ผลของอะดีโนซีนไตรฟอสเฟตต่อการแปรสภาพเป็นเซลล์เนื้อเยื่อแข็งของเซลล์เนื้อเยื่อในฟันน้ำนมมนุษย์

นางสาวอรนุช เตชาธาราทิพย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาช่องปาก
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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THE EFFECT OF ADENOSINE TRIPHOSPHATE ON OSTEOGENIC DIFFERENTIATION IN HUMAN DENTAL PULP CELLS FROM DECIDUOUS TEETH

Miss Oranuch Techatharatip

A Dissertation Submitted in Partial Fulfillment of the Requirements
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By	Miss Oranuch Techatharatip
Field of Study	Oral Biology
Thesis Advisor	Professor Doctor Prasit Pavasant, Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Dentistry
(Associate Professor Doctor Suchit Poolthong, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Doctor Jeerus Sucharitakul, Ph.D.)

.....Thesis Advisor
(Professor Doctor Prasit Pavasant, Ph.D.)

.....Examiner
(Assistant Professor Doctor Waleerat Sukarawan, Ph.D.)

.....Examiner
(Associate Professor Doctor Thanaphum Osathanoon, Ph.D.)

.....External Examiner
(Associate Professor Doctor Nirada Dhanesuan, Ph.D.)

อรนุช เตชาธาราทิพย์ : ผลของอะดีโนซีนไตรฟอสเฟตต่อการแปรสภาพเป็นเซลล์เนื้อเยื่อแข็งของเซลล์เนื้อเยื่อในฟันน้ำนมมนุษย์ (THE EFFECT OF ADENOSINE TRIPHOSPHATE ON OSTEOGENIC DIFFERENTIATION IN HUMAN DENTAL PULP CELLS FROM DECIDUOUS TEETH) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ทพ. ดร. ประสิทธิ์ ภวสันต์, 70 หน้า.

เมื่อมีแรงดันภายในโพรงฟันเซลล์เนื้อเยื่อในโพรงฟันตอบสนองโดยการหลั่งอะดีโนซีนไตรฟอสเฟต (เอทีพี) โดยปริมาณเอทีพีที่หลั่งออกมาแปรผันตามขนาดของแรง นอกจากนี้เอทีพีมีผลต่อการควบคุมการแปรสภาพของเซลล์ต้นกำเนิดไปเป็นเซลล์ไขมัน และเซลล์กระดูก อย่างไรก็ตามยังไม่มีการศึกษาถึงผลของเอทีพีภายนอกเซลล์ต่อคุณสมบัติในการเป็นเซลล์ต้นกำเนิดและการแปรสภาพเป็นเซลล์เนื้อเยื่อแข็งของเซลล์เนื้อเยื่อในฟันน้ำนมมนุษย์ ดังนั้นการศึกษานี้จึงสนใจถึงผลของเอทีพีภายนอกเซลล์ต่อคุณสมบัติในการเป็นเซลล์ต้นกำเนิดและการแปรสภาพเป็นเซลล์เนื้อเยื่อแข็งของเซลล์เนื้อเยื่อในฟันน้ำนมมนุษย์ โดยเลือกใช้ความเข้มข้นต่ำ (0.1 ไมโครโมลาร์) และสูง (10 ไมโครโมลาร์) เพื่อจำลองขนาดของแรงน้อยและมากตามลำดับ

เซลล์เนื้อเยื่อในฟันน้ำนมมนุษย์ถูกนำมาเพาะเลี้ยงในอาหารเลี้ยงเซลล์หรืออาหารเลี้ยงเซลล์สำหรับเหนี่ยวนำเป็นเซลล์เนื้อเยื่อแข็งที่มีหรือไม่มีเอทีพีที่มีความเข้มข้น 0.1, 1 และ 10 ไมโครโมลาร์ ทำการตรวจวัดระดับการแสดงออกของยีนที่เกี่ยวข้องกับเซลล์ต้นกำเนิด การสร้างเนื้อเยื่อแข็ง และการควบคุมการสะสมแร่ธาตุด้วยวิธีรีเวอร์สทรานสคริบชัน เรียลทาร์มโพลีเมอร์เรสเชนรีแอคชัน วัดปริมาณการสะสมแร่ธาตุในห้องปฏิบัติการด้วยวิธีการย้อมอะลิซาริน เรด เอส และตรวจวัดระดับการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเตส (เอแอลพี) และ เอคโตนิวคลีโอไทด์ ไพโรฟอสฟาเตส หรือ ฟอสโฟไดเอสเตอเรส (อีเอ็นพีพี) ซึ่งเป็นเอนไซม์ควบคุมระดับฟอสเฟตและไพโรฟอสเฟต

ผลการศึกษาแสดงให้เห็นว่าในสภาวะอาหารเลี้ยงเซลล์ปกติทั้งความเข้มข้นของเอทีพีต่ำและสูงมีผลต่อการเพิ่มระดับการแสดงออกของยีนที่เกี่ยวข้องกับเซลล์ต้นกำเนิดและการสร้างเนื่อเยื่อแข็ง ส่วนในสภาวะอาหารเลี้ยงเซลล์สำหรับเหนี่ยวนำเป็นเซลล์เนื้อเยื่อแข็งให้ผลแตกต่างกันโดยเอทีพีที่มีความเข้มข้น 0.1 ไมโครโมลาร์เหนี่ยวนนำให้เกิดการสะสมแร่ธาตุเพิ่มขึ้น ในขณะที่เอทีพีความเข้มข้น 10 ไมโครโมลาร์ยับยั้งการสะสมแร่ธาตุในห้องปฏิบัติการ นอกจากนี้เอทีพีความเข้มข้น 10 ไมโครโมลาร์ยังเพิ่มการแสดงออกของยีนและการทำงานของเอนไซม์อีเอ็นพีพี

โดยสรุป เอทีพีมีผลต่อการแสดงออกของยีนที่เกี่ยวข้องกับเซลล์ต้นกำเนิดและการสร้างเนื้อเยื่อแข็งและมีผลในการกระตุ้นหรือยับยั้งการสะสมแร่ธาตุในห้องปฏิบัติการโดยขึ้นกับสภาวะอาหารเลี้ยงเซลล์และความเข้มข้นของเอทีพี ในสภาวะอาหารเลี้ยงเซลล์ปกติ เอทีพีทั้งสองความเข้มข้นมีผลต่อการเพิ่มระดับการแสดงออกของยีนที่เกี่ยวข้องกับเซลล์ต้นกำเนิดและการสร้างเนื้อเยื่อแข็ง อย่างไรก็ตามในสภาวะอาหารเลี้ยงเซลล์สำหรับเหนี่ยวนำเป็นเซลล์เนื้อเยื่อแข็งเอทีพีทั้งสองความเข้มข้นมีผลต่อการสะสมแร่ธาตุในห้องปฏิบัติการแตกต่างกัน โดยเอทีพีความเข้มข้นต่ำมีผลในการกระตุ้น ส่วนเอทีพีความเข้มข้นสูงมีผลยับยั้งการสะสมแร่ธาตุในห้องปฏิบัติการ ดังนั้นจึงอาจสรุปได้ว่าเอทีพีถูกหลั่งจากเซลล์เนื้อเยื่อในฟันน้ำนมมนุษย์ภายหลังได้รับแรงกดเชิงกล และเอทีพียังมีผลต่อการควบคุมความเป็นเซลล์ต้นกำเนิดและการแปรสภาพของเซลล์เนื้อเยื่อในฟันน้ำนมมนุษย์

สาขาวิชา ชีววิทยาช่องปาก ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5476059432 : MAJOR ORAL BIOLOGY
KEYWORDS: ADENOSINE TRIPHOSPHATE (ATP) / OSTEOGENIC DIFFERENTIATION / IN VITRO MINERALIZATION / STEM CELLS ISOLATED FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED

ORANUCH TECHATHARATIP: THE EFFECT OF ADENOSINE TRIPHOSPHATE ON OSTEOGENIC DIFFERENTIATION IN HUMAN DENTAL PULP CELLS FROM DECIDUOUS TEETH. ADVISOR: PROF. DR. PRASIT PAVASANT, Ph.D., 70 pp.

Background: Adenosine triphosphate (ATP) is released in response to intrapulpal pressure and its released amount depends on the magnitude of pressure. ATP is involved in the regulation of differentiation of stem cells into adipocytes and osteoblasts. However, it is unknown whether extracellular ATP influences the stemness properties and osteogenic differentiation of stem cells from human exfoliated deciduous teeth (SHEDs).

Objective: To investigate the effect of extracellular ATP in a low (0.1 μM) and high (10 μM) concentration on the stemness properties and osteogenic differentiation of SHEDs.

Material and methods: SHEDs were cultured in either growth medium or osteogenic medium in the presence or absence of 0.1, 1 or 10 μM ATP. The level of expression of stem cell markers, osteogenesis and mineralization modulating genes were determined by Real-time PCR. *In vitro* mineralization was determined by alizarin red S staining. The activity level was assessed of alkaline phosphatase (ALP) and ectonucleotide pyrophosphatase/phosphodiesterase (ENPP), a phosphate (Pi)/pyrophosphate (PPi) modulating enzyme.

Results: In growth medium, both concentrations of ATP increased the mRNA expression of pluripotent and osteogenic markers. In osteogenic medium a different effect was found. Here, 0.1 μM of ATP enhanced the *in vitro* mineralization, whereas 10 μM of ATP inhibited this process. Under the latter conditions ATP stimulated the mRNA expression and activity of ENPP; an enzyme that regulates the Pi/PPi ratio.

Conclusion: Depending on the growth condition and concentration, ATP may stimulate stemness and *in vitro* mineralization or inhibit the latter process. In normal medium, both concentrations of ATP stimulated gene expression of pluripotent and osteogenic markers. However, in osteogenic medium a biphasic effect was found on *in vitro* mineralization; a low concentration had a stimulating whereas a high concentration had an inhibiting effect. We propose that ATP released due to mechanical stress modulates stemness and differentiation of SHEDs.

Field of Study: Oral Biology Student's Signature .....

Academic Year: 2016 Advisor's Signature .....

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSP	bone sialophosphoprotein
BMP	bone morphogenetic protein
BzATP	selective P2X7 receptor agonist
DMP1	Dentin matrix acidic phosphoproteins
DSPP	Dentin sialophosphoprotein
ENPP	Ectonucleotide pyrophosphatase/phosphodiesterase-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IL	Interleukin
KN62	selective P2X7 receptor antagonist
MRS2179	selective P2Y1 receptor antagonist

MRS2365	selective P2Y1 receptor agonist
OCN	Osteocalcin
OCT4	Octamer4
OM	osteogenic medium
OPN	Osteopontin
OSX	Osterix
PPAR-ɣ	Peroxisome proliferator-activated receptor gamma
Pi	phosphate
PPi	pyrophosphate
PiT1	Type III sodium/phosphate cotransporters
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SHEDs	stem cells from human exfoliated deciduous teeth
SOX2	(Sex determining region-Y)-box2

UDP	uridine diphosphate
UTP	uridine triphosphate

CHAPTER I

INTRODUCTION

It has been demonstrated that, after injury or irritation, blood vessels within the tooth pulp will be dilated, resulting in an increase of interstitial fluid pressure [Heyeraas and Berggreen, 1999]. Since the dental pulp is surrounded by mineralized dentin, expansion of the tissue is limited. Therefore, cells in dental pulp chambers are normally exposed to pressure [Heyeraas and Berggreen, 1999; Yu and Abbott, 2007]. Previously, we have shown that in response to compressive pressure, adenosine triphosphate (ATP) was released by dental pulp cells from both permanent and deciduous teeth [Govitvattana et al., 2015; Satrawaha et al., 2011]. Thus, dental pulp cells may always be in an environment with fluctuating levels of ATP.

ATP is the main energy source for the majority of cellular functions. However, ATP also functions as an important extracellular signaling molecule that regulates cell proliferation, cell differentiation, muscle contraction and intercellular communication [Burnstock, 2006]. It has been reported that ATP will be released from damaged or necrotic cells and can influence cellular behavior in an autocrine or paracrine fashion [Brandao-Burch et al., 2012]. Besides the damaged or necrotic cells, we have demonstrated that, upon mechanical stimulation, cells from dental tissue, such as periodontal ligament and dental pulp cells, released ATP and perhaps other types

of nucleotides to regulate cellular behavior through a P2-signaling dependent pathway [Govitvattana et al., 2013; Luckprom et al., 2011; Wongkhantee et al., 2008]. In general, ATP signals through the P2 receptors on the plasma membrane. P2 receptors are grouped in the P2X family of ionotropic cation channels and the P2Y family of G-protein-coupled receptors. All receptors are primarily activated by their physiological agonist ATP, but differ in their rates of inactivation and sensitivity to ATP [Khakh et al., 2001; North, 2002].

Extracellular ATP is involved in the regulation of several physiological processes such as bone and cartilage remodeling, microvascular remodeling and the differentiation of mesenchymal stem cells into adipocytes or osteoblasts [Ciciarello et al., 2013; Rumney et al., 2012]. The role of extracellular ATP on regulating adipogenic and osteogenic differentiation has been reported. Previous study demonstrated that pretreatment with 1 mM ATP before adipogenic induction in human bone marrow-derived mesenchymal stem cell promoted adipogenic differentiation by increasing lipid accumulation and adipogenic marker gene [Ciciarello et al., 2013]. The effect of extracellular ATP on regulating osteogenic differentiation may depend on the concentration of ATP. Evidence indicated that high levels of ATP (100 μM) induced osteogenesis, whereas low levels of ATP (0.1-10 μM) inhibited osteoblast function and increased osteoclast development and activity [Ayala-Pena et al., 2013; Costessi et al., 2005; Hoebertz et al., 2002].

Extracellular matrix mineralization is tightly linked to the inorganic phosphate (P_i)/ pyrophosphate (PP_i) ratio found in the tissue. P_i forms a complex with calcium, and forms hydroxyapatite crystals [Hayashi and Nagasawa, 1990]. In contrast, PP_i is a potent, mineral-binding small molecule inhibitor of crystal nucleation and growth at micromolar concentrations [Orriss et al., 2012; Terkeltaub, 2001]. The homeostatic regulation of local P_i and PP_i levels by bone cells is thus an important part of their function related to mineralization. Extracellular P_i and PP_i levels are controlled by the following enzymes and cotransporters: ectonucleotidepyrophosphatase/phosphodiesterase (ENPP), progressive ankylosis homolog protein (ANKH) and alkaline phosphatase (ALP) [Foster et al., 2008].

Stem cells from human exfoliated deciduous teeth (SHEDs) were identified to be a population of highly proliferative, clonogenic cells capable of differentiating into a variety of cell types including neuronal cells, adipocytes, osteo/odontoblasts, endothelial cells and hepatocyte-like cells [Ishkitiev et al., 2010; Miura et al., 2003; Nourbakhsh et al., 2011; Sakai et al., 2010]. Moreover, these cells expressed mRNA and protein markers of embryonic and mesenchymal stem cells [Govitvattana et al., 2013; Miura et al., 2003; Nowwarote et al., 2015]. One of our previous studies indicated that SHEDs responded to extracellular ATP by upregulating *REX-1* expression via the P2Y1 receptor [Govitvattana et al., 2015]. The release of ATP was triggered by

stress-induced IL-6 secretion and the level of ATP release depended on the dose of IL-6 and magnitude of stress. An increased magnitude of mechanical stress resulted in an increased amount of released ATP [Govitvattana et al., 2013; Govitvattana et al., 2015]. Thus, mechanical stress appears to play a role in the regulation of stemness of SHEDs. However, a possible role of ATP on regulating stemness and osteogenic differentiation by SHEDs has not been elucidated.

The purpose of this study was to investigate the effect and underlying mechanisms of extracellular ATP in low (0.1 μM) and high (10 μM) concentrations on the stemness properties and osteogenic differentiation of SHEDs. This knowledge will increase understanding of the role of extracellular ATP to maintain pulpal homeostasis and dentin repair.

Research questions

1. Does extracellular ATP have any influence on the stemness properties of SHEDs?
2. Does extracellular ATP affect the osteogenic differentiation of SHEDs?

Objectives and hypothesis

Objective 1: To examine the influence of extracellular ATP on the stemness properties

Hypothesis 1: Extracellular ATP has influence on the stemness properties

Experimental design:

1.1 SHEDs were cultured in the presence or absence of extracellular ATP at concentration 0.1 - 10 μM for 1 day. Then, the RNA were extracted for

quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis for mesenchymal stem cell marker *(CD90)* embryonic stem cell marker (*REX1*, *NANOG*, *OCT4, SOX2*), osteogenic (*RUNX2*, *OSX*, ALP, OCN) mRNA expression.

1.2 SHEDs were cultured in the presence or absence of extracellular ATP at concentration 0.1 - 10 μM for 1, 3 and 7 days. Then, the cell proliferation assay was performed.

1.3 SHEDs were cultured in the presence or absence of extracellular ATP at concentration 0.1 - 10 μM for 14 days. Then, the colony forming unit assay was performed.

Objective 2: To examine the effect and mechanism of extracellular ATP on the osteogenic differentiation ability of SHEDs.

Objective 2.1: To examine the effect of extracellular ATP on osteogenic differentiation of SHEDs

Hypothesis 2.1: Extracellular ATP inhibits osteogenic differentiation of SHEDs

2.1.1 SHEDs were induced with osteogenic medium (OM) in the presence or absence of ATP at concentration 0.1, 1 or 10 μM for 10 days. Then, *in vitro*

mineralization was visualized with alizarin red staining and destained with 10% Cetylpyridinium chloride monohydrate.

2.1.2 SHEDs were induced with OM in the presence or absence of ATP at concentration 0.1, 1 or 10 μM for 1 and 3 days. Then, the RNA was extracted for qRT-PCR analysis for *RUNX2*, *OSX*, *DMP1*, *OCN, ALP, ANKH* and *ENPP* mRNA expression.

2.1.3 SHEDs were induced with OM in the presence or absence of ATP at concentration 0.1 and 10 μM for 3 days. Then, ALP activity and total protein were measured.

2.1.4 SHEDs were induced with OM in the presence or absence of ATP at concentration 0.1 and 10 μM for 3 days. Then, the ENPP activity and total protein were measured.

Objective 2.2: To investigate the mechanism of extracellular ATP on osteogenic differentiation in SHEDs

Hypothesis 2.2: Extracellular ATP inhibits osteogenic differentiation in SHEDs via purinergic signaling

Experimental design:

2.2.1 SHEDs were induced with OM in the presence or absence of ATP at concentration 0.1, 1 or 10 μM and with/without purinergic receptor antagonists and agonists (Suramin, KN62, NF449, BzATP, **α,β**-meATP) for 10 days. Then, *in vitro* mineralization was visualized with alizarin red staining and destained with 10% Cetylpyridinium chloride monohydrate.

2.2.2 SHEDs were induced with OM in the presence or absence of ATP at concentration 0.1-10 μM and with/without purinergic receptor antagonists and agonists (Suramin, KN62, NF449, BzATP, **α,β**-meATP) for 1 and 3 days. Then, the RNA will be extracted for qRT-PCR analysis for *OSX* and *ENPP* mRNA expression.

Expected benefit

This study clarifies the role of ATP in human dental pulp cells and the biological response and the mechanism of SHEDs to maintain pulpal homeostasis and dentin repair.

Limitation

SHEDs are primary cells which have a large variation of characteristics such as genetic in each patient, sex. These experiments were performed *in vitro*, thus the results might not explain what occur *in vivo* or physiological condition. ATP is easily degraded by ectonucleotidase

enzyme. Therefore, the cells were cultured for several days; the result might come from the effects of ATP and its products.

Keywords

Adenosine triphosphate (ATP)

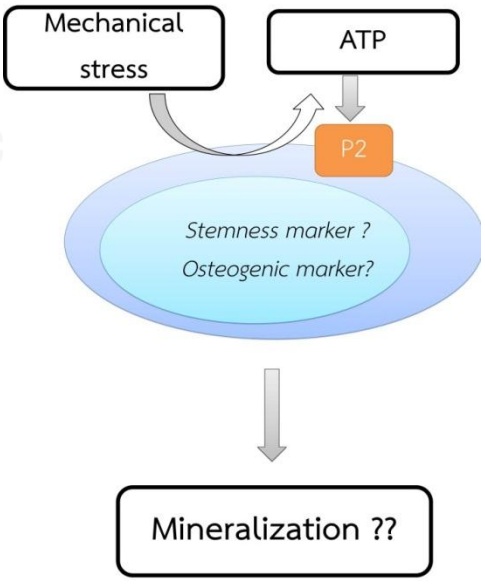
osteogenic differentiation

stem cells from human exfoliated deciduous teeth (SHEDs)

Research design

Laboratory experimental research

Conceptual framework



CHAPTER II

REVIEW OF RELATED LITERATURES

Adenosine-5'-triphosphate (ATP)

Adenosine 5-triphosphate (ATP) is present in every living cell of the human body. Chemically, ATP consists of adenosine, an adenine ring and ribose sugar, and three phosphate groups. The purine base (adenine ring) is attached to the 1'carbon atom and the three phosphate groups are attached to 5'carbon atom of the pentose sugar (ribose), as shown in Figure 2.1. ATP is highly soluble in water and is quite stable in solutions between pH 6.8 and 7.4, but is rapidly hydrolyzed at extreme pH. Consequently, ATP is best stored as an anhydrous salt. In alkali solution, ATP is hydrolyzed to AMP and pyrophosphate.

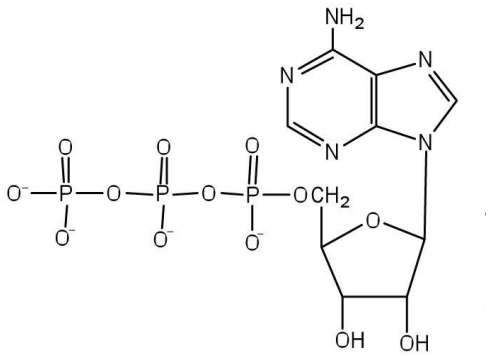


Figure 2.1 Molecular structure of ATP. ATP consists of an adenine base, a ribose sugar and a phosphate chain.

In physiological conditions, the average concentration of ATP varies from 0.4-6 μM in human blood cells [Traut, 1994]. Plasma ATP concentrations range between 0.15 and 3.9 μM [Harkness et al., 1984] It is produced by a wide variety of enzymes, including ATP synthase, from inorganic phosphate, adenosine diphosphate (ADP) or adenosine monophosphate (AMP) and various phosphate group donors. In contrast, nucleotides are rapidly degraded by ectonucleotidases, such as CD39 and CD73, which transform ATP sequentially into ADP, AMP and finally adenosine [Deaglio and Robson, 2011]. Therefore, ATP is continuously recycled in an organism and cells obtain energy via hydrolysis of the high-energy phosphate bond.

A well-known role of ATP is as an energy source in living cells. In addition, ATP plays a role in intracellular signal transduction, and is used as a substrate in signal transduction pathways by kinases that phosphorylate proteins and lipids [Cotrina et al., 2000; Kamenetsky et al., 2006]. It is also used by adenylate cyclase and is transformed to the secondary messenger molecule, cyclic AMP (cAMP), which is involved in triggering calcium signals by the release of calcium from intracellular storages [Kamenetsky et al., 2006]. This form of signal transduction is particularly important in brain function.

In addition, ATP is also an extracellular signaling molecule that performs many essential roles in the cells. ATP, ADP and adenosine are recognized by purinergic receptors. In humans, this

signaling role is important in a wide variety of biological processes, including inter alia afferent signaling (including pain), regulation of renal blood flow, vascular endothelium, inflammatory responses, cell proliferation, cell differentiation, muscle contraction and intercellular communication [Burnstock, 2006].

Purinergic receptors

A large family of membrane-bound receptors mediates cell signaling by ATP and adenosine. Purinergic receptors have been classified into two families, P1 and P2 receptors, which respond to adenosine and ATP, respectively (Figure 2.2 and Table 2.1).

P1 receptors are member of the rhodopsin-like family of G-protein coupled receptors (GPCR). Adenosine can interact with at least four cell surface P1 receptors, identified as A1, A2A, A2B, and A3 receptors, and influence a variety of physiological functions. The endogenous ligand is adenosine (AMP). Besides adenosine itself, its breakdown product inosine has also been shown to exert an agonist action on A1 and A3 receptors, but not on A2 receptors. However, this agonist action of inosine appears to have a low efficacy compared with adenosine, especially at A3receptors [Fredholm et al., 2001]. P1 receptors differently regulate the production of cAMP. The A2A and A2B types could activate adenylate cyclase and increase the levels of cytoplasmic cAMP, whereas A1 and A3 receptors inhibit adenylate cyclase [Baroja-Mazo et al., 2013;

Burnstock, 1976; Burnstock, 2006; Burnstock, 2009]. These receptors induce various downstream reactions on calcium and kinases [Klinger et al., 2002].

The P2 receptor family is divided into the P2X family of ionotropic cation channels and the P2Y family of GPCRs, with a number of different subtypes which have variable affinities for ATP, ADP, uridine triphosphate (UTP), uridine diphosphate (UDP) and UDP–glucose. P2X receptors are ligand-gated ion channels, of which currently seven subtypes have been characterized (P2X1–7) [Khakh et al., 2001; North, 2002]. All are primarily activated by their physiological agonist ATP but are different in their rates of inactivation and sensitivity to ATP. The P2X7 receptor requires the highest concentration of ATP for activation and inactivates the slowest [North, 2002]. Activation of ionotropic P2X receptors opens cationic channels, allowing extracellular Na^{+} and Ca^{2+} influx and K^{+} efflux, resulting in cellular hyperpolarization. For the P2Y receptors, eight subtypes have been identified (P2Y1, 2, 4, 6, 11–14) [Jacobson et al., 2002; von Kugelgen and Wetter, 2000]. P2Y receptors can be subdivided into two groups based on their coupling to specific G-proteins and their sequence homology [Abbracchio et al., 2006; Boeynaems et al., 2003]. Group 1 couple mainly to Gq protein and consists of specific purinergic receptors (P2Y1, P2Y11), specific pyrimidinergic receptors (P2Y4, P2Y6) and receptors of mixed specificity (P2Y2). Group 2 couple to Gi protein and contains two specific ADP receptors (P2Y12, P2Y13) and a

receptor for UDP–glucose (P2Y14). The metabotropic P2Y receptor stimulation leads to the activation of phospholipase C, production of inositol triphosphate, increase in cytosolic Ca^{2+} because of its release from intracellular storages, as well as the activation of adenylate cyclase [Baroja-Mazo et al., 2013]. This variety of agonists and effectors ensures that purinergic signaling can affect several processes in different tissues.

Recently, gene silencing techniques have been proven useful to verify the functions of purinergic receptors. Various roles of P2X and P2Y have been reported. P2X1 receptors enhance platelet functions and loss of P2X1 receptors reduces thrombus formation of small arteries [Gachet, 2008; Hechler et al., 2003]. P2X2 and P2X3 subunits play an important role in the initiation of sensory signaling in pathways subserving taste, chemoreception, visceral distension, and neuropathic pain [Bo et al., 1999; Cockayne et al., 2005; Honore et al., 2002; Spyer et al., 2004]. P2X4 receptors are involved in the function of vascular endothelium that regulates blood pressure and vascular remodeling [Yamamoto et al., 2006]. P2X7 receptors are related to immune system and bone remodeling. Activation of P2X7 receptors induces the release of pro-inflammatory cytokines from macrophages [Ferrari et al., 2006]. P2X7 receptor knockout mice show abnormal bone remodeling in the form of a reduced periosteal circumference and decreased cortical bone content [Ke et al., 2003]. In addition, P2X1 and P2X7 mediate the

inhibition of bone mineralization by increasing ENPP activity and pyrophosphate in primary rat osteoblast cells [Orriss et al., 2012]. P2Y2 receptors play roles in leukocyte chemotaxis and tissue infiltration. P2Y12 receptors regulate microglia chemotaxis and P2Y6 receptors stimulate their phagocytic activity [Haynes et al., 2006; Koizumi et al., 2007]. Moreover, P2Y1, P2Y2, P2Y6 and P2Y13 receptors are involved in bone turnover. P2Y1 knockout mice show decrease bone mass [Orriss et al., 2011]. P2Y2 knockout mice show increase bone mass [Orriss et al., 2011]. P2Y6 receptors stimulate the formation of osteoclasts from precursor cells and enhance the resorptive activity of mature osteoclasts [Orriss et al., 2011]. P2Y13 knockout mice show reduction in bone formation and bone resorption because the number of osteoblasts and osteoclasts is lower than that of wild type mice [Orriss et al., 2011; Rumney et al., 2012].

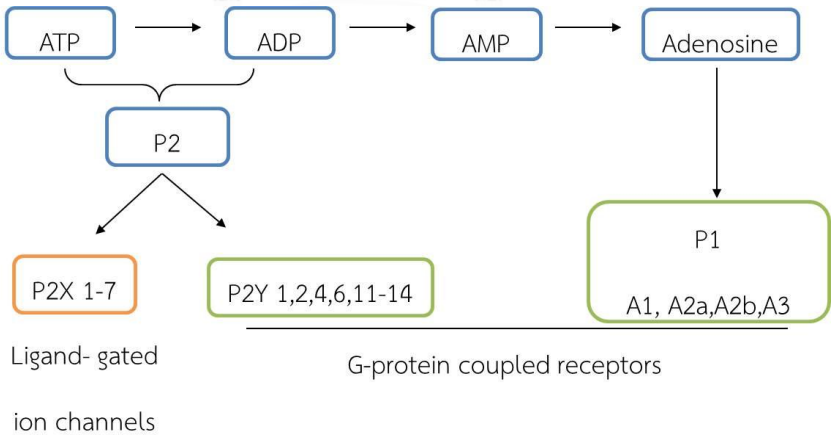


Figure 2.2 A schematic overview of purinergic receptors and their affinities

Table 2.1 Properties of P2Y receptor [Boeynaems et al., 2012]

Group	Receptor	Agonist (human)	G protein
A	P2Y1	ADP	Gq
	P2Y2	ATP=UTP	Gq(+Gi)
	P2Y4	UTP	Gq(+Gi)
	P2Y6	UDP	Gq
	P2Y11	ATP	Gq + Gs
B	P2Y12	ADP	Gi
	P2Y13	ADP	Gi
	P2Y14	UDP, UDP-glucose	Gi

Dentinogenesis

Tooth development arises from interactions between the oral epithelium and migrated cells from the cranial neural crest. While the epithelial cells differentiate into ameloblasts, the outer layer of mesenchymal cells that line the dental pulp differentiate into odontoblasts. Odontoblasts express specific proteins that form the organic components of the dentin, a unique

extracellular mineralized matrix. The composition of dentin consists of inorganic and organic structure. The main composition of the inorganic structure is hydroxyapatite, while its organic structure is more complex than the inorganic structure. Collagenous and non-collagenous proteins have been detected in the dentin. The main collagen found in dentin is type I collagen, whereas type III, IV, and V collagens are detected in lower quantities. Smaller amounts of non-collagenous proteins are also detected in the extracellular matrix of dentin. These include decorin, biglycan [Steinfort et al., 1994], osteonectin [Reichert et al., 1992], osteocalcin [Bronckers et al., 1987], osteopontin [Butler, 1989], bone sialoprotein [Chen et al., 1993], dentin matrix protein-1 [Butler and Ritchie, 1995], dentin phosphoprotein (DPP) [MacDougall et al., 1985], and dentin sialoprotein (DSP) [Butler et al., 1992]. DSP and DPP are encoded by the dentin sialophosphoprotein gene (DSPP) which is important in initiation and regulation of dentin mineralization [Suzuki et al., 2009]. DPP is synthesized and secreted by odontoblasts via the odontoblastic process at the mineralization front. It is strongly associated with the mineral phase of dentin, being soluble only after demineralization of the extracellular matrix. DSP is localized in dental tissues and its expression is regulated by differentiating odontoblasts, with a transient expression in presecretory ameloblasts.

Dentin repair

Dentin plays a major role in protecting the underlying pulp. Odontoblasts, which constitute the outermost cellular layer of the dental pulp, provide both communication and an isolation barrier between the dentin and the underlying pulp. Under normal conditions, these cells act as sensor cells to external stimuli. The presence of odontoblasts at the pulp periphery is very important for the dental pulp homeostasis. These cells secrete secondary dentin at a rate of 5 microns/day. However, when they are irritated by external stimuli, they form reactionary dentin over 4–6 weeks. Reactionary dentin, which is formed much faster than secondary dentin, is regarded as an important defense mechanism of the pulp–dentin complex in response to either pathological or physiological insults.

Following mild injuries to the pulp, most primary odontoblasts survive and are stimulated to synthesize and secrete a reactionary dentin matrix. This leads to the focal secretion of a new matrix at the pulp–dentin interface and intratubularly contributes to the histological appearance of dentinal sclerosis at the injury site and a decrease in dentin permeability [Pashley, 1985]. The alteration of odontoblastic layers and dental pulp tissue occurs following severe injuries, such as deep injuries, during cavity preparation with rotary instrument and after placement of toxic material directly onto the pulp tissue. Under this situation, the odontoblastic nuclei can be aspirated into the dentin tubules hundreds of micrometers away from their normal

position in the odontoblastic layer. The disappearance of these cells by apoptosis alters the pulp protection mechanism [Mitsiadis et al., 2008]. The transmission of external stimuli to the pulp tissue can be partially responded by the transient receptor ion channels expressed by the odontoblasts during initial stimulation [El Karim et al., 2011] and by receptors expressed by the dental pulp cells at a later stage. This mechanism is also associated with an increased vascularity and initiation of innate immune responses in the area. This is attributed to the ability of dental pulp cells to secrete inflammatory cytokines and growth factors that stimulate progenitor/stem cells to migrate to the injury site [Tecles et al., 2005] and differentiate to a new generation of odontoblast-like cells responsible for the deposition of a specific type of tertiary dentin termed reparative dentin. These progenitor cells are defined as dental pulp stem cells and appear as a minor subpopulation within the pulp. Dental pulp stem cells from permanent teeth are determined as DPSCs. In primary teeth, these cells are defined as stem cells from human exfoliated deciduous teeth (SHEDs) and. Both DPSCs and SHEDs provide characteristic stem cell properties as they are self-renewed, highly proliferative with clonogenic efficiency, and possess the capability for multi-lineage differentiation [Gronthos et al., 2002; Ishkitiev et al., 2010; Miura et al., 2003; Nourbakhsh et al., 2011; Sakai et al., 2010].

Dental pulp tissues are commonly exposed to pressure because they are surrounded by rigid dentinal walls. Accordingly, even a modest increase in pulpal fluid volume will raise the tissue pressure, which may compress blood vessels, leading to ischemia and necrosis. Pulpal inflammation leads to an increase in interstitial fluid pressure in dental pulp by vasodilatation and increase in vessel permeability [Heyeraas and Berggreen, 1999]. It has been reported that during cavity preparation with rotary instruments, an approximately 4-fold increase in intrapulpal pressure in the interstitial fluid surrounding of dental pulp cells is produced. Mechanical stress, which is an essential biological reaction, occurs not only during therapeutic orthodontic tooth movement, pathological occlusal trauma or dental caries but also during restorative procedures or even normal physiological masticatory activities. It induces inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, IL-10, TNF-α, antioxidant enzymes such as Heme oxygenase-1, Superoxide dismutase, etc. and ATP release from dental pulp cells and periodontal ligament cells [Govitvattana et al., 2013; Kanjanamekanant et al., 2013; Satrawaha et al., 2011]. In human periodontal ligament cells, mechanical stress induces OPN expression via ATP, which mediates the signal through the P2Y1 receptor [Wongkhantee et al., 2008]. The role of mechanical stress in human dental pulp has been shown to influence release of inflammatory cytokines IL-6, which is a multifunctional cytokine, which acts both pro- and anti-inflammatory [Satrawaha et al., 2011]. Moreover, mechanical stress plays a role in the regulation of stemness in SHEDs via IL-6-ATP-REX-

1 interaction, which mediates the signal through Janus kinase (JAK) dependent pathway and P2Y1 receptor [Govitvattana et al., 2013; Govitvattana et al., 2015]. In addition, P2X7 and P2Y6 receptor antagonist upregulate *REX-1* mRNA expression [Govitvattana et al., 2015]. Therefore, the P2 receptor may involve in the regulation of stemness [Govitvattana et al., 2015].

DPSCs and SHEDs were identified to be a population of highly proliferative, clonogenic cells capable of differentiating into a variety of cell types including neural cells, adipocytes, osteo/odontoblasts, endothelial cell and hepatocyte-like cells [Govindasamy et al., 2010; Gronthos et al., 2002; Ishkitiev et al., 2010; Miura et al., 2003; Osathanon et al., 2014; Sakai et al., 2010]. Previous studies showed that 100 μM of ATP promotes osteoblast differentiation in rat primary osteoblasts via PI3K/AKT signaling pathway activation. After culturing osteoblasts in the presence of 100 μM ATP, bone mineralization, ALP activity and the mRNA levels of *ALP, BMP-2, BMP-4, BMP-5* and *BSP* are increased [Ayala-Pena et al., 2013]. In human bone marrow-derived mesenchymal stem cell, 1mM of ATP stimulates osteogenic differentiation by promoting mineralization and gene expression level of *RUNX-2* [Ciciarello et al., 2013]. In addition, ATP promotes adipogenesis by increasing lipid accumulation and gene expression level of *PPAR-*γ which mediate through P2Y1 and P2Y4 receptors [Ciciarello et al., 2013]. In human dental pulp cells, 1-100 μM of ATP promotes proliferation of human dental pulp cells and high

concentrations of ATP (≥400 μM) can inhibit human dental pulp cell growth and promote expression of odontoblast differentiation-related genes and *in vitro* mineralization [Wang et al., 2016].

Tissue mineralization

Mineralization is a process which an organic substance becomes impregnated by inorganic substance. Mineralization of bone, dentin and cementum occurs in a collagen-rich-extracellular matrix. Osteoblasts, odontoblasts and cementoblasts secrete non-collagenous matrix protein, such as osteopontin, bone sialoprotein, osteonectin and osteocalcin, to bind with collagen fibrils. Non-collagenous proteins provide regulate the induction of mineralization and control hydroxyapatite crystal growth by binding to mineral surfaces. Osteopontin and bone sialoprotein are essential for the initiation of bone mineralization. In contrast, osteonectin and osteocalcin, which are present in the fully mineralized matrix, play role in controlling the size and speed of crystal formation. In addition, osteocalcin acts as a chemoattractant for osteoclasts, while both osteopontin and bone sialoprotein facilitate the binding of osteoclasts via the arg-gly-asp motif [Roach, 1994]

Extracellular matrix mineralization in bone is tightly linked to the phosphate (P_i)/ pyrophosphate (PP_i) ratio found in this tissue. PP_i is a potent, mineral-binding small molecule

inhibitor of crystal nucleation and growth at micromolar concentrations [Orriss et al., 2012; Terkeltaub, 2001]. PPi induces *OPN* mRNA expression, which inhibits hydroxyapatite growth. In addition, PPi inhibits *BSP, ALP* and *OCN* mRNA expression in MC3T3 [Deaglio and Robson, 2011]. The homeostatic regulation of local P_i and PP_i levels by bone cells is thus an important part of their function related to mineralization. The major ectoenzyme responsible for generation of extracellular PP_i is ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1; also known as NPP1 and NPPS). ENPP1, which is a membrane-bound enzyme, generates pyrophosphate through the hydrolysis of nucleotides and nucleotide sugars [Evans et al., 1973]. Intracellular PP_i can also be exported into the extracellular compartment by the membrane transporter ANK (progressive ankylosis or ANKH). The ANKH protein is detectable on the surfaces of the osteoblasts and chondrocytes. Both ENPP1 and ANKH are expressed at high levels in bone, cartilage, and teeth [Johnson et al., 2003]. In contrast, Type III sodium/phosphate cotransporters (PiT1) which plays role in cotransporter in matrix calcification, utilize the free energy provided by the Na^+ concentration gradient to mediate the influx of Pi across the cell membrane [Suzuki et al., 2006]. ALP, a well characterized marker of the osteoblast lineage, is capable of hydrolyzing PP_i into P_i which promote mineralization (Fig. 2.3). In osteoblast cultures, ALP is also responsible for the generation of P_i from phosphate esters such as β-glycerophosphate, which is commonly used as a source of organic phosphate for mineralization.

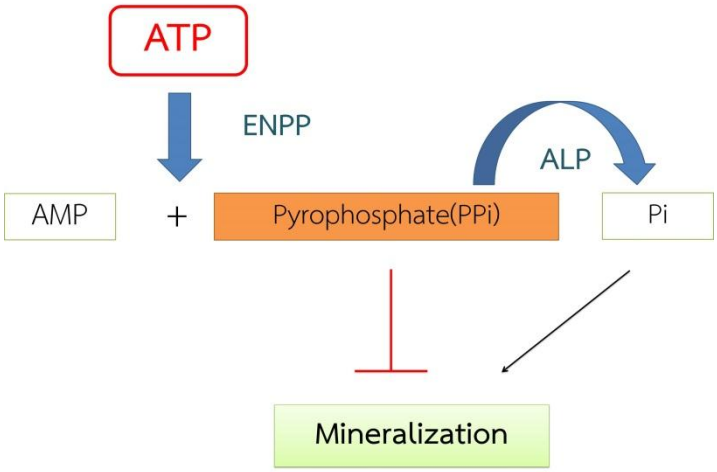


Figure 2.3 A schematic overview of regulation of mineralization by phosphate and pyrophosphate

CHAPTER III

RESEARCH METHODOLOGY

Patient selection and sample collection

Human deciduous dental pulp cells

Sample

Human dental pulp tissues obtained from the non-carious deciduous teeth of children

Ethical considerations

Human deciduous dental pulp cells

1. The study had approved from the ethical committee, Faculty of the Dentistry, Chulalongkorn University. (Study code: HREC-DCU 2016-009)
2. The study process causes no harm to the patients as the extracted deciduous teeth used in the experiment are teeth that nearly exfoliated or prolonged ones with the presence of the permanent succedaneous teeth, which was the indication for tooth extraction in children.
3. The legal guardians were informed about the details of the study process before signing the consent forms.

Inclusion criteria

Human dental pulp obtained from the non-carious deciduous teeth of

- children 5-12 years old.
- children with no systemic disease.
- children with no local anesthetic allergy.

Study variables

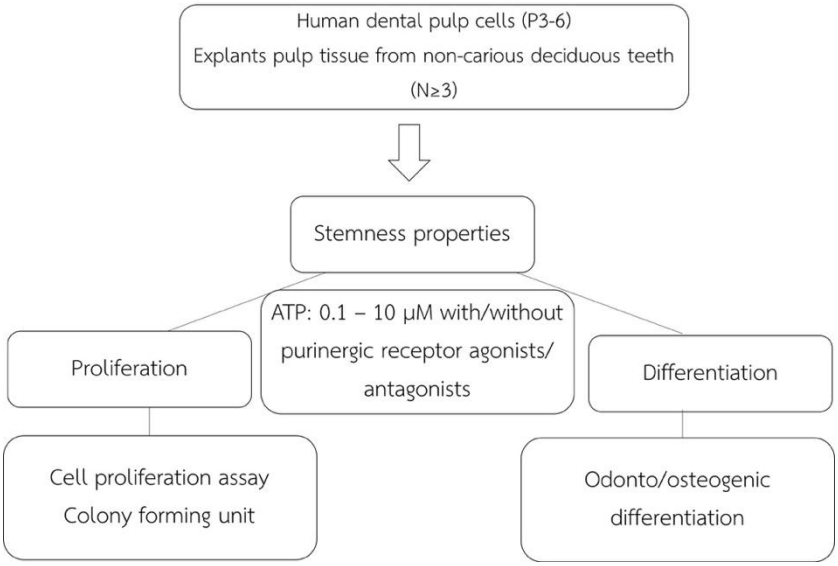
Independent variables

Concentration of ATP

Dependent variables

Odonto/osteogenic differentiation

Experimental design



Materials and Methods

Cell isolation and culture

Extracted teeth were briefly washed with phosphate buffered saline. Pulps were removed and cut into 1x1 mm sections and placed on a culture dish (Corning, New York, NY). The explants were maintained in growth medium which was composed of Dulbecco's Eagle Medium (DMEM) (Gibco, BRL, Carlsbad, CA) containing 10% fetal bovine serum (HyClone, Thermo Scientific, Logan, UT) with 2 mM L-glutamine (Gibco BRL, Carlsbad, CA) and 100 Units/ml Penicillin, 100 µg/ml Streptomycin and 0.25 µg/ml Amphotericin B (Multicell, Wiscent Inc, Quebec, Canada) in a humidified atmosphere, 37°C and 5% CO_2. Only cells from passage 3-6 were used in further experiments. To evaluate the effect of ATP (Sigma-Aldrich Chemical, St. Louis, MO), ATP at concentration 0.1 - 10 µM were added to growth medium (GM) and osteogenic induction medium (OM). ATP was added into the medium every other day in the same time that medium was changed. Cells in GM or OM were used as a control group. All experiments were performed in triplicate using cells prepared from three different preparations.

SHEDs characterization

The isolated cells were characterized by using flow cytometry. Expression of surface markers (CD44, CD90, CD105 and CD45) was evaluated. The multilineage differentiation protocol were investigated according to previous reports [Govitvattana et al., 2013; Nowwarote et al., 2015;

Nowwarote et al., 2017]. Briefly, cells were cultured in OM or adipogenic induction medium (AM) which consisted of GM supplemented with 0.1 mg/ml insulin, 1 mM dexamethasone, 1 mM IBMX, and 0.2 mM indomethacin. Osteogenic and adipogenic marker gene expression were determined using real-time quantitative polymerase chain reaction (PCR). Alizarin red S and Oil Red O staining were used to evaluate mineral deposition and intracellular lipid accumulation, respectively. Cells cultured in GM served as the control.

Flow cytometry

Cells were stained with FITC-conjugated anti-CD44 antibody (BD Biosciences Pharmingen, USA), PerCP-Cy™5.5-conjugated anti-CD90 antibody (BD Biosciences Pharmingen), PE-conjugated anti-CD105 antibody (BD Biosciences Pharmingen), and PerCP-conjugated anti-CD45 antibody (BD Biosciences Pharmingen). The stained cells were analyzed on a FACSCalibur regarding the Cellquest software (BD Biosciences, USA). The presented values were illustrated as mean fluorescence intensity (MFI).

Colony forming unit assay

SHEDs were seeded into 60-mm-diameter culture dishes at density of 500 cells per dish and maintained in the cultured medium supplemented with or without ATP at concentration 0.1 - 10 μM. After 14 days, cells were fixed with 10 % buffer formalin (MERCK, Darmstadt, Germany) for 10 min, washed twice with PBS and stained with coomassie blue (Sigma-Aldrich Chemical, St. Louis,

MO, USA). Aggregates of approximately 50 cells will be scored as a colony and counted under microscope (Axiovert40CFL, CarlZeiss, Gottingen, Germany).

Cell proliferation assay

Cells were seeded at a density of 12,500 cells/well in 24-well-plates and maintained in the GM and OM supplemented without or with 0.1 or 10 μM ATP. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed at day 1, 3, and 7. Cells were treated with 1 mg/ml MTT solution (USB Corporation, Cleveland, OH) for 10 min at 37 ºC. Formazan product was dissolved in solubilization/stop solution. Using a microplate reader (ELx800; BIO-TEK, Winooski, VT), the optical densities were measured at 540 nm.

Luciferin-Luciferase bioluminescence assay

Cells were treated with mechanical stress (0–2.5 g/cm^2) for 2 h. ATP released into the culture medium and a series of known concentration of ATP standard were measured using a luciferase based bioluminescence detection kit for ATP (ENLITEN® kit, the ATP Assay System; Promega, Madison, WI, USA). Briefly, the Enliten Luciferase/Luciferin (L/L) medium was mixed with the sample at 1:1 ratio (PACKARD, Promega, Madison, WI, USA). The emitted light was immediately measured at 560 nm by H1 Hybrid multi-Mode microplate reader (Biotek, Winooski, Vermont, USA). A standard curve was performed using a series of ATP standard.

Osteogenic differentiation

Cells were seeded at a density of 2.5 x 10^4 cells/well in 24-well-plate and maintained in GM. After 2 days, the OM which consisted of GM supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich Chemical), 100 nM dexamethasone (Sigma-Aldrich Chemical) and 10 mM β-glycerophosphate (Sigma-Aldrich Chemical) were changed (defined as day 0). Cells were cultured for 1, 3 and 10 days without or with 0.1-10 μM ATP in OM. Medium was changed every 2 days.

Real-time quantitative polymerase chain reaction

Expression of embryonic stem cell markers and osteogenic and mineralization associated genes were determined using real-time quantitative polymerase chain reaction (real-time PCR). Total cellular RNA was extracted with Trizol reagent (Roche Diagnostics, IN). RNA samples (1 μg) were converted to cDNA using the ImPromII Reverse Transcription System (Promega, WI). Polymerase chain reaction (PCR) was performed using SYBR Green I Master kit (Roche, IN) in a LightCycler® Nano (Roche, IN). The amplification profile was: 95 ºC/10 s, 60 ºC/10 s and 72 ºC/20 s for 45 cycles and followed by melting curve analysis cycle. Reaction product was quantified with GAPDH as reference gene. Primer sequences were shown in Table 3.1.

In vitro mineralization assay

Cells were cultured in OM without or with 0.1-10 μM of ATP for 10 days. Cells were fixed with ice-cold methanol for 10 min, washed with deionized water, and stained with 1% Alizarin Red S

solution (Sigma-Aldrich Chemical). The amount of calcium deposition was quantified by destaining with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich Chemical) in 10 mM sodium phosphate. The absorbance of product was measured used microplate reader at 570 nm.

ALP and ENPP activity assay

Cells were cultured in OM without or with 0.1 or 10 μM of ATP for 3 days. For ALP, cells were lysed in alkaline lysis buffer. Aliquots were incubated at 37ºC in a solution containing 2 mg/ml *p*-nitrophenol phosphate (Invitrogen, Frederick, MD), 0.1 M 2-amino-2methyl-1-propanol and 2 mM MgCl2. After 15 min, 50 mM NaOH were added to stop the reaction. For ENPP, cells were lysed in a buffer containing 1% Triton-X100 in 0.2 M Tris base with 1.6 mM MgCl, pH 8.1. Following centrifugation at 500g, the ENPP activity of collected supernatants was measured using 5 mM p-nitrophenyl-thymidine 5′-monophosphate as a substrate. The presence of *p*-nitrophenol was measured at an absorbance of 410 nm with microplate reader. Total cellular protein was determined by using a Pierce bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL). The enzyme activity was normalized to the total cellular protein.

Inhibitors and agonists

SHEDs were seeded at a density of 2.5 x 10^4 cells/well in 24-well-plates. Cells were maintained in GM for 2 days and the medium was replaced by OM. The inhibitors were added 30 min prior to ATP application. Inhibitors were used as follows: 15 μM suramin (P2YR antagonist, Sigma-Aldrich

Chemical t) 2.5 μM KN62 (P2X7 antagonist, Tocris Bioscience, Minneapolis, MN, USA), 1 μM NF449 (P2X1 antagonist, Tocris Bioscience), ARL67156 trisodium salt (ENPP inhibitor, Tocris Bioscience). Agonists were used as follows: 1, 10 μM BzATP triethylammonium salt (P2X7 agonist, Tocris Bioscience), 1, 10 μM α,β-Methyleneadenosine 5' triphosphate trisodium salt (α,β-meATP; P2X1 agonist, Tocris Bioscience). 0.1 Unit Alkaline phosphatase enzyme (Sigma-Aldrich Chemical) was used. Inhibitors and agonists was added into the medium every other day in the same time that medium was changed.

Statistical analysis

All experiments were performed at least three times, with the data being represented as mean ± standard deviation. All data were analyzed by one-way analysis of variance (ANOVA) using statistical software (SPSS, Chicago, IL). Scheffé's test was used for post hoc analysis. The differences at $p<0.05$ was considered as a statistical significant difference.

Table 3.1 The oligonucleotide sequences of primers

Gene	Accession no.	Primer sequence	size (bp)
P2X1	NM_002558.3	Forward: 5' GCTACGTGGTGCAAGAGTCA 3' Reverse: 5' GTAGTTGGTCCCGTTCTCCA 3'	215
P2X2	NM_174872.2	Forward: 5' GCTCCTTTCCATCTCACTGG 3' Reverse: 5' GGAAGTGAGCAGCCCTGTA 3'	237
P2X3	NM_002559.3	Forward: 5' ACAGCCAGGGACATGAAGAG 3' Reverse: 5'AGCCGGGTGAAGGAGTATTT 3'	209
P2X4	NM_001261397.1	Forward: 5' GAGATTCCAGATGCGGACC 3' Reverse: 5' GACTTGAGGTAAGTAGTGG 3'	215
P2X5	NM_002561.3	Forward: 5' CTGGTCGTATGGGTGTTCCT 3' Reverse: 5' CTGGGCTGGAATGACGTAGT 3'	159
P2X6	NM_005446.3	Forward: 5' ACTCTGTGTGGAGGGAGCTG 3' Reverse: 5' GGCAAGTGGGTGTCAGAACT 3'	151
P2X7	NM_002562.5	Forward: 5' AAGCTGTACCAGCGGAAAGA 3' Reverse: 5' GCTCTTGGCCTTCTGTTTTG 3'	202
P2Y1	NM_002563.4	Forward: 5' AAAACTAGCCCCCTGCAACT 3' Reverse: 5' GATCTGATGCCGGATGAACT 3'	153
P2Y2	NM_176072.2	Forward: 5' CCACCTGCCTTCTCACTAGC 3' Reverse: 5' TGGGAAATCTCAAGGACTGG 3'	163

Gene	Accession no.	Primer sequence	size (bp)
P2Y4	NM_002565.3	Forward: 5' CACCCACTTCGGGCACTAC 3' Reverse: 5' CCTGGCAAGGGCTGATACA 3'	284
P2Y6	NM_001277208.1	Forward: 5' AGCTGGGCATGGAGTTAAGA 3' Reverse: 5' GCTGACTGGGACCTCTCAAG 3'	139
P2Y11	NM_002566.4	Forward: 5' CCTCTACGCCAGCTCCTATG 3' Reverse: 5' CACTGCGGCCATGTAGAGTA 3'	211
P2Y12	NM_176876.2	Forward: 5' TTTGCCCGAATTCCTTACAC 3' Reverse: 5' ATTGGGGCACTTCAGCATAC 3'	192
P2Y13	NM_176894.2	Forward: 5' CCCCTGGTACACTTGGAAGA 3' Reverse: 5' TACAGAGGAGGGGGTGATTG 3'	125
P2Y14	NM_014879.3	Forward: 5' TCTTTGGGCTCATCAGCTTT 3' Reverse: 5' TCCGTCCCAGTTCACTTTTC 3'	213
ANKH	NM_054027.4	Forward: 5' GAGGTGACAGACATCGTGG 3' Reverse: 5' CCTTTAAATCAAGGCCTCTTTCATTAC 3'	177
ALP	NM_000478.3	Forward: 5' CGAGATACAAGCACTCCCACTTC 3' Reverse: 5' CTGTTCAGCTCGTACTGCATGTC 3'	120
CD90	NM_00628.3	Forward : 5' GAAGACCCCAGTCCAGATCCA 3' Reverse : 5' TGCTGGTATTCTCATGGCGG 3'	229

Gene	Accession no.	Primer sequence	size (bp)
DMP1	NM_004407.3	Forward: 5' ATGCCTATCACAACAAACC 3' Reverse: 5' CTCCTTTATGTGACAACTGC 3'	102
ENPP1	NM_006208.2	Forward: 5' AAATATGCAAGCCCTCTTTGT 3' Reverse 5′ TTTAGAAGGTGGTTAAGACTTCCATGA 3'	162
NANOG	NM_001287698.1	Forward: 5' ATGCCTCACACGGAGACTGT 3' Reverse: 5' AAGTGGGTTGTTTGCCTTTG 3'	103
OCT4	NM_001285987.1	Forward: 5' TCGAGAACCGAGTGAAGG 3' Reverse: 5' GAACCACACTCGGACACA 3'	125
OSX	NM_001173467.1	Forward: 5' GCCAGAAGCTGTGAAACCTC 3' Reverse: 5' GCTGCAAGCTCTCCATAACC 3'	161
OCN	NM_199173.4	Forward: 5' CTTTGTGTCCAAGCAGGAGG 3' Reverse: 5' GCCGTAGAAGCGCCGATAGGC 3'	166
PPAR-γ	NM_138711.3	Forward 5′ CCAGTGGTTGCAGATTACAAGTATG 3′ Reverse:5′TTGTAGAGCTGAGTCTTCTCAGAATAATAAG 3'	110
REX1	NM_001304358.1	Forward: 5' TGGGAAAGCGTTCGTTGAGA 3' Reverse: 5' CACCCTTCAAAAGTGCACCG 3'	90

Gene	Accession no.	Primer sequence	size (bp)
RUNX2	NM_001024630.3	Forward: 5' ATGATGACACTGCCACCTCTGA 3' Reverse: 5' GGCTGGATAGTGCATTCGTG 3'	167
SOX2	NM_003106.3	Forward: 5' ACCAGCTCGCAGACCTACAT 3' Reverse: 5' ATGTGTGAGAGGGGCAGTGT 3'	320
GAPDH	NM_001289746.1	Forward: 5' CACTGCCAACGTGTCAGTGGTG 3' Reverse: 5' GTAGCCCAGGATGCCCTTGAG 3'	121

CHAPTER IV

RESULTS

SHEDs characterization

The stem cell properties of cells explanted from dental pulp tissues of human exfoliated deciduous teeth were examined. The isolated cells expressed the mesenchymal stem cell markers CD44 (95.39%±1.19), CD90 (92.93%±4.94), and CD105 (99.81%±0.05) (Fig. 4.1A–C), while hematopoietic related surface marker CD45 (1.36%±0.58) were not detected (Fig. 4.1D). When culturing the cells in induction medium, the cells were able to differentiate into osteogenic and adipogenic lineages. The *RUNX2* and *ALP* mRNA expression were significantly increased after 3 days of osteogenic induction (Fig. 4.1E, F). At day 14, a significant increase in mineral deposition was found (Fig. 4.1G). For adipogenic differentiation, the cells were maintained in AM for 16 days. The mRNA expression of the adipogenic marker gene, Peroxisome proliferator-activated receptor gamma (PPAR-γ), was significantly increased (Fig. 4.1H), corresponding with increased accumulation of intracellular lipid accumulation as evaluated by Oil Red O staining (Fig. 4.1I). These results demonstrated that the cells isolated from pulp tissues of human exfoliated deciduous teeth exhibit mesenchymal stem cell characteristics. Therefore, we referred to these cell populations as SHEDs in the rest of this study.

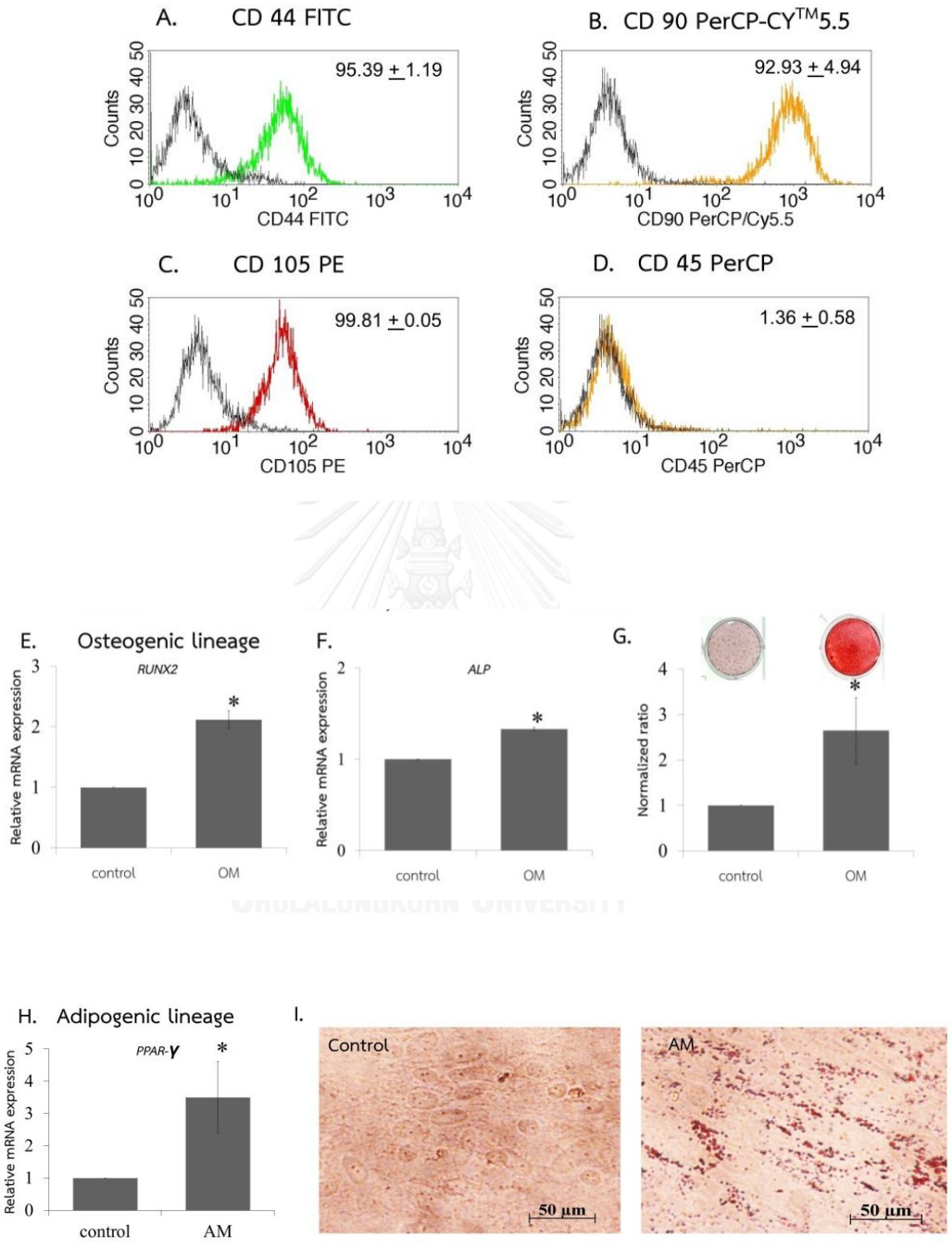


Figure 4.1 SHEDs characterization. Mesenchymal stem cell surface marker levels were analyzed using flow cytometry (positive; CD44 **(A)**, CD90 **(B)**, and CD105 **(C)** and negative; CD45 **(D)**).

Multipotential differentiation, cells were induced with induction medium. For osteogenic differentiation, the mRNA expression of osteogenic marker genes, *RUNX2* **(E)** and *ALP* **(F)**, at day 3 were determined by real-time RT-PCR. The increase of mineral deposition was detected by alizarin red staining at day 14 **(G)**. For adipogenic induction, cells expressed the adipogenic marker gene *PPAR-*γ as analyzed by real-time RT-PCR **(H)**. The intracellular lipid droplets accumulation was demonstrated at day 16 using oil red O staining **(I)**. (* $p<0.05$ versus control; growth medium)

Mechanical stress induced releasing of ATP in a force dependent manner

In a previous study of our group have shown that compressive force induced releasing of ATPby SHEDs [Govitvattana et al., 2015]. To measure concentration of ATP release, SHEDs were treated with mechanical stress (0–2.5 g/cm^2) for 2 h using a luciferin–luciferase bioluminescence assay. The light intensity was emitted by SHEDs about 200000-400000. Then, Light intensity was determined with ATP standard curve. The concentration of ATP released after cell compression was about 0.01-0.1 μM in a force dependent manner. Therefore, we used 0.1 μM to mimic low mechanical stress and 10 μM to mimic high mechanical stress.

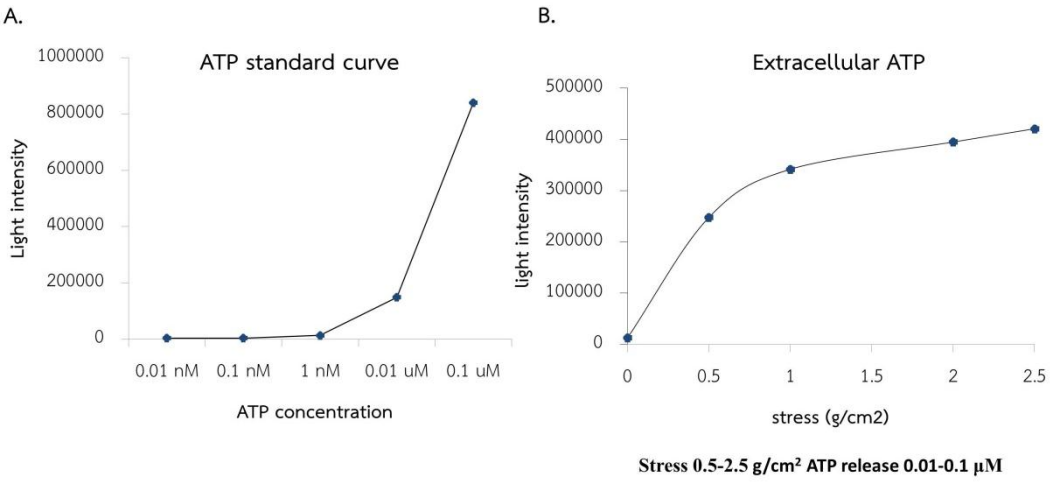


Figure 4.2 Concentration of ATP released. A series of known concentration of ATP was measured using a luciferin–luciferase bioluminescence assay **(A)**. SHEDs were treated with mechanical stress (0–2.5 g/cm^2) for 2 h. ATP release was then measured using a luciferin–luciferase bioluminescence assay **(B)**.

ATP increased mRNA expression of pluripotent and osteogenic markers in SHEDs

To determine the influence of ATP on stemness and osteogenic differentiation gene expression, cell were cultured in GM in the presence or absence of extracellular ATP at a concentration of 0.1 - 10 µM for 1 day. The result from real-time PCR revealed that all three concentrations of ATP significantly up-regulated the expression of *NANOG* and CD90 in a dose-dependent manner(Fig. 4.3A).The expression of *OCT4,REX1* and *SOX2* were significantly up-regulated in the culture with 1

and 10 μM ATP (Fig. 4.3A). Interestingly, ATP also dose-dependently increased the expression of the osteogenic markers *RUNX2, OSX, OCN, ALP,* and *DMP1* (Fig. 4.3B).

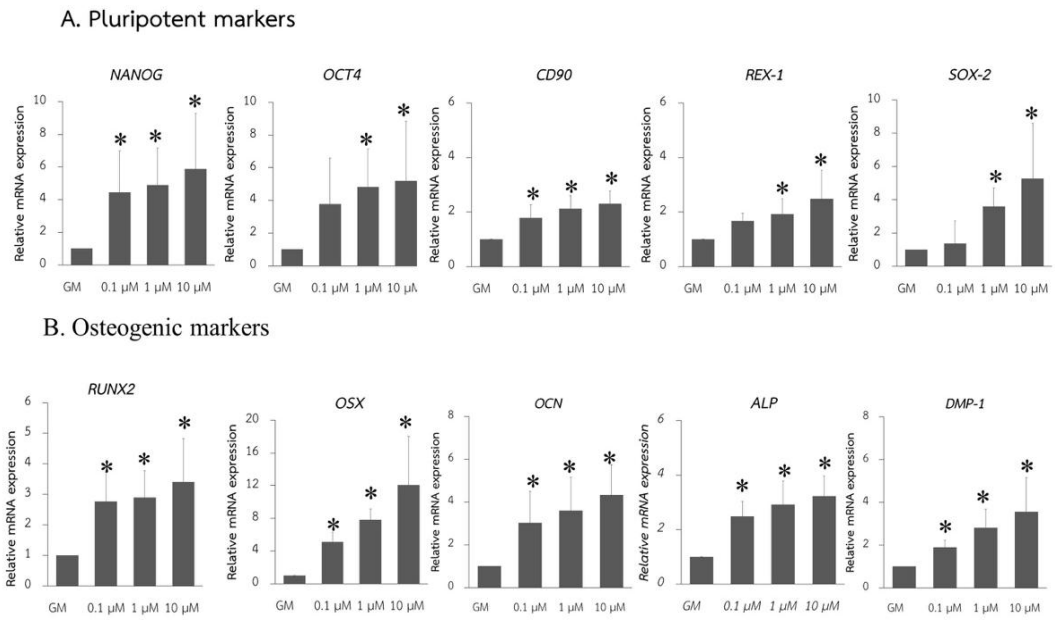


Figure 4.3 The effect of ATP on the expression of pluripotent and osteogenic markers in growth medium condition. SHEDs were cultured in GM in the presence or absence of extracellular ATP at a concentration of 0.1 - 10 μM for 1 day. The mRNA expression of the pluripotent markers **(A)***NANOG, OCT4, CD90, REX1* and *SOX2* and osteogenic markers **(B)** *RUNX2, OSX, OCN, ALP* and *DMP1* were determined by real-time RT-PCR. (* *p<0.05* versus control; medium alone)

Next, the effect of ATP on cells cultured in osteogenesis inducing medium (OM) was examined. Cells were cultured in OM with and without 0.1, 1 or 10 μM ATP. PCR analyses showed

that, after 24 hours, all three concentrations of ATP significantly up-regulated the stem cell markers, *NANOG*, *REX1* and *SOX2*, but had no effect on *CD90* and *OCT4* expression (Fig. 4.4A). Moreover, all concentrations of ATP significantly up-regulated mRNA levels of *OSX* and *DMP1* after 1 day in culture (Fig. 4.4B). However, at day 3, the up-regulation of *OSX* was observed only in cells stimulated with 0.1 μM ATP. The expression level of *RUNX2* was significantly up-regulated only in cells treated with 0.1 and 1 μM ATP at day 1 but not at day 3. The expression of *OCN* was significantly increased on day 3 in cells stimulated with 0.1 μM ATP (Fig. 4.4B)

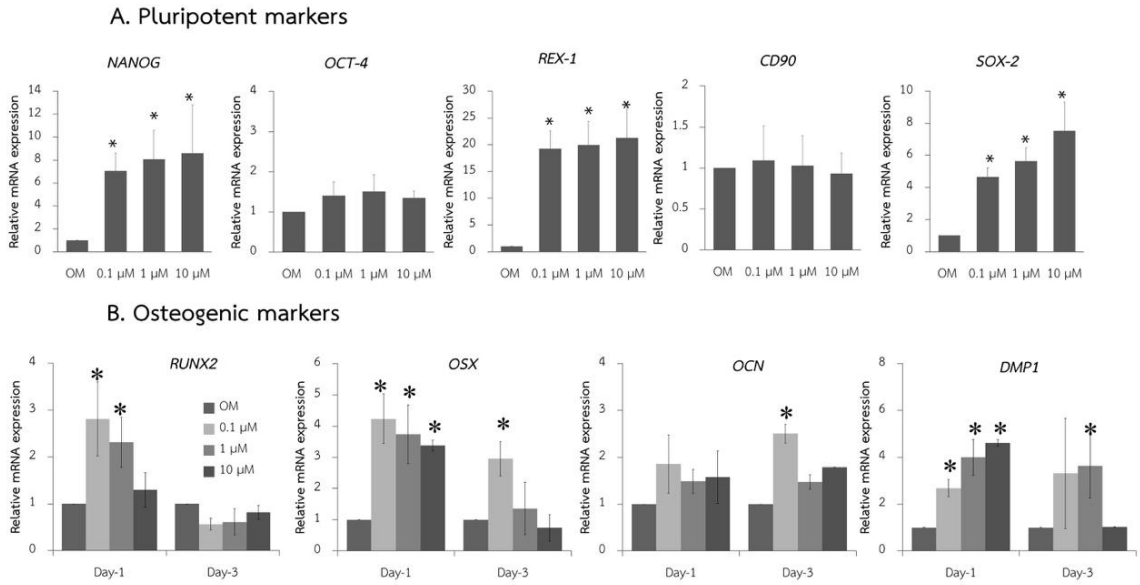


Figure 4.4 The effect of ATP on the expression of pluripotent and osteogenic markers in osteogenic medium condition. SHEDs were cultured in OM in the presence or absence of extracellular ATP at a concentration of 0.1 or 10 μM for 1 and 3 days. The mRNA expression of

the pluripotent **(A)** and osteogenic **(B)** markers were determined by real-time RT-PCR. (* *p<0.05* versus control)

Effect of ATP on cell proliferation and colony forming unit

To investigate the effect of ATP on cell proliferation, SHEDs were cultured in GMand OM in the presence or absence of extracellular ATP at a concentration 0.1 - 10 μM for 1, 3 and 7 days. There was no significant difference in cell viability/proliferation as assessed by the MTT assay between cells cultured in GM and OM with or without ATP (Fig. 4.5A, B).

To determine the effect of ATP on self-renewal property, SHEDs were cultured in low density maintained in the cultured medium supplemented with or without ATP at concentration 0.1 - 10 μM. Although ATP increased mRNA expression of embryonic stem cell marker, there was no significant difference in colony forming unit (Fig 4.5C).

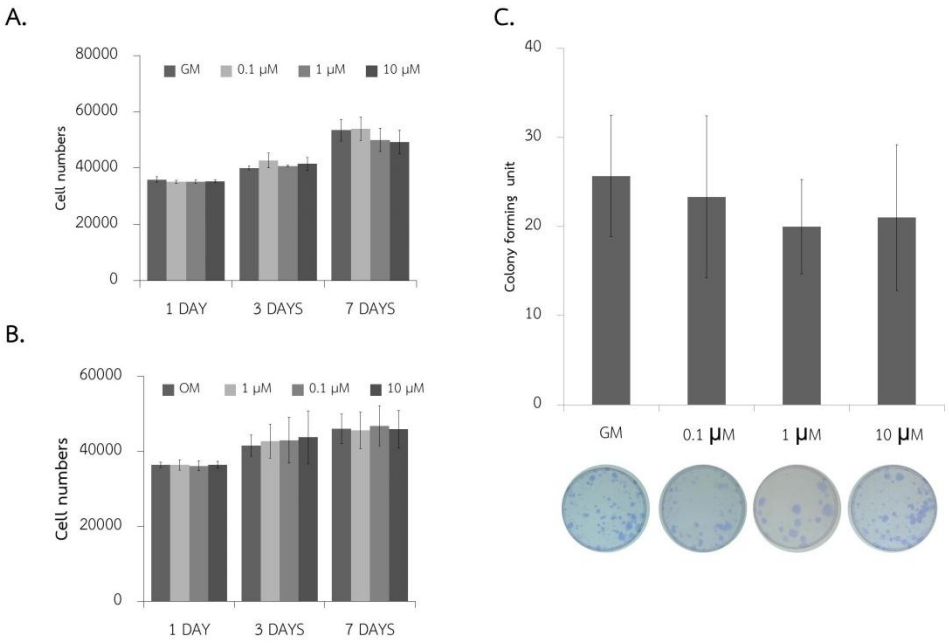


Figure 4.5 The effect of ATP on SHEDs proliferation. MTT analysis was performed at 1, 3 and 7 days after culturing in GM **(A)** and OM **(B)** in the presence or absence of extracellular ATP at a concentration 0.1 - 10 μM. Representative figures of the colony forming unit ability in the presence or absence of extracellular ATP at concentration 0.1 - 10 μM were evaluated by coomassie blue staining at day 14**.** Number of colony was counted and shown in the graph **(C)**

Biphasic effect of ATP on *in vitro* calcification

Next, the effect of ATP on *in vitro* calcification by SHEDs was examined. SHEDs were cultured in OM for 10 days without or with 0.1, 1 or 10 μM ATP and the calcification was assessed by alizarin red s staining. The lowest concentration of ATP proved to increase calcium deposition significantly (Fig 4.6). In the presence of 1 μM ATP only a slight, but not significant increase in

mineralization was seen. However, in the presence of 10 μM ATP hardly any mineral was deposited (Fig 4.6).

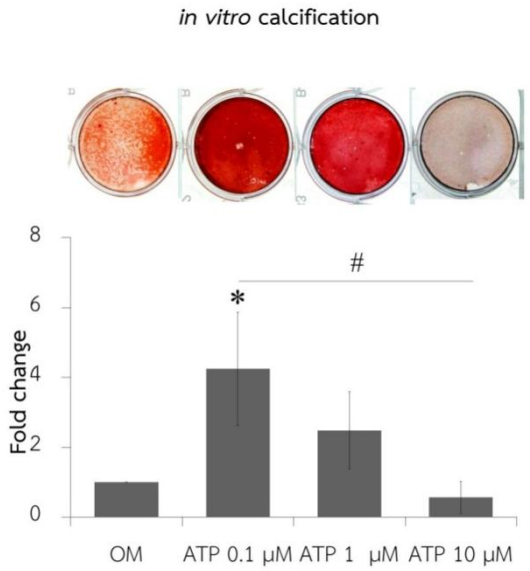


Figure 4.6 The effect of ATP on in vitro mineralization. SHEDs were cultured in OM in the presence or absence of extracellular ATP at a concentration of 0.1-10 μM for 10 days. *In vitro* mineral deposition was determined by alizarin red S staining. (* $p<0.05$ versus control, # $p<0.01$ versus 0.1 μM of ATP)

High concentration of ATP inhibits *in vitro* mineralization by SHEDs via up-regulation of mineralization associated genes

To investigate the effect of ATP on genes related to the process of mineralization (*ANKH, ENPP* and *ALP*), SHEDs were cultured in OM for 1 and 3 days without or with 0.1 - 10 μM ATP. The

results showed that after 24 h, only 10 μM ATP significantly increased expression of *ANKH* and *ENPP* mRNA; no effect was seen with the lower concentration of ATP (Fig 4.7 B, C). Corresponding with the mRNA level of *ENPP*, in the presence of 10 μM of ATP significantly increased the ENPP activity at day-3 (Fig 4.7E). ATP treatment did not affect the mRNA level of ALP and/or its activity (Fig 4.7A, D).The increased of ENPP by 10 μM ATP corresponded with the inhibitory effect on *in vitro* mineralization. To confirm the effect of ENPP/pyrophosphate on mineral deposition, non specific inhibitor of ectonucleotidases (ARL67156) or exogenous ALP were added in the cells culture in OM medium with 10 μM ATP for 10 days. The result showed that both ARL67156 and exogenous ALP rescued the inhibitory effect of 10 μM ATP by inducing the *in vitro* mineralization (Fig. 4.7F). The results supported that the inhibitory effect of 10 μM ATP on mineral deposition occurred via the increase of extracellular pyrophosphate.

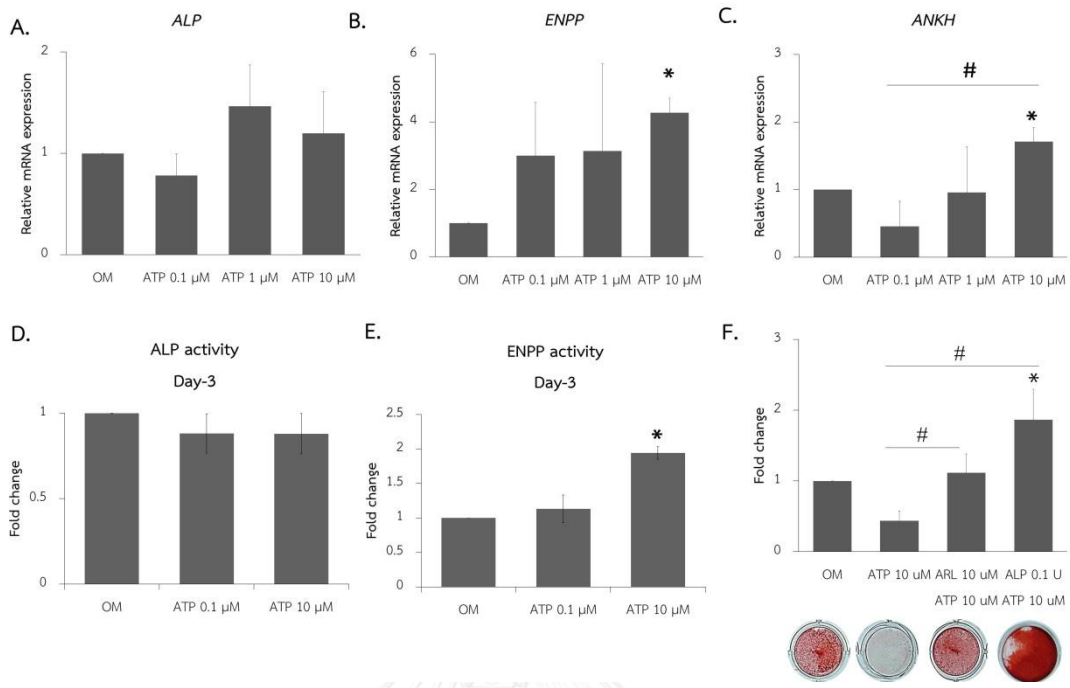


Figure 4.7 Involvement of pyrophosphate in ATP-modulated SHEDs osteogenic differentiation and *in vitro* mineralization. SHEDs were cultured in OM in the presence or absence of extracellular ATP at a concentration of 0.1 or 10 μM for 1 and 3 days. The mRNA expression of mineralized associated genes, *ALP* (A), *ENPP* (B) and *ANKH* (C) were determined by real-time PCR on day-1 (* *p<0.05* versus control, *# p<0.01* versus 0.1 μM of ATP). ALP (D) and ENPP (E) activities were determined on day-3 (* *p<0.05* versus control).

SHEDs express mRNA of all P2 receptors

SHEDs expressed mRNA of all P2 receptors. The mRNA levels of all P2 receptors were shown as fold change in expression relative to the P2X1 receptor. The rank order of mRNA expression was

P2X4>P2X6>P2X7>P2Y6>P2Y2>P2Y1>P2X5>P2Y14>P2Y13>P2Y4>P2Y11>P2Y12>P2X3>P2X1>P2X2 receptors (Fig 4.8).

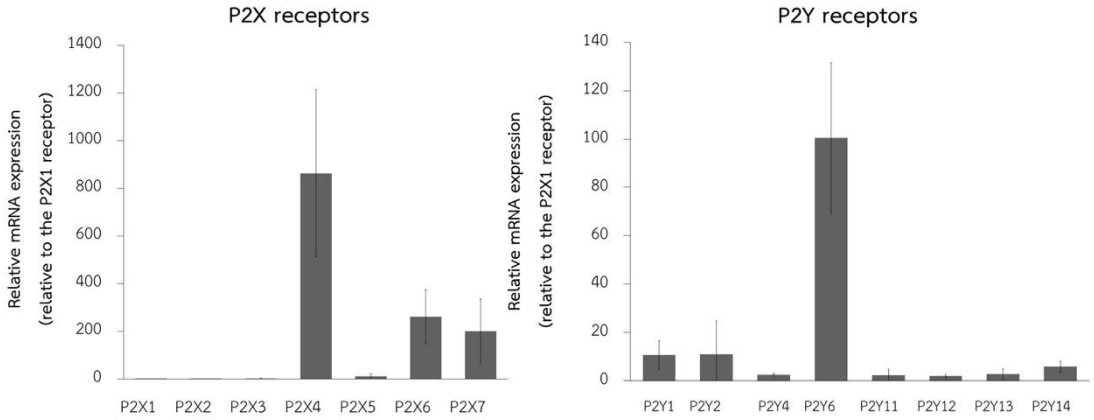


Figure 4.8 The mRNA expression of all P2 receptors were determined by real-time PCR and shown as fold change in expression relative to the P2X1 receptor.

P2Y receptors are not involved in ATP-modulated osteogenic differentiation and in vitro mineral deposition

To investigate which type of purinergic receptors were involved in ATP-modulated osteogenic differentiation and in vitro mineral deposition. First, we used suramin,which is a P2YRs antagonist, to clarify P2XRs or P2YRs involved in this phenomenon. PCR analyses showed that suramin could not inhibit ATP-induced *OSX* and *ENPP* expression (Fig 4.9A, B). In addition, suramin could not rescue the inhibitory effect on mineral deposition caused by 10 μM ATP at day 10 (Fig 4.9C). The result suggested that P2XRs were involved in ATP-modulated osteogenic differentiation and in vitro mineral deposition.

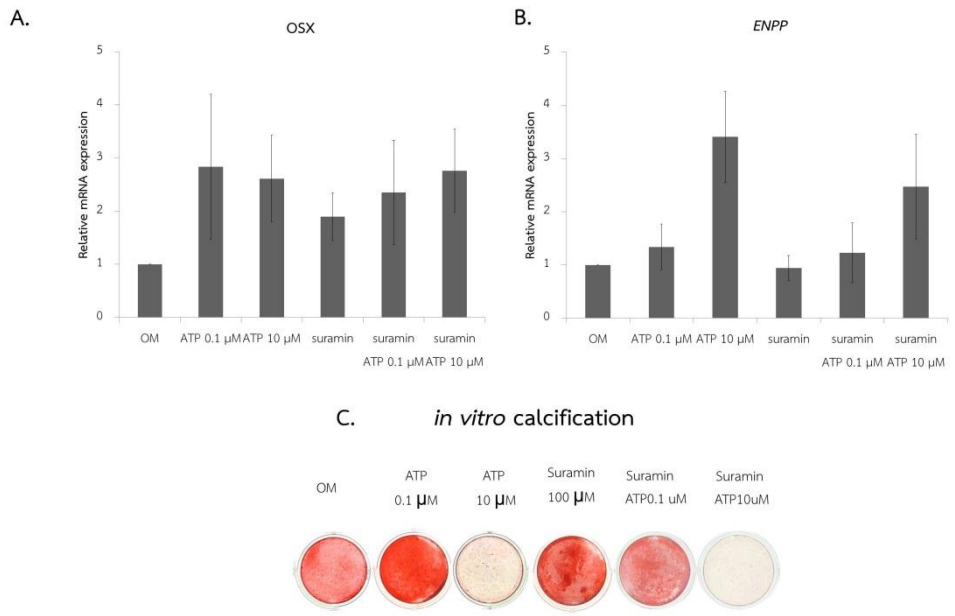


Figure 4.9 The effect of suramin on SHEDs. Cells were cultured in OM with or without 0.1 or 10 μM ATP. Suramin was added 30 min prior to ATP application. The mRNA expression of *OSX* **(A)** and *ENPP* **(B)** were determined by real-time PCR on day-1. *In vitro* mineral deposition was determined by alizarin red S staining at day-10.

P2X7 and P2X1 are involved in ATP-modulated osteogenic differentiation and in vitro mineral deposition

To investigate which P2XR were involved in ATP-modulated osteogenic differentiation and *in vitro* mineral deposition. Therefore,P2X1 and P2X7 has been reported to mediated the inhibition of bone mineralization [Orriss et al., 2012; Sindhavajiva et al., 2017], the specific agonist (BzATP) and antagonist to P2X7 (KN62) and agonist (α,β-meATP) and antagonist of P2X1 (NF449) was used. PCR analyses showed that both KN62 and NF449 inhibited 10 μM ATP-induced *ENPP* expressions. The

inhibition also found in 0.1 μM ATP-induced *OSX* expression. In addition, KN62 inhibited 10 μM ATP-induced *OSX* expression. However, the dose of NF449 used in this study could not inhibit the 10 μM ATP-induced *OSX* expression (Fig. 4.10A, B). Addition of P2X1 antagonist (NF 449), but not P2X7 antagonist (KN62), abolished the inhibitory effect on mineral deposition caused by 10 μM ATP at day 10 (Fig. 4.10C).

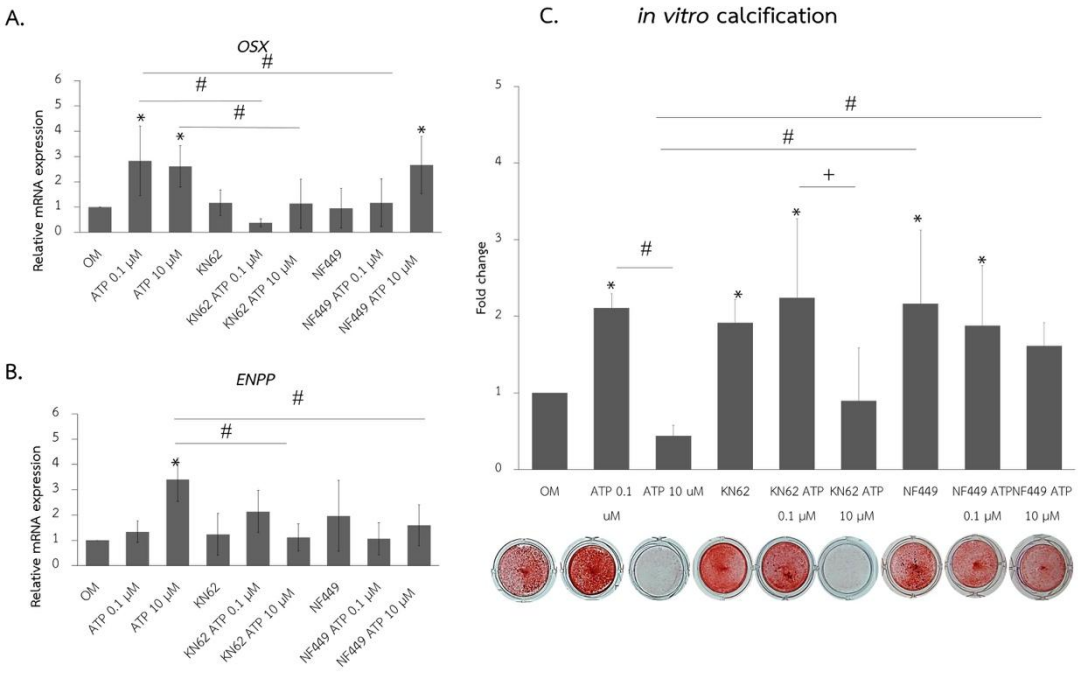


Figure 4.10 The effect of P2X1 and P2X7 antagonists on SHEDs. Cells were cultured in OM with 0.1 or 10 μM ATP. To investigate the involvement of P2XR, specific antagonists to P2X7 and P2X1 (antagonist to P2X7: KN62, P2X1: NF449) was added in the culture. The mRNA expression of *OSX* **(A)** and *ENPP* **(B)** were determined by real-time PCR on day-1. *In vitro* mineral deposition

was determined by alizarin red S staining at day-10. Graph showed the amount of mineral deposition was quantitated by destaining with 10% cetylpyridinium chloride monohydrate in 10 mM sodium phosphate (**C**). (* *p<0.05* versus control, # *p<0.05* versus 0.1 or 10 μM of ATP, +*p<0.05* versus KN62 with 0.1 μM of ATP)

To further confirm the role of P2X7 and P2X1 in ATP-modulated osteogenic differentiation and *in vitro* mineralization, cells were cultured in OM in the presence of both P2X7 (1, 10 μM BzATP) and P2X1 (1, 10 μM **α,β**-meATP) agonists compared to the effect of 0.1 or 10 μM ATP. The result showed that all doses of agonists significantly up-regulated mRNA expression of *OSX* (Fig. 4.11A). Interestingly, 10 μM of P2X1 and P2X7 agonists increased the *ENPP* expression significantly (Fig. 4.11B) and inhibited mineral deposition at day 10, similar to the effect of 10 μM ATP (Fig. 4.11C). Moreover, addition of 1 μM **α,β**-meATP, but not 1 μM BzATP, increased calcium deposition similar to the effect of 0.1 μM ATP (Fig. 4.11C). These results indicated that, at least, P2X1 and P2X7 participated in the ATP regulate osteogenic differentiation and calcification.

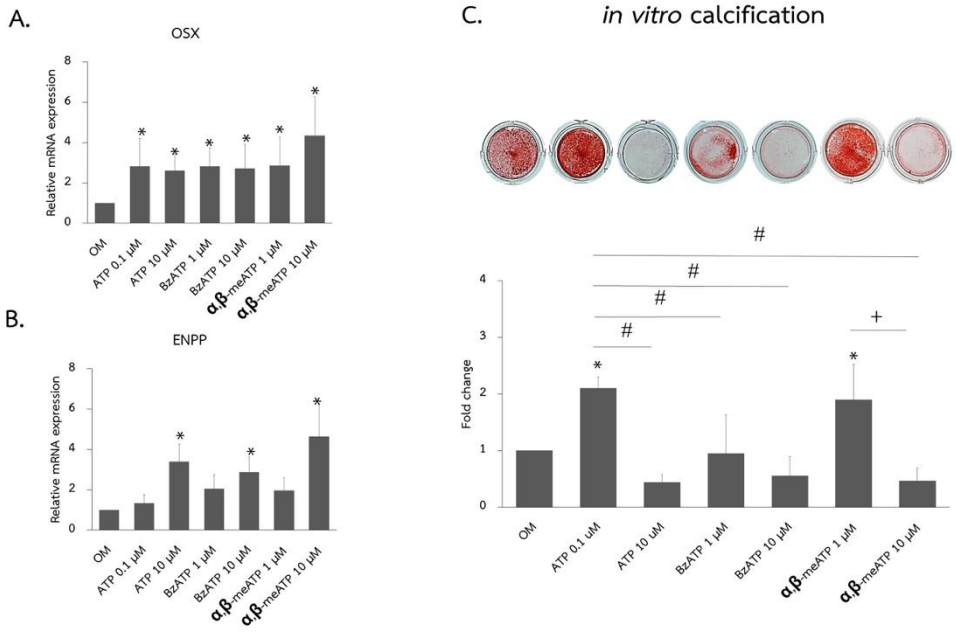


Figure 4.11 The effect of P2X1 and P2X7 agonists on SHEDs. Cells were cultured in OM with 0.1 or 10 μM of specific agonists to P2X7 and P2X1 compared to the effect of 0.1 or 10 μM ATP (agonist to P2X7: BzATP, P2X1: **α,β**-meATP). The mRNA expression of *OSX* **(A)** and *ENPP* **(B)** were determined by real-time PCR on day-1. *In vitro* mineral deposition was determined by alizarin red S staining at day-10 (**C**) Graph showed the amount of mineral deposition was quantitated by destaining with 10% cetylpyridinium chloride monohydrate in 10 mM sodium phosphate (**C**).

(* *p<0.05* versus control, *# p<0.05* versus 0.1 μM of ATP, +*p<0.05* versus 1 μM **α,β**-meATP)

CHAPTER V

DISCUSSION AND CONCLUSIONS

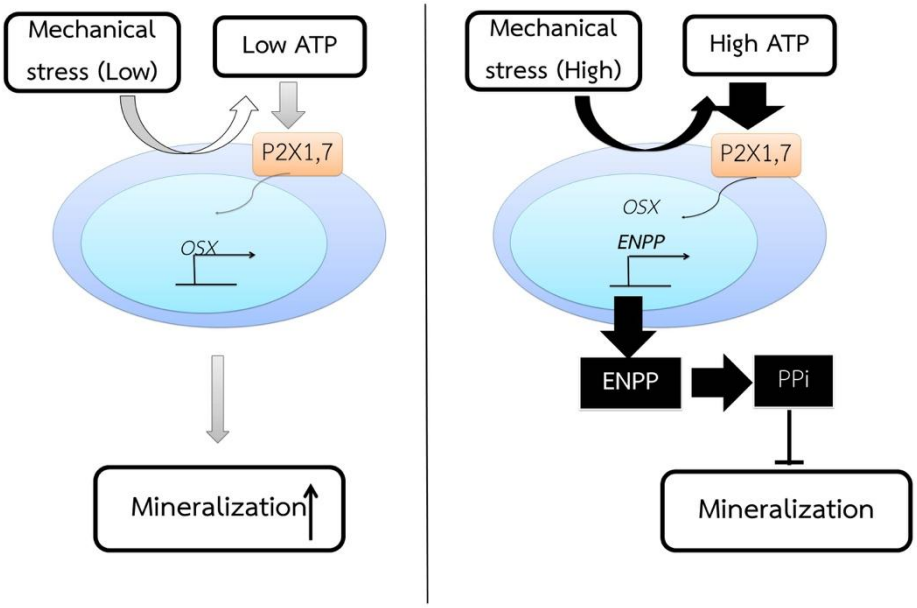


Figure 5.1 The diagram demonstrated the proposed model for the regulation of SHEDs behavior by mechanical stress via ATP.

The present study demonstrated that exogenous ATP up-regulated several stem cell markers as well as osteogenesis differentiation related genes of SHEDs. Interestingly, the effect of ATP on *in vitro* mineralization proved to be biphasic. Low concentrations promoted mineral deposition whereas a high concentration blocked this process.

Cells from the tooth and periodontium regularly receive mechanical stimulation generated by normal physiological masticatory activity and supraphysiological forces of the oral cavity resulting from a high restoration or orthodontic forces, for example. This probably induces ATP release from the cells present in the tooth-associated tissues. In a previous study we have shown this to occur for dental pulp cells [Govitvattana et al., 2015]. The concentration of ATP released after cell compression was about 0.01-0.1 μM in a force dependent manner (Fig 4.2). In the present study, we used 0.1 μM to mimic low mechanical stress and 10 μM to mimic high mechanical stress. In general, dental pulp cells play an important role in maintaining the tissue homeostasis including secondary and tertiary dentin formation. We propose that ATP plays an essential role in these processes, due to its ability to modulate expression of stem cell-related as well as osteogenesis- related genes. Next to that ATP can modulate mineral deposition by regulating the phosphate balance via ALP-ENPP expression.

Our study demonstrated that in growth medium all concentrations of ATP increased the mRNA expression of osteogenesis related genes, *RUNX2*, *OSX*, *OCN, ALP* and *DMP1,* although, with different levels of induction. In osteogenic medium, the mRNA expression of osteogenesis related genes was different. Therefore, it appears that the different media differently affect ATP-modulated gene expression. An important difference between the two media is the presence of β-glycerophosphate in OM. The presence of this compound implies that the level of phosphate is higher in OM than in GM. Therefore, it has been suggested that the level of phosphate regulated expression of osteogenic genes[Schäck et al., 2013], we speculate that the different mRNA expression patterns was caused by β-glycerophosphate in OM.

A concentration-dependent effect of ATP on osteogenesis and calcification has been reported previously for other cell types. In contrast to our findings, in rat primary osteoblasts 100 μM of ATP stimulated osteogenic differentiation related genes and *in vitro* mineral deposition [Ayala-Pena et al., 2013; Costessi et al., 2005; Laiuppa and Santillan, 2016]. However, 1-10 μM of ATP inhibited *in vitro* mineral deposition and stimulated the formation and resorptive activity of osteoclasts [Hoebertz et al., 2002; Orriss et al., 2007]. In human bone marrow mesenchymal stem cells, 0.1 -100 μM of ATP induced osteogenic differentiation by increasing ALP activity, OCN production and bone nodule formation [Sun et al., 2013]. Human permanent dental pulp cells

cultured in GM with 10 μM of ATP, the nucleotide promoted cell proliferation whereas 800 μM of ATP arrested cell proliferation and enhanced odontoblast differentiation and *in vitro* mineral deposition [Wang et al., 2016]. It is possible that these contradictory effects are due to the use of different cell types and species. In addition, our results showed that all P2X and P2Y receptors were expressed in SHEDs. Human permanent dental pulp cells expressed only P2X3, P2X4, P2X5, P2X7 and all P2Y receptors [Wang et al., 2016]. Therefore, a different pattern of P2R expression might be another possible explanation. Further studies on the specific P2R that respond to ATP in these different cell types will probably help to offer an explanation for these intriguing differences.

ATP also modulated the expression of a number of stem cell markers. In the presence of ATP mRNA expression was up-regulated of *NANOG, OCT4, REX1, CD90* and *SOX-2*. A comparable up-regulation was found in previous studies in which the cells were mechanically stimulated [Govitvattana et al., 2013; Govitvattana et al., 2015]. The increase of these stem cell markers may associate with the osteogenic differentiation potential of SHEDs. With respect to NANOG and SOX2, it has been shown that overexpression of these genes by human bone marrow derived mesenchymal stem cells resulted in increased osteogenic differentiation compared with control cells [Go et al., 2008]. Moreover, it has been shown that a CD90 positive population of stem cells

demonstrated increased osteogenic differentiation [Chung et al., 2013; Yamamoto et al., 2014]. Therefore, we hypothesize that an increased expression of stem cell markers induced by ATP might associate with osteogenic differentiation potential of SHEDs.

The decrease of mineral deposition by a relatively high dose ATP can be explained by the expression of enzymes essential for mineralization: ANKH and ENPP. The results showed that 10 μM of ATP up-regulated mRNA expression of *ANKH* and *ENPP* which coincided with an increased ENPP activity. Indeed, extracellular pyrophosphate has been shown to be an inhibitor of mineralization and has been shown to play a role in the regulating the calcification process [Foster et al., 2008; Orriss et al., 2012; Terkeltaub, 2001]. Therefore, to confirm the effect of pyrophosphate, ARL67156, a non-specific ectonucleotidase inhibitor, or ALP, were added in the 10 μM ATP culture. The results showed that adding either ARL67156 or ALP reversed the inhibition of mineral deposition inhibition induced by 10 μM ATP. Taken together, our results support that ATP, depending on its concentrations, modulate *in vitro* mineral formation by SHEDs via up-regulating expression and activity of ENPP, which results in an imbalance of the PPi/Pi ratio.

The effect of the P2XR agonists and antagonists suggested that P2X1 and P2X7 were involved in the ATP modulated SHEDs osteogenic differentiation and *in vitro* mineralization. The

use of the P2X1 and P2X7 agonists induced *OSX* and *ENPP* mRNA expression similar to that of the ATP treatments. Moreover, the P2X1 and P2X7 antagonists inhibited the ATP-induced *ENPP* expression. Although the P2X1 antagonist did not inhibit the 10 μM ATP-induced OSX expression, this may be due to an inappropriate dose and type of antagonists. In addition, ATP is a universal agonists, the effect of ATP comes from activating all P2XR and P2YR. The chemical inhibitors blocked specific receptor, it might activate other P2XR. More studies are required for a better understanding of which specific P2XR regulates the osteogenic effect of extracellular ATP.

Interestingly, similar to the effect of 10 μM ATP, the use of 10 μM P2X1 and P2X7 agonists inhibited *in vitro* mineral deposition. However, the P2X1 and P2X7 antagonists showed different effects on *in vitro* mineral deposition. Although the P2X1 antagonist rescued mineralization, the P2X7 antagonist did not. Further investigation is required to distinguish the roles of P2X1 and P2X7 in mineralization. However, the results suggest that the biphasic effect of ATP on mineralization occurred via the P2X1 and P2X7 signaling pathways.

ATP also modulated the expression of a number of stem cell markers. In the presence of ATP mRNA expression was up-regulated of *NANOG, OCT4, REX1, CD90* and *SOX-2*. A comparable up-regulation was found in previous studies in which the cells were mechanically stimulated [Govitvattana et al., 2013; Govitvattana et al., 2015]. The increase of these stem cell markers may

associate with the osteogenic differentiation potential of SHEDs. With respect to NANOG and SOX2, it has been shown that overexpression of these genes by human bone marrow derived mesenchymal stem cells resulted in a much higher osteogenic differentiation potential when compared to control cells [Go et al., 2008]. Moreover, it has been shown that a CD90 positive population of stem cells had higher rate in osteogenic differentiation [Chung et al., 2013; Yamamoto et al., 2014]. Therefore, we hypothesize that an increased expression of stem cell markers induced by ATP might associate with osteogenic differentiation potential of SHEDs.

In conclusion, this study provided evidence that ATP participates in the functional activity of SHEDs. ATP modulated expression of pluripotent markers and osteogenesis differentiation related genes. Moreover, a biphasic effect of ATP on *in vitro* mineral deposition was recorded, probably by its regulation of the level of pyrophosphate; a potent inhibitor of mineralization (Fig. 5.1).

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APPENDIX

VITA

MIss Oranuch Techatharatip was born in Bangkok on March 2, 1982. She graduated the degree of Doctor of Dental Surgery (D.D.S.) with second class honors from Faculty of Dentistry, Chulalongkorn University in 2006. After graduation, she worked as a dentist at Chumpae hospital, Khon Kaen, Thailand from April 2006-April 2008. She studied in the Master of Sciences Program (M.Sc.) at Pediatric department, Faculty of Dentistry, Chulalongkorn University from 2008-2011. Since 2011, she has joined as a faculty member in the Pediatric department, Faculty of Dentistry, Chulalongkorn University. She enrolled in the Doctor of Philosophy Program in Oral Biology, Faculty of Dentistry, Chulalongkorn University. Her research work was performed at Research Unit of Mineralized Tissue, Faculty of Dentistry, Chulalongkorn University.