อิทธิพลของแรงกดเชิงกลแบบเป็นจังหวะต่อการแปรสภาพเป็นเซลล์กระดูกของเซลล์คล้ายเซลล์กระดูกที่เพาะเลี้ยงจากกระดูกขากรรไกรล่างของมนุษย์

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THE INFLUENCE OF INTERMITTENT COMPRESSIVE FORCE ON OSTEOGENIC DIFFERENTIATION OF HUMAN MANDIBULAR-DERIVED OSTEOBLAST-LIKE CELLS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Prosthodontics
Department of Prosthodontics
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Thesis Title	THE INFLUENCE OF INTERMITTENT COMPRESSIVE FORCE ON OSTEOGENIC DIFFERENTIATION OF HUMAN MANDIBULAR-DERIVED OSTEOBLAST-LIKE CELLS
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พิมพ์รำไพ โรจนกิจ สินธวาชีวะ : อิทธิพลของแรงกดเชิงกลแบบเป็นจังหวะต่อการแปรสภาพเป็นเซลล์กระดูกของเซลล์คล้ายเซลล์กระดูกที่เพาะเลี้ยงจากกระดูกขากรรไกรล่างของมนุษย์ (THE INFLUENCE OF INTERMITTENT COMPRESSIVE FORCE ON OSTEOGENIC DIFFERENTIATION OF HUMAN MANDIBULAR-DERIVED OSTEOBLAST-LIKE CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทพ. ดร. แมนสรวง อักษรนุกิจ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ทพ. ดร. ประสิทธิ์ ภวสันต์, 65 หน้า.

แรงเชิงกลมีบทบาทต่อการแปรสภาพเป็นเซลล์กระดูกของเซลล์คล้ายเซลล์กระดูก ชนิดและขนาดของแรงที่แตกต่างกันส่งผลที่แตกต่างกันต่อเซลล์คล้ายเซลล์กระดูก ผลของแรงเชิงกลนี้เกี่ยวของกับพิวริเนอจิกทู รีเซบเตอร์ซึ่งถูกกระตุ้นโดยอะดีโนซีน ไตรฟอสเฟตที่เซลล์คล้ายเซลล์กระดูกหลั่งออกเมื่อได้รับแรงเชิงกล อย่างไรก็ตามยังไม่มีการศึกษาถึงผลของแรงกดเชิงกลแบบเป็นจังหวะในเซลล์กระดูกของมนุษย์ การศึกษานี้มีวัตถุประสงค์ที่จะศึกษาผลของแรงกดเชิงกลแบบเป็นจังหวะต่อการแปรสภาพเป็นเซลล์กระดูกของเซลล์คล้ายเซลล์กระดูกที่เพาะเลี้ยงจากกระดูกขากรรไกรล่างของมนุษย์ โดยศึกษาถึงความเกี่ยวข้องของพิวริเนอจิก ทูเอกซ์เซเว่น รีเซบเตอร์ในกระบวนการนี้ด้วย เซลล์กระดูกที่เพาะเลี้ยงจากกระดูกขากรรไกรล่างของมนุษย์ได้รับแรงกดเชิงกลแบบเป็นจังหวะเป็นเวลา 20 ชั่วโมง และเพื่อที่ศึกษาผลของการกระตุ้นพิวริเนอจิก ทูเอกซ์เซเว่น เซลล์จึงถูกกระตุ้นด้วย 2’(3’)-*O*-(4-เบนโซอิว) เบนโซอิว-อะดีโนซีนไตรฟอสเฟต จากนั้นการแสดงออกระดับเอ็มอาร์เอ็นเอของยีนที่เกี่ยวข้องกับการแปรสภาพเป็นเซลล์กระดูกและสัญญาณวินท์-เบตาคาทีนินถูกตรวจวัดด้วยวิธีรีเวอร์สทรานสคริปชันโพลีเมอร์เรสเชนรีแอคชัน การสร้างเนื้อเยื่อแข็งในห้องปฏิบัติการถูกย้อมด้วยอะริซาริน เรดเอส และเพื่อยืนยันผลของการกระตุ้นพิวริเนอจิก ทูเอกซ์เซเว่น การสร้างโปรตีนพิวริเนอจิก ทูเอกซ์เซเว่นถูกยับยั้งด้วยวิธีสมอลอินเตอร์เฟียริ่งอาร์เอ็นเอ ตำแหน่งของเบตาเคทีนินภายในเซลล์ถูกย้อมด้วยวิธีอิมมูโนฟลูออเรสเซนส์ ผลการศึกษาพบว่า แรงกดเชิงกลแบบเป็นจังหวะสามารถกระตุ้นการหลั่งอะดีโนซีน ไตรฟอสเฟต และการแปรสภาพเป็นเซลล์กระดูกของเซลล์คล้ายเซลล์กระดูกที่เพาะเลี้ยงจากกระดูกขากรรไกรล่างของมนุษย์ โดยการกระตุ้นนี้เกี่ยวข้องกับการเพิ่มขึ้นของสัญญาณวินท์-เบตาคาทีนิน เนื่องจากพบการเพิ่มปริมาณเบตาคาทีนินในนิวเคลียส และการกระตุ้นนี้เป็นผลมาจากการหลั่งโดยอะดีโนซีน ไตรฟอสเฟต อย่างไรก็ตาม เมื่อศึกษาถึงผลของการกระตุ้นพิวริเนอจิก ทูเอกซ์เซเว่น รีเซบเตอร์ พบว่า การแสดงออกระดับเอ็มอาร์เอ็นเอของยีนที่เกี่ยวข้องกับการแปรสภาพเป็นเซลล์กระดูกและสัญญาณวินท์-เบตาคาทีนินลดลง เมื่อเซลล์ถูกกระตุ้นพิวริเนอจิก ทูเอกซ์เซเว่น รีเซบเตอร์ และเมื่อใส่โปรตีนวินท์ทรีเอซึ่งเป็นโปรตีนที่เกี่ยวข้องกับสัญญาณวินท์-เบตาคาทีนินเข้าไปด้วย วินท์ทรีเอสามารถเพิ่มระดับการแสดงออกระดับเอ็มอาร์เอ็นเอของยีนที่เกี่ยวข้องกับการแปรสภาพเป็นเซลล์กระดกได้ โดยสรุป แรงกดเชิงกลแบบเป็นจังหวะกระตุ้นการแปรสภาพเป็นเซลล์กระดูกของเซลล์คล้ายเซลล์กระดูกที่เพาะเลี้ยงจากกระดูกขากรรไกรล่างของมนุษย์โดยผ่านการทำงานของอะดีโนซีน ไตรฟอสเฟต อย่างไรก็ตามการกระตุ้นพิวริเนอจิก ทูเอกซ์เซเว่น รีเซบเตอร์ยับยั้งการแปรสภาพเป็นเซลล์กระดูกของเซลล์คล้ายเซลล์กระดูกที่เพาะเลี้ยงจากกระดูกขากรรไกรล่างของมนุษย์ โดยกระบวนการนี้เกี่ยวข้องกับการยับยั้งสัญญาณวินท์-เบตาคาทีนิน

