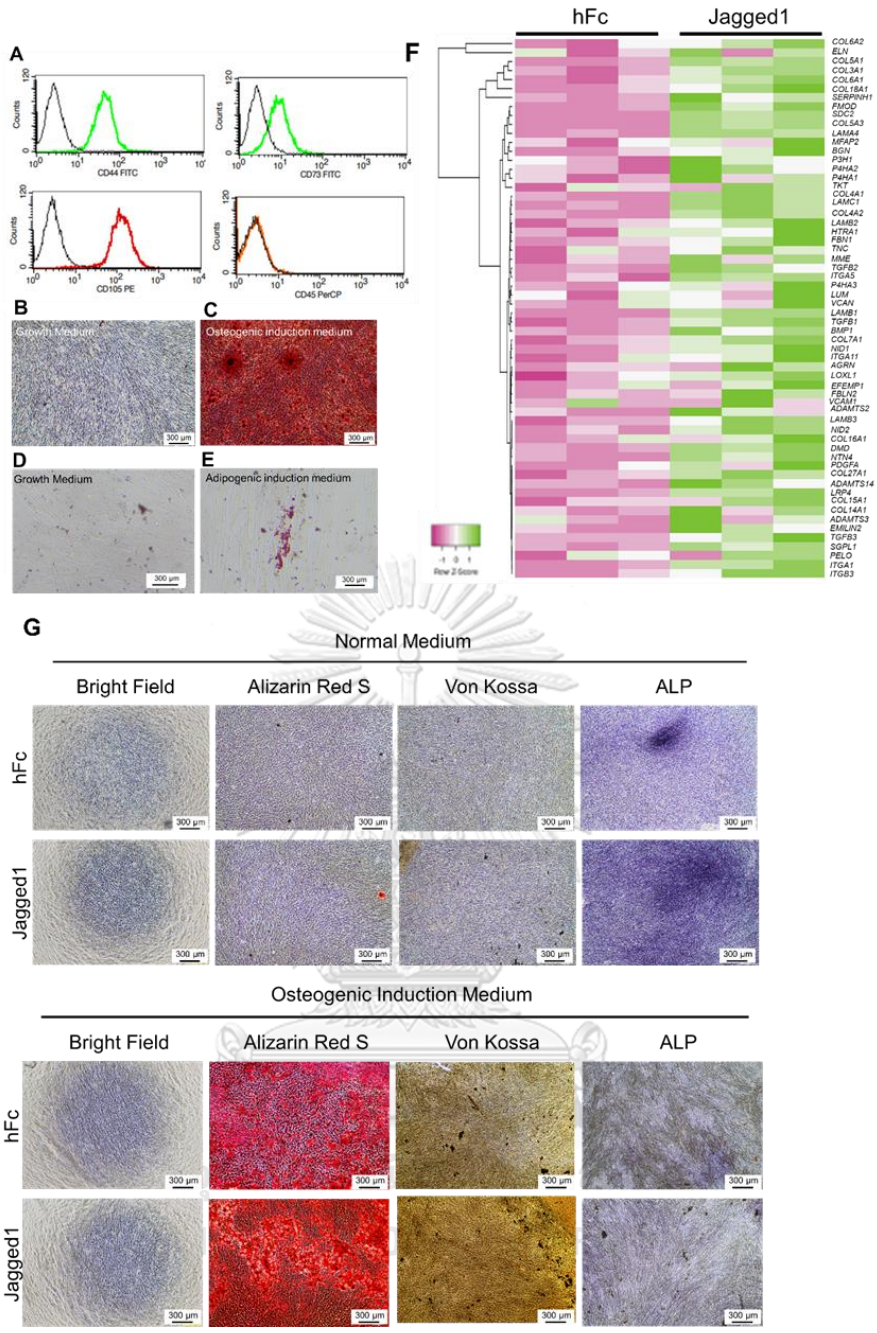

Figure 2 Human dental pulp stem cells (hDPSCs) were characterised by flow cytometry to examine surface protein marker expression (A). The mineralisation was examined using Alizarin Red S staining on day 14 after osteogenic induction (B-C). The intracellular lipid accumulation was detected using Oil Red O staining on day 16 after adipogenic induction (D-E). The effect of Jagged1 on hDPSCs was examined. Bioinformatic analysis of RNA sequencing data of genes related to ECM organisation was illustrated by heatmap (F). Morphology, mineralisation, and alkaline phosphatase enzymatic activity (ALP) were examined (G).

4.4 Characteristics and morphological appearance of decellularised ECM (dECM) derived from Jagged1-treated hDPSCs

After culturing hDPSCs on Jagged1- or hFc-treated surfaces with normal medium or OM for 21 days, we investigated the characteristics of ECM after conducting the decellularisation process. The bright-field microscope observation indicated the absence of cells. The dECM derived from the normal medium condition, both Jagged1 and hFC dECM-N, showed negative for ARS, Von Kossa, and ALP staining. In contrast, when maintaining the hDPSCs culture in the osteogenic induction condition (dECM-OM), the calcium deposition remained in Jagged1-treated dECM-OM compared to that of hFc-treated dECM-OM control after eliminating cellular components (Fig.3A). Scanning electron microscopy (SEM) analysis of Jagged1-treated hDPSCs-derived dECMs revealed the massive well-arranged fibrillar ECM networks in both normal medium and OM conditions (Fig.3B). ECM proteins were visualised after decellularisation by immunofluorescent staining. We found that fibronectin and type I-collagen were preserved in dECM derived from Jagged1- and hFc-treated surfaces.

Moreover, the absence of DAPI nuclei staining in dECM-N and dECM-OM indicated that the decellularising procedure was effective in removing genetic materials (Fig.3C). Proteoglycans accumulation in dECM was also observed by Alcian Blue staining after decellularisation in order to detect glycosaminoglycans (GAGs). The result showed that GAGs deposition was increased in Jagged1-treated dECMs in both normal medium and OM conditions when compared with the hFc control (Fig.3D). To investigate the chemical elements of dECM, we used energy dispersive X-ray spectrometry (EDX). The types of chemical components exhibited on surfaces were similar in both dECM derived from Jagged1- and hFc-treated hDPSCs with normal medium and OM conditions (Fig.3E). The findings confirmed that dECMs were deprived of cellular components but retained the main ECM proteins.

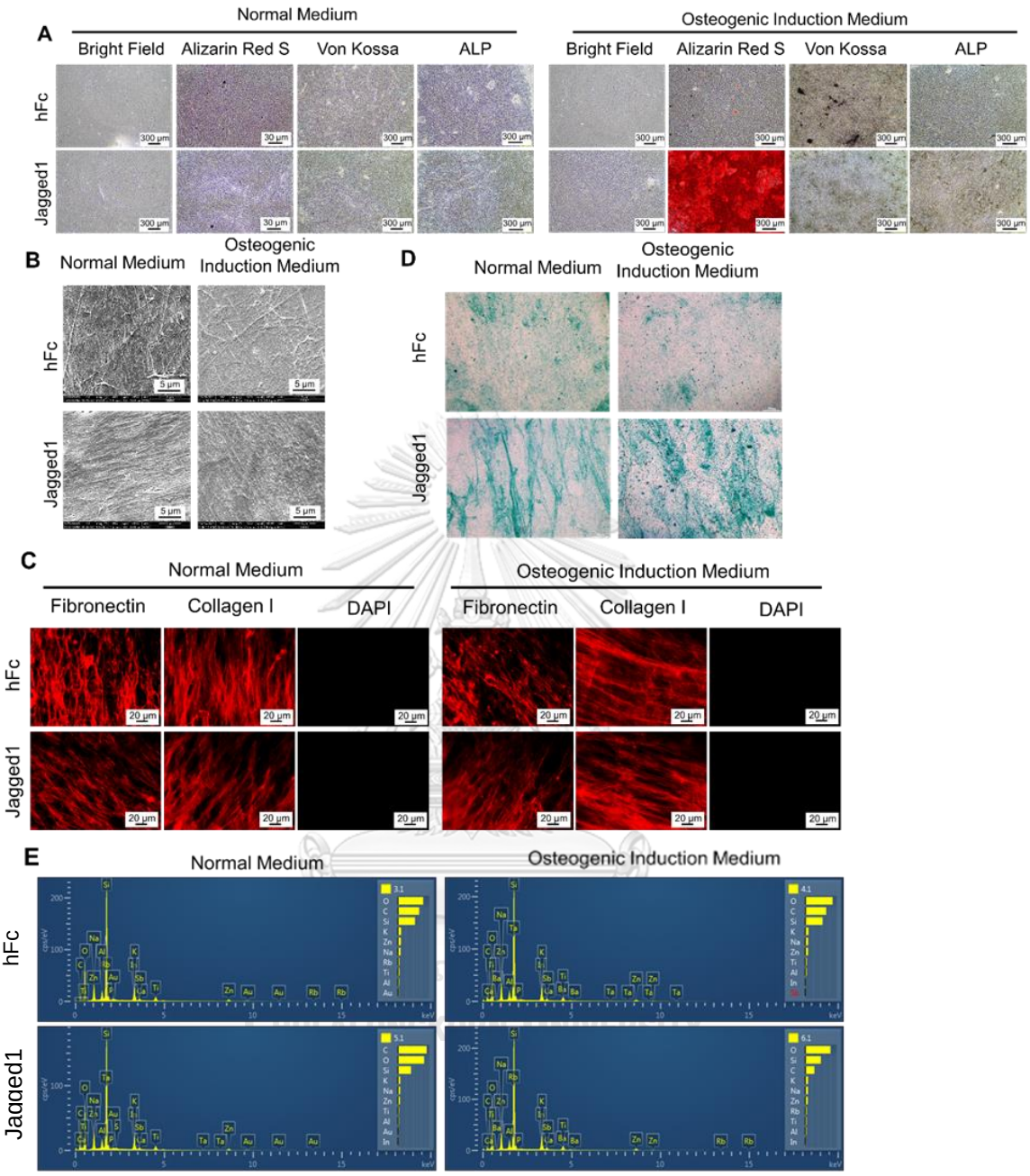

Figure 3 Characterization of decellularized extracellular matrix (dECM). Morphology, mineralisation, and alkaline phosphatase enzymatic activity (ALP) were examined (A). The ultrastructure of dECM was observed using scanning electron microscopic analysis (B). Fibronectin and type I collagen were determined using immunofluorescence staining (C). The genetic component was stained using DAPI (C).

Proteoglycan structures were examined by Alcian blue staining (D). The chemical composition of dECM was examined using energy-dispersive X-ray spectrometry (E). dECM-N; decellularised extracellular matrix derived from maintaining cells in normal medium, dECM-OM; decellularised extracellular matrix derived from maintaining cells in osteogenic medium.

4.5 Biological responses of SCAPs on dECM derived from Jagged1-treated hDPSCs

Stem cells isolated from apical papilla (SCAPs) were characterised by surface marker expression and multilineage differentiation ability. Isolated SCAPs expressed CD44, CD73, and CD105. Still, they lacked CD45 expression (Fig.4A). Osteogenic and adipogenic differentiation of SCAPs was confirmed by the positive staining for mineral nodules by ARS staining (Fig.4B-C) and intracellular lipid droplets accumulation by Oil Red O staining (Fig.4D-E), respectively.

The biological responses of SCAPs on dECM derived from Jagged1-treated hDPSCs were next determined. SCAPs were reseeded on either Jagged1-or hFc-treated hDPSCs-derived dECMs. Cell viability of SCAPs was assessed on day1 3, and 7 of culture by MTT assay. Gelatin-coated surfaces were used for the control. Even though gelatin is irreversibly denatured collagen, it still has molecular structure and properties close to native collagen and has recently been widely utilised as a biomaterial scaffold (Tondera et al. 2016; Bello et al. 2020). Normal cell proliferation of SCAPs was observed in all control and tested surfaces (Fig.4F). Thus, Jagged1- and hFc-treated hDPSCs-derived dECMs had no cytotoxicity and proliferative effects on SCAPs.

Cellular attachment and spreading were observed after seeding SCAPs on dECMs for 30 min, 24h, and 7 days in the growth medium. The cytoskeletal protein organisation, f-actin, was visualised by phalloidin immunofluorescent staining. SCAPs attached to all dECM surfaces at 30 min without spreading. However, the well-organized f-actin arrangement in SCAPs was not noticeably different in either Jagged1-treated hDPSC-derived dECM-N or dECM-OM on day7 (Fig.4G). SEM analysis further demonstrated that SCAPs attached, flattened, and spread with tiny filopodia

on Jagged1-treated hDPSC-derived dECM-OM after 30 min, while on other surfaces the cell shape was still round (Fig.4H). At 24h, SCAPs flattened and elongated on all dECM surfaces and later completely spread to form a monolayer covering the surface on day7 (Fig.4H). These results suggested that all dECMs originated from Jagged1- and hFc-treated surfaces are biocompatible for SCAPs proliferation, attachment, and spreading in vitro.

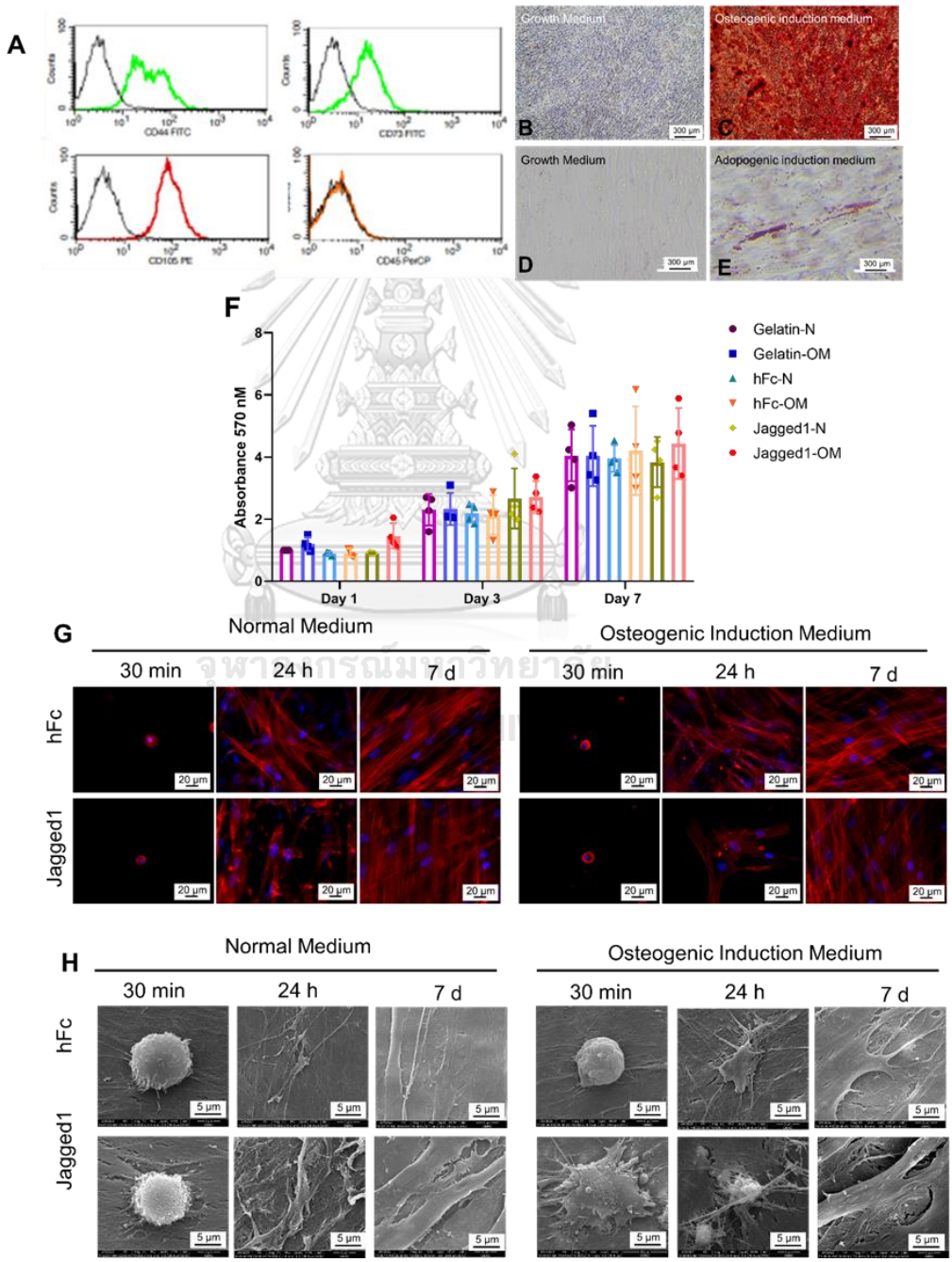

Figure 4 Stem cells isolated from apical papilla (SCAPs) were characterized by flow cytometry to examine surface protein marker expression (A). The mineralization was examined using Alizarin Red S staining at day 14 after osteogenic induction (B-C). The intracellular lipid accumulation was detected using Oil Red O staining at day 16 after adipogenic induction (D-E). The cell viability of SCAPs on dECM was determined using a MTT assay. The data were presented as mean±SEM and each dot represented the value from each donor (F). Cell attachment and actin arrangement were examined using phalloidin staining at 30 min, 24 h, and 7 d (G). Cell spreading was observed using scanning electron microscopic analysis (H). dECM-N; decellularized extracellular matrix derived from maintaining cells in normal medium, dECM-OM; decellularized extracellular matrix derived from maintaining cells in osteogenic medium.

4.6 dECM derived from Jagged1-treated hDPSCs promote the mineralization of SCAPs

To evaluate the osteogenic differentiation potential of SCAPs on Jagged1-treated hDPSCs-derived dECMs from normal medium (dECM-N) or OM (dECM-OM) conditions, the mineralization was observed by ARS staining on day7. SCAPs culturing on Jagged1-treated hDPSC-derived dECM-OM in the growth medium significantly increased the calcium deposition compared with those on hFc-treated and Jagged1-treated hDPSC-derived dECM-N (Fig.5A-B). When maintained SCAPs with OM for 7 days, those cells exhibited robustly increased in vitro mineralization. Cells on Jagged1-treated hDPSC-derived dECM-OM exhibited significantly higher mineralization compared with those cells on hFc-treated hDPSC-derived dECM-N (Fig.5C-D).

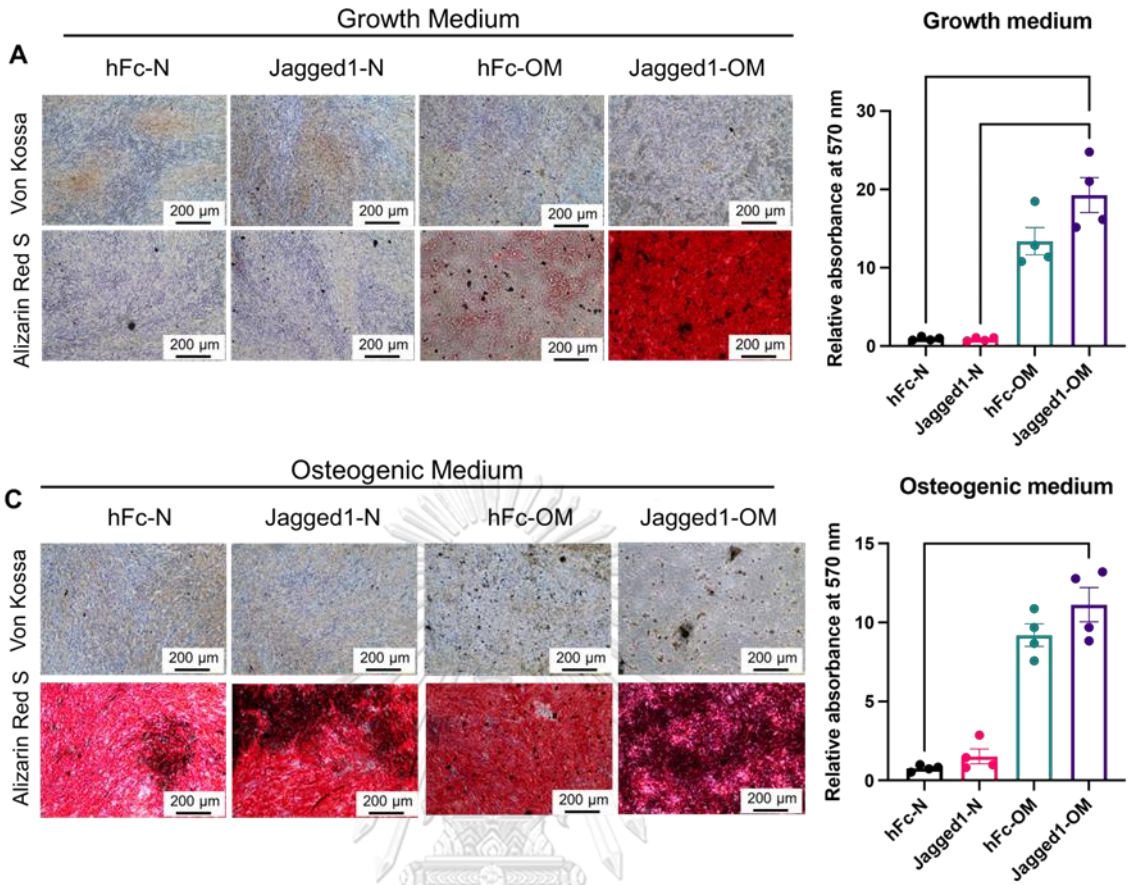

Figure 5 Jagged1-treated hDPSCs dECM promoted mineralization ability of SCAPs. SCAPs were seeded on dECM and maintained in growth medium (A) or osteogenic induction medium (C). Mineralization was observed using Von Koss and Alizarin Red S staining. The deposited Alizarin Red S was solubilized and the relative absorbance at 570 nm was illustrated (B and D). Bars indicated a statistically significant difference.

CHAPTER V
DISCUSSION AND CONCLUSION

The ECM scaffold is a biomaterial candidate for prospective clinical application in tissue regeneration, repair, and remodelling of both skeletal and non-skeletal tissues (Lin et al. 2020; Alaribe, Manoto, and Motaung 2016). Decellularized brain tissue (bECM) rapidly augmented the development of mature neuronal networks (Lam et al. 2019). The adipose tissue-derived decellularized ECM also provided favourable graft properties for adipose tissue engineering when combined with adipose-derived stem cells to repair the soft tissue defects (Wang et al. 2013). Additionally, the ECM scaffold is an excellent source of bioactive proteins for mineralized tissue formation, including bone morphogenetic protein 2 (BMP2), transforming growth factor-beta (TGFβ), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) (Sonoyama et al. 2006; Ravindran et al. 2014). Promotion of cell survival and proliferation after recellularized on ECM scaffolds by increased telomerase activity were previously indicated (Sonoyama et al. 2006).

Activation of the canonical Notch signalling pathway by Jagged1 promotes the odonto/osteogenic differentiation of various dental stem cells. Indirect Jagged1 immobilized surfaces markedly enhanced the osteogenic marker genes expression such as *RUNX2, OSX, OCN, COL1, OPN, BMP2,* and *DSPP* in hDPCs, hPDLSCs, and human bone-derived cells (Manokawinchoke et al. 2017; Osathanon et al. 2019). Importantly, the differential gene expression profile of hDPSCs cultured on Jagged1 immobilized surfaces demonstrated the upregulation of several genes involved in ECM organization, focal adhesion, and ECM-receptor interaction (Manokawinchoke et al. 2017). Upregulation of some matrix metalloproteinase genes, *ADAMTS2, ADMATS3, ADAMTS14,* which play a role in ECM proteins degradation and ECM remodelling also supported the osteogenic differentiation and mineralization of hDPSCs. Previous studies revealed the pivotal functions of MMPs in osteogenic, chondrogenic, adipogenic and endothelial differentiation (Mannello et al. 2006), mineral deposition

and calcium nodule during the differentiation process of MSCs (Almalki and Agrawal 2016). Moreover, ECM originating from hDPSCs served as an excellent material for inducing odontogenic differentiation of stem cells from the oral tissues (Ravindran, Huang, and George 2014). Thus, hDPSCs-derived ECM could be a promising biomaterial for utilization in regenerative dentistry, such as tooth and bone repair.

In this study, we generated and characterized the dECMs from immobilized Jagged1-treated hDPSCs and subsequently illustrated their osteoinductive activity on SCAPs. Previous reports showed that human gingival stem cells (hGSCs) could well attach, spread, and proliferate on hDPSCs-derived dECMs (Nowwarote et al. 2021). In addition, hDPSCs-derived ECM markedly enhanced osteogenic differentiation of the hGSCs (Nowwarote et al. 2021). Decellularization eliminates the genetic components while preserving proteins and ECM properties by chemical and enzymatic, physical, or combinative methods (Gilpin and Yang 2017). This procedure should be designed to trigger neither cytotoxicity nor immune response (Rieder et al. 2004). Thus, mechanical properties and reduced immunogenicity are critical considerations for further clinical applications of decellularized scaffold (Gilpin and Yang 2017). dECMs in this study are biocompatible for recellularized SCAPs. Moreover, the criteria for evaluating the appropriate decellularization included no visible nuclear material by staining with DAPI, less than 50 ng of dsDNA per mg of dry weight, and less than 200 bp of DNA fragment (Gilbert, Sellaro, and Badylak 2006). In addition, the protein components remaining in ECM after the decellularization should be assessed such as collagen, fibronectin, and glycosaminoglycans (Crapo, Gilbert, and Badylak 2011; Guneta et al. 2017). In accordance with those requirements, dECM-N and dECM-OM derived from Jagged1-treated hDPSCs were negative for DAPI staining and contained the classical ECM structure proteins, type I-collagen, and fibronectin, as well as those of the hFc-treated controls. Proteoglycans, which are composed of GAGs, is an abundant glycoprotein found in the ECM. It is important for the biological functions of ECM and is involved in ECM interaction with extracellular ligands for signalling transduction as well as stem cell homeostasis (Kusindarta and Wihadmadyatami 2018; Kular, Basu, and Sharma 2014). We observed that hDPSC-derived dECMs treated on Jagged1 immobilized surfaces showed increased GAGs deposition, but

there was no difference between normal and OM conditions. This suggested that the Jagged1-activated Notch signalling pathway participated in several gene expression and translational processes of ECM proteins. The potentiality of proteoglycans and GAGs in facilitating biological processes of stem cells was previously reported, which is a promising tool for developing as a bioactive material in bone regeneration (Chen et al. 2021).

Characterization of dental pulp stem cells in the *Jagged1*-knockout mouse model is not achievable due to embryonic lethality (Xue et al. 1999). However, we alternatively blocked the Notch signaling pathway or knocked down the *NOTCH2* receptor by γ-secretase inhibitor or short hairpin RNA (shRNA), respectively. In both molecular strategies, Jagged1-induced osteogenic marker genes and mineralization were significantly eliminated in hDPSCs (Manokawinchoke et al. 2017).

The interactions between cells and ECM influenced cell responses and function. Because cell adhesion is important for cell communication and regulation, as well as tissue development and maintenance (Khalili and Ahmad 2015), biological scaffolds should encourage cell adhesion to the substrate that further modulates cell behaviors such as cell proliferation, migration, spreading, and differentiation. We showed that Jagged1-treated dECM-OM promoted early cell adhesion and spreading of SCAPs compared to other dECMs by 30 min. It has been reported that cell shape and spreading area are related to the osteogenic differentiation potential (Jiao et al. 2020). Early cell adhesion and spreading correspond with increased osteogenic differentiation of human mesenchymal stromal cells on bone substituted materials (Barradas et al. 2013). Hence, the early spreading of SCAPs on Jagged1-treated hDPSCs derived dECM-OM could be related to its higher mineralisation when cultured in an osteogenic induction medium.

SCAPs are the neural crest-derived cells located underneath dental pulp tissues at the apex of the developing roots (Sonoyama et al. 2008). Since the dental papilla contributes to dentine and dental pulp tissue formation, the potential of SCAPs for tooth repair or endodontic regeneration should be expected. Regenerative endodontic treatment regenerates the pulp-dentin complex (Raddall, Mello, and Leung 2019). In this therapy were used the advantage of biomaterials scaffold is to

improve the treatment outcome. The scaffold was used to transplant into the root canal, which led to the migration of SCAPs into the scaffold to induce revascularisation and root development (Widbiller and Schmalz 2021; Raddall, Mello, and Leung 2019). The previous studies demonstrated that SCAP-mediated tissue regeneration suggests promising cell-based therapy for root regeneration. *In vivo*, SCAPs added into a root-shaped HA/TCP block with PDLSCs supported the restoration of tooth function in the swine (Sonoyama et al. 2006). The previous studies also reported that Jagged1 is essential for odonto/osteogenic differentiation and osteoblast development (Manokawinchoke et al. 2017; Hill et al. 2014). Dental pulp-derived ECM provided a proper environmental niche to assist the cell replication and regeneration of dental pulp tissues (Zhang et al. 2017). Decellularized dental pulp scaffold supported proliferation and differentiation to odontoblast-like cells of SCAPs (Song et al. 2017). Therefore, we hypothesised that Jagged1-derived dECM could have the capability to enhance odontogenesis/osteogenesis of dental stem cells, including SCAPs. Interestingly, we found the higher mineralisation of SCAPs after reseeding on Jagged1-OM dECMs by ARS staining in both normal medium and OM. The hFc-treated hDPSCs dECM did not show the remaining mineral content.

This observation was consistent with a previous study showing that decellularised process could eliminate the mineral content in dECM of hDPSCs culture in OM medium (Nowwarote et al. 2021). However, in the present study, we noted that decellularisation could not eliminate mineral components in Jagged-1 treated hDPSCs dECM. This observation could imply the distinct characteristics of ECM in Jagged1-treated hDPSCs as the mineral crystals could tightly bind to ECM structure. An additional investigation should be employed to elucidate the underlying mechanism.

Further studies are needed to specify the property of Jagged1-treated dECM and the mechanism to augment the mineralisation of SCAPs and other dental stem cells, for example, the role of ECM proteins. Indeed, the development of cell-derived ECM scaffold structures with other materials to stabilise the mechanical and physical properties and improve the recellularisation and regeneration are also required. In summary, Jagged1-treated hDPSCs dECM could be beneficial to develop as the novel

allograft for alternative dental therapeutics such as regenerative endodontic procedures or pulp capping material.

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