ภาควิชา ทันตกรรมประดิษฐ์ ลายมือชื่อนิสิต .....

สาขาวิชา ทันตกรรมประดิษฐ์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5476052032 : MAJOR PROSTHODONTICS
KEYWORDS: INTERMITTENT COMPRESSIVE FORCE / ADENOSINE TRIPHOSPHATE (ATP) / PURINERGIC 2X7 RECEPTOR / HUMAN OSTEOBLASTS / BONE MINERALIZATION / WNT SIGNALING

PIMRUMPAI ROCHANAKIT SINDHAVAJIVA: THE INFLUENCE OF INTERMITTENT COMPRESSIVE FORCE ON OSTEOGENIC DIFFERENTIATION OF HUMAN MANDIBULAR-DERIVED OSTEOBLAST-LIKE CELLS. ADVISOR: ASSOC. PROF. DR. MANSUANG ARKSORNNUKIT, Ph.D., CO-ADVISOR: PROF. DR. PRASIT PAVASANT, Ph.D., 65 pp.

Mechanical force can regulate osteoblast differentiation in osteoblasts. Different types of force have different effects on osteoblasts. These effects are associated with the activation of purinergic receptors, especially purinergic 2X7 receptor (P2X7R). However, the molecular response of human osteoblasts to intermittent compressive force (ICF) associated with P2X7 receptors has not been clarified. In this study, the influence of ICF on human mandibular-derived osteoblast-like cells (hMOBs) was investigated. The role of ICF-induced ATP through P2X7R was examined. The hMOBs were obtained from non-pathologic mandibular bone. Cells were subjected to ICF for 20 h. The hMOBs were cultured in osteogenic medium in the absence or presence of 0.5-5 μM 2'(3')-*O*-(4-benzoyl) benzoyl-ATP (BzATP), a selective P2X7R agonist. The mRNA expression was investigated by quantitative real time polymerase chain reaction. *In vitro* mineral deposition was investigated by Alizarin Red S staining. Transfection of small interfering RNA was performed to confirm the effect of P2X7R activation. WNT/β-catenin signaling was detected by immunofluorescence staining for β-catenin. The results demonstrated that ICF increased the *OSX* and *ALP* mRNA expression and enhanced *in vitro* mineralization. In addition, *WNT3A* mRNA expression and β-catenin nuclear translocation were also increased. These effects were related to ICF-induced ATP. However, BzATP significantly attenuated *in vitro* mineralization and *RUNX2* and *OSX* mRNA expression in osteogenic medium-induced hMOBs. Knockdown P2X7R expression by siRNA rescued BzATP-inhibited *RUNX2* and *OSX* expression. Interestingly, a reduction of *WNT3A* mRNA expression and blockage of osteogenic medium-induced β-catenin nuclear translocation were also found in BzATP treated group. The addition of recombinant human WNT3A abolished the effect of BzATP-reduced osteogenic marker gene expression and *in vitro* mineralization. In conclusion, our results demonstrated that ICF-induced ATP enhanced osteoblast differentiation and WNT/β-catenin signaling pathway. P2X7R pathway participates in BzATP-inhibited osteoblast differentiation of hMOBs. This inhibitory effect was associated with inhibition of the WNT/β-catenin signaling pathway.

Department:	Prosthodontics	Student's Signature	.....
Field of Study:	Prosthodontics	Advisor's Signature	.....
Academic Year:	2016	Co-Advisor's Signature	.....

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CHAPTER I

INTRODUCTION

Mechanical force plays an important role in bone remodeling. The studies demonstrated that various types and magnitudes of mechanical force have diverse effects on osteoblast differentiation. (Robling, Castillo et al. 2006, Sanchez, Gabay et al. 2009, Rumney, Sunters et al. 2012, Hecht, Liedert et al. 2013, Orriss, Key et al. 2013, Tripuwabhrut, Mustafa et al. 2013, Kariya, Tanabe et al. 2015). Tension force promoted osteogenesis by enhancing osteogenic genes and *in vitro* mineralization in MC3T3-E1 (Kariya, Tanabe et al. 2015) .Compressive stress, as found in orthodontic treatment, could modulate the remodeling of periodontal ligament (PDL) and alveolar bone by enhancing bone resorption. (Davidovitch 1991, Henneman, Von den Hoff et al. 2008, Tripuwabhrut, Brudvik et al. 2010) Studies have revealed that, under compressive stress, osteoblasts produce several inflammatory mediators related to bone resorption, such as prostaglandin E2 (PGE2), interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor-α (TNF-α). (Sanuki, Mitsui et al. 2007, Sanchez, Gabay et al. 2009) Moreover, *in vitro* study in human osteoblast-like cells (hOBs) demonstrated that runt-related transcription factor 2 (Runx2) mRNA expression, an important transcription factor in bone formation, was inhibited by compressive force. Moreover, receptor activator of nuclear κB ligand (RANKL) expression, one of the markers for osteoclastogenesis, was also upregulated. (Tripuwabhrut, Mustafa et al. 2013) Although, the effects of many types of force on osteoblasts have been investigated, the effect of intermittent compressive force on human alveolar-derived osteoblasts, which is found in masticatory system, has not been investigated.

The efflux of adenosine 5'-triphosphate triphosphate(ATP) in osteoblasts can be induced by many types of mechanical force (Romanello, Pani et al. 2001, Genetos, Geist et al. 2005, Rumney, Sunters et al. 2012, Hecht, Liedert et al. 2013, Kariya, Tanabe et al. 2015). ATP is rapidly released after mechanical force was applied (Hecht, Liedert et al. 2013, Kariya, Tanabe et al. 2015) .In primary rat osteoblasts ,

ATP increased transient intracellular Ca2+ level (Oriss, Key et. al. 2012), and inhibited *in vitro* mineralization (Hoebertz, Mahendran et al. 2002, Orriss, Utting et al. 2007, Orriss, Key et al. 2013). Tension force-induced ATP upregulated Runx2, osterix (Osx), ALP activity, and *in vitro* mineralization via purinergic X7 receptor, one of the receptors for ATP (Kariya, Tanabe et. al. 2015).

Purinergic 2 or P2 receptors are known to be the receptors for nucleotides including ATP. The human genome contains 7 P2X and 8 P2Y receptor genes (Orriss, Syberg et al. 2011). Among, P2 receptors, the effect of P2X7 receptor (P2X7R) activation on osteoblast differentiation has been investigated (Panupinthu, Rogers et al. 2008, Grol, Panupinthu et al. 2009, Orriss, Key et al. 2012). Functional P2X7R was expressed *in situ* in murine and human osteoblasts (Panupinthu, Rogers et al. 2008, Agrawal, Henriksen et al. 2017). The level of P2X7R protein increased in primary rat calvarial osteoblasts during proliferation and differentiation (Orriss, Key et al. 2012). However, there is contradictory data about the effect of P2X7R on osteoblast functions. The study showed that activation of P2X7R promoted osteogenic differentiation and *in vitro* mineralized nodule formation in rat and murine calvarial osteoblasts. (Panupinthu, Rogers et al. 2008, Rodrigues-Ribeiro, Alvarenga et al. 2015) Nevertheless, when P2X7R, in primary rat calvarial osteoblast, was activated, *in vitro* mineralization and alkaline phosphates (ALP) activity decreased. (Orriss, Key et al. 2012) This contradictory effect of P2X7 receptors can be found in *in vivo* studies of P2X7 receptors knockout (KO) mice. P2rx7-/- mice demonstrated significant reduction in total and cortical bone content of femoral bone (Ke, Qi et al. 2003) while P2rx7-/- mice in the other study (Gartland, Buckley et al. 2003) showed no significant skeletal bone phenotype with exception of thicker cortical bone.

The association of mechanical force and P2X7R has been investigated. Skeletal sensitivity to mechanical loading was reduced by up to 73% in $P2rx7^{-/-}$ mice (Li, Liu et al. 2005). Further *in vitro* study showed that fluid shear stress (FSS) induced ATP and PGE2 release in osteoblasts from WT mice and MC3T3-E1, but not in osteoblasts from P2X7R KO mice. PGE2 release in WT mice osteoblasts and MLO-Y4 osteocytes could be suppressed by P2X7R blockage. (Li, Liu et al. 2005) *In vitro* study that focused on effect of tensile force demonstrated that tensile force

applied on MC3T3 could activate P2X7R and induced osteogenic differentiation and *in vitro* mineralization. (Kariya, Tanabe et al. 2015)

WNT signaling is a well-known pathway that plays important role in osteogenic differentiation by osteoblasts. WNT signaling occurs via canonical and non-canonical pathways (Tompkins 2011). When canonical or WNT/β-catenin signaling pathway is activated, the level of β-catenin in the cytoplasm is increased. β-catenin is then subsequently translocated to the nucleus and binds to T cell factor/lymphoid enhancer factor. This binding induces expression of genes involved in osteogenic differentiation (Behrens, von Kries et al. 1996, Minear, Leucht et al. 2010). Among WNT molecules which are associated with WNT/β-catenin signaling, WNT3a protein is found in osteoblasts and plays a role in the osteoblast differentiation (Hu, Hilton et al. 2005). The association of P2X7R activation and WNT signaling was investigated in MC3T3-E1 (Grol, Brooks et al. 2016). This study demonstrated that P2X7R activation by BzATP prolonged and potentiated WNT/β-catenin signaling.

Although there are studies focusing on the influence of mechanical force through P2X7R on osteoblasts, there is a lack of study focusing the influence of intermittent compressive force (ICF) on osteogenic differentiation in primary human osteoblasts. Therefore, this study aims to investigate the influence of ICF on ATP efflux and osteoblast differentiation in human mandibular-derived osteoblasts (hMOBs). The effect of ATP/P2X7R activation on osteoblast differentiation and WNT/β-catenin signaling pathway in hMOBs is also investigated. This knowledge would be beneficial to give an alternative modality to enhance bone regeneration especially in mandibular arch.

Research question

Does ICF have an influence on osteoblast differentiation of hMOBs?

Objectives and hypotheses

Objective 1: To investigate an effect of ICF on ATP efflux and osteoblast differentiation in hMOBs

Hypothesis: ICF induces ATP efflux and enhance osteoblast differentiation in hMOBs.

Objective 2: To investigate molecular mechanism of ATP/P2X7R activation in hMOBs in term of osteoblast differentiation regulation

Hypothesis: ATP regulates osteoblast differentiation of hMOBs through P2X7 receptors.

Benefits of our study

This study clarified the molecular mechanism of osteoblast differentiation regulation by ICF in hMOBs. This knowledge would be beneficial to give an alternative modality to enhance bone regeneration especially in mandibular arch.

Limitations

This study was performed *in vitro*. Therefore, the results of this study may not reflect *in vivo* situation. Characteristics of primary hMOBs used in this study may have a large variation due to variation among each individual.

Keywords

Human mandibular-derived osteoblasts

Intermittent compressive force

Osteoblast differentiation

Purinergic 2X7 receptor

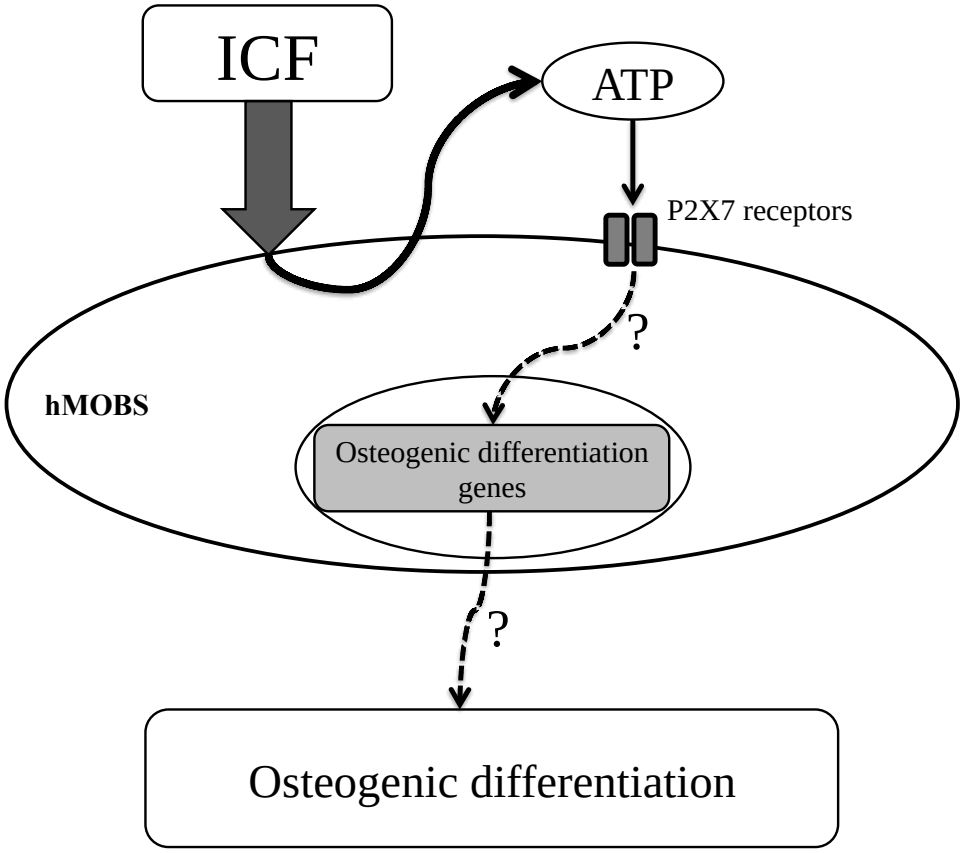
Adenosine 5’-triphosphate (ATP)

WNT-signaling pathway

Research design

Laboratory experimental research

Conceptual framework



CHAPTER II

REVIEW OF RELATED LITERATURE

Osteoblast differentiation and bone remodeling

Osteoblasts and osteocytes differentiate from mesenchymal stem cells (MSCs), which are pluripotent cells. MSCs can differentiate into different tissue specific cells including osteoblasts, chondrocytes, fibroblast, adipocytes and myocytes (Yamaguchi and Kahn 1991). The differentiation to different cell types is due to signals that are given to MSCs. Runx2 is a master transcription factor that can drive MSCs into osteogenic differentiation (Yamaguchi and Kahn 1991). During differentiation process, there are several osteogenic gene markers expressed by osteoblasts such as Runx2, Osx, ALP, type 1 collagen (Col 1A1), osteopontin (OPN) and osteocalcin (OCN) (Rucci 2008) (Fig. 2.1).

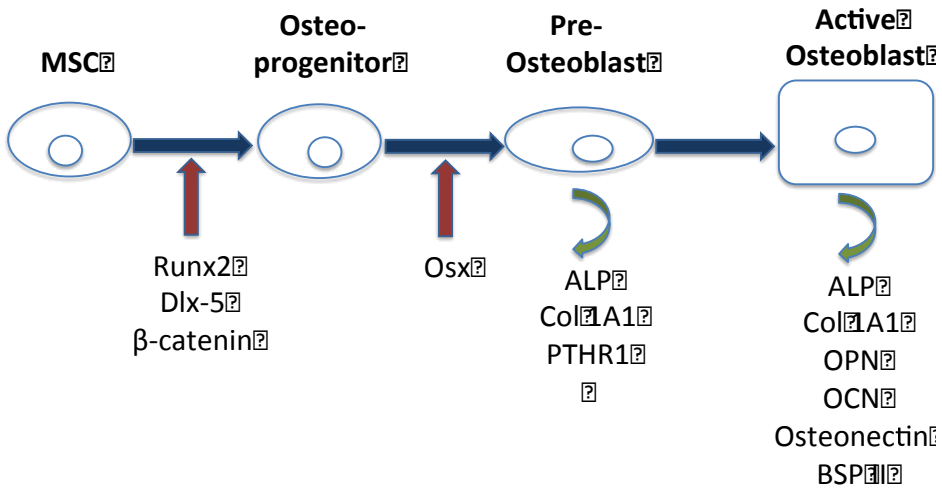


Figure 2.1 Osteoblastogenesis process (modified from (Rucci 2008)

Runx2 and Osx are the important regulators in osteoblast differentiation ((Ducy, Zhang et al. 1997, Nakashima, Zhou et al. 2002) Runx2 is a transcriptional factor which can bind to the promoter region of gene osteoblast differentiation including OPN and OCN. The study in Runx2 KO mice demonstrated the arrested osteoblast differentiation in both endrochondral and intramembranous skeleton

(Komori, Yagi et al. 1997, Otto, Thornell et al. 1997). Osx is a Sp 1transcription family member. The studies showed that Osx can be regulated by Runx 2and Bone morphogenetic protein 2(Matsubara, Kida et al. 2008). Osx KO mice showed no bone formation and complete absence of osteoblasts suggesting an important role of Osx in osteogenesis (Nakashima, Zhou et al. 2002).

The remodeling of the bone is an important dynamic process to maintain the structure of the bone throughout lifetime. This process is regulated by different types of bone cells, which are osteoblasts, osteocytes and osteoclasts. Osteoblasts and osteocytes are responsible for forming and maintaining the bone, while osteoclasts are responsible for resorption of bone (Rucci 2008). The balance of bone resorption and bone formation is essential to maintain a constant bone mass. Therefore, when this balance is disturbed by systemic or local factors, the gain/loss of bone mass will occur. The examples of systemic factors are hormones and systemic disease. Parathyroid hormone can stimulate bone resorption in order to maintain serum calcium level (Li, Amling et al. 1998). Osteoclasts in osteopetrosis, a rare genetic disease, are unable to resorb bone and leads to an increase of bone mass (Rucci 2008).

Effect of mechanical force on bone remodeling

Different magnitudes and types of mechanical force that is applied on the bone can induce various changes in bone. In orthodontic treatment, compressive force that is applied on alveolar bone can cause bone resorption, while bone deposition will occur on tensile side (Krishnan and Davidovitch 2006). *In vitro* and *in vivo* studies have been performed to investigate the molecular mechanism when mechanical forces were applied. The study in MC3T3-E 1cells demonstrated that tension force promoted osteogenesis by enhancing osteogenic genes and *in vitro* minrealization (Kariya, Tanabe et al. 2015) .When osteoblasts are subjected to compressive mechanical force, many cytokines and other mediators will be released from osteoblasts. There are several inflammatory mediators related to bone resorption, such as PGE2, IL1, IL-6, IL-8, and TNF-α. These mediators can induce resorptive function in osteoclasts resulted in bone resorption (Sanuki, Mitsui et al. 2007, Sanchez, Gabay et al. 2009).

Although2 g/cm^2 of static compressive force upregulated type I collagen and ALP expression at both mRNA and protient levels in human alveolar osteoblasts, Runx2 mRNA was inhibited (Tripuwabhrut, Mustafa et al. 2013) .When static compressive was increase to 4g/cm^2, receptor activator of nuclear factor kB ligand (RANKL), an important mediator for osteoclatogenesis, tegerinwas enhanced and osteopro(OPG) expression was suppressed. Nevertheless, compressive force can not only inhibit but also induce osteogenic differentiation in osteoblasts. Optimum magnitude (1 g/cm^2) of continuous compressive force upregulated Runx2 and Osx, distal-less homeobox 5 (Dlx-5) mRNA expression in rat ROS17/2.8 cell line (Yanagisawa, Suzuki et al. 2008). Bone sialoprotein (BMP) 2, 4, 6, 7 protein levels were also found to be increased (Mitsui, Suzuki et al. 2006).

While majority of the studies focused on continuous compressive force, a few studies investigated the influence of intermittent compressive force, mimicking masticatory function. ICF applied on mouse calvarial osteoblasts increased ALP activity and *in vitro* mineralization (Klein-Nulend, Veldhuijzen et al. 1987, Roelofsen, Klein-Nulend et al. 1995). When intermittent compressive loading (2.5 N, 10 Hz) was given to 3D osteoblast-osteocyte co-culture, PGE2 release was increased 30 min post-load and type I pro-collagen was increased at Day1 and Day5 (Vazquez, Evans et al. 2014). The findings suggested that optimal intermittent compressive force could enhance osteoblast mineralization. These data emphasized that different magnitudes and types of mechanical force could affect bone cells in various ways.

The summary of the effect of mechanical force on osteoblasts is shown in Table 2.1.

Table 2.1 Effect of mechanical force on osteoblasts

Cell Type	Species	Mechanical force	Effect	Reference
Primary alveolar	Human	Static compression (2, 4 g/cm2)	RUNX2 ,OPG ↓ RANKL ↑	Tripuwapbut 2013
ROS17/2.8	Rat	Static compression (1 g/cm2)	Runx2, Osx, Dlx5 ↑	Yanakisawa 2008
Saos-2	Human	Static compression (1 g/cm2)	Bmp2, 4, 6, 7 ↑	Mitsuni 2006
Primary calvarial	Mouse	Intermittent compression (13 kPa, 0.3 Hz)	ALP activity ↑ *In vitro* mineralization ↑	Klein-Nulend 1987 Roelofsen 1995
MC3T3-E1 /MLO-Y4 co-culture	Mouse	Intermittent compression (2.5 N, 10 Hz)	PGE2 Release Col 1a1 ↑	Vazquez 2014
MC3T3-E1	Mouse	6% Tension force	Runx2 ,Osx ↑	Kariya2015

Adenosine 5'-triphosphate (ATP)

ATP is a nucleotide that consists of a nitrogenous base (adenine), a sugar (ribose) and a chain of 3 ionised groups (phosphates) bound to the ribose. ATP has both intracellular and extracellular physiological roles (Bodin and Burnstock 2001). Intracellular concentration of ATP is about 3-5 mM at basal condition (Fitz 2007). Osteoblasts can release ATP by 2 mechanisms: lytic and non-lytic mechanisms (Rodrigues-Ribeiro, Alvarenga et al. 2015). While lytic mechanism occurs when there is an injury to osteoblasts and results in high local concentration of ATP. Non-lytic mechanism occurs when certain stimuli, such as mechanical stress, is given to

osteoblasts. When ATP is released outside of the cells, it has autocrine and narrow paracrine effects due to rapid kinetic degradation and dispersed by blood/fluid flow (Fitz 2007).

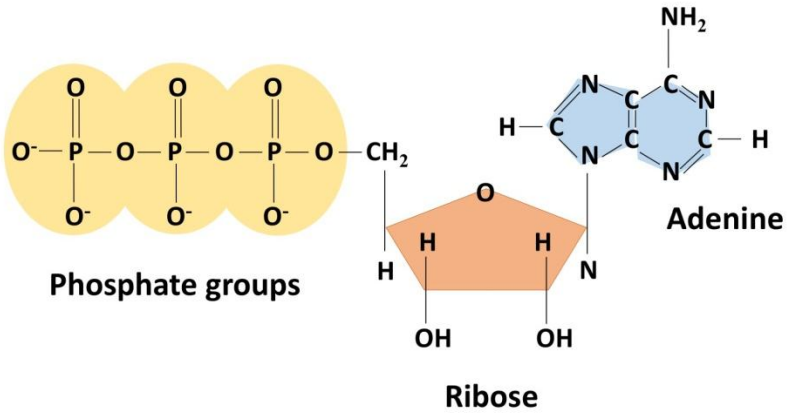


Figure 2.2 The structure of ATP

Mechanical forces such as compressive, tensile, fluid shear stress can induce ATP efflux of osteoblasts. Different concentrations of ATP efflux from osteoblasts and osteocytes were reported. Tensile force stimulation induced extracellular ATP efflux of MC3T3-E1 at 6-7x10^{-13}mol/μg protein. MLO-Y4 osteocyte-like cell line showed acute extracellular ATP release at 4-342 nM after fluid pulse was applied (Kringelbach, Aslan et al. 2015). However, there is controversy about the effect of ATP on osteoblasts in term of osteogenic differentiation and mineralization. Concentrations of ATP at 10 and 100 μM could inhibit *in vitro* mineralization in rat calvarial osteoblasts (Hoebertz, Mahendran et al. 2002). However, when tensile force was applied and ATP was release, *in vitro* mineralization was enhanced (Kariya, Tanabe et al. 2015). The differences of the results might be due to the activation of ATP on different types of receptors.

Purinergic 2 Receptors (P2 receptors)

P2 receptors are known to be the receptors for nucleotides including ATP. The human genome contains 7 P2X and 8 P2Y receptor genes. While P2Y receptors are G-protein coupled receptors, P2X receptors are ion channel protein. The functions of P2 receptors have been studied and the functional effects of P2 signaling on osteoblasts are shown in Table 2.2 and Fig. 2.3.

Table 2.2 Functional effects of P2 signaling on osteoblasts .

Functional effects of P2 signaling on osteoblasts
1. Enhance cell proliferation (Nakamura, Uezono et al. 2000)
2. Increase IL-6 synthesis (Ihara, Hirukawa et al. 2005)
3. Induce osteoblastic membrane blebbing (Panupinthu, Zhao et al. 2007)
4. Induce production of lipid mediators (Panupinthu, Rogers et al. 2008)
5. Inhibit bone mineralization (ATP, UTP $\geq$ 1 μM) (Hoebertz, Mahendran et al. 2002, Orriss, Utting et al. 2007)
6. Modulate osteoblast response to systemic factor (e.g., parathyroid hormone) (Bowler, Buckley et al. 2001, Buckley, Wagstaff et al. 2001)

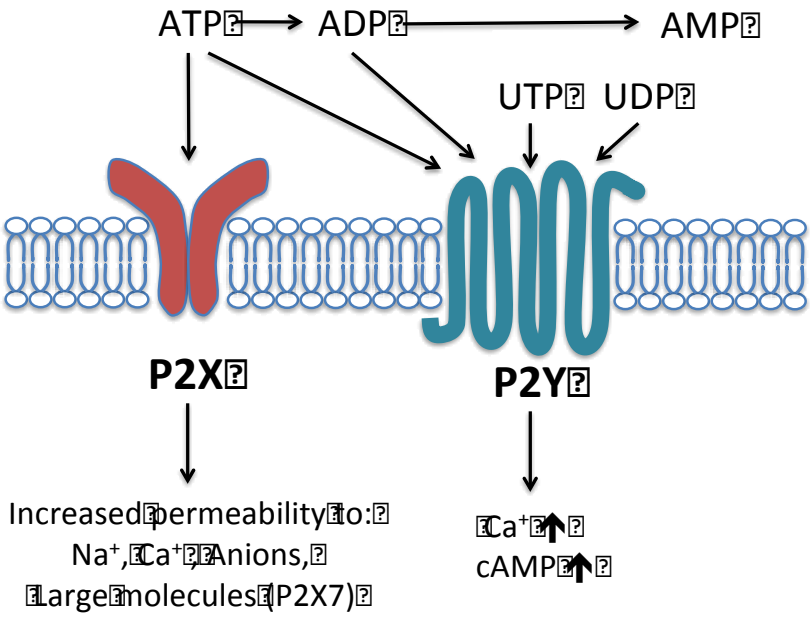


Figure 2.3 P2X and P2Y receptors

P2X receptor consists of 7 subtypes: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7. All of P2X receptor subtypes are expressed by osteoblasts in different level during osteoblast differentiation and each subtype has different effect on osteoblast differentiation of osteoblasts (Orriss, Key et al. 2012, Rumney, Wang et al. 2012).

P2X7 receptors in bone cells

Among P2X receptor subtypes, P2X7 receptor subtype has been found to play an important role in osteoblast differentiation and mineralization. Primary rat osteoblasts expressed P2X7 protein most in proliferating and differentiating stages (Orriss, Key et al. 2012). The activation of P2X7 receptors can result in pore formation at the membrane of osteoblasts. The study compared the morphological changes between human osteosarcoma cell lines, SaOS-2 and Te85 cells, and primary human bone-derived cells (HBDCs) (Gartland, Hipskind et al. 2001). When bone cells were treated with BzATP, a selective P2X7 agonist ATP, flow cytometry

demonstrated increased uptake of ethidium bromide, indicating membrane pore formation, in SaOs-2 cell line and HBDCs and would lead to P2X7 receptor-induced apoptosis. However, this change could not be found in Te85 cell line. It implied the variety of the effect of P2X7 receptor activation in different cell lines.

In vivo studies in knock-out (KO) mice showed contradictory effect of P2X7 receptors. Significant reduction in total and cortical bone content was found in $P2rx7^{-/-}$ mice. Nevertheless, the significant difference in length could not be found (Ke, Qi et al. 2003). Further study was performed to investigate the effect of mechanical loading on skeletal bone of $P2rx7^{-/-}$ mice (Li, Liu et al. 2005). The results showed that the osteogenic response to mechanical loading was suppressed in $P2rx7^{-/-}$ mice. Mechanical strain was required to initiate a response, which is increase in bone formation rate, in KO mice. On the other hand, the other in vivo study in $P2rx7^{-/-}$ mice demonstrated no significant skeletal phenotype except thicker cortical bones than wild-type (WT) mice (Gartland, Buckley et al. 2003, Grol, Panupinthu et al. 2009). This difference could be due to the different techniques to generate KO mice.

Recent study in primary human osteoblasts demonstrated that P2X7R were involved in cell death, growth and cellular signaling (Agrawal, Henriksen et al. 2017). The authors concluded that only mature human trabecular-derived osteoblasts expressed functional P2X7R. These receptors were partially responsible for the mechanical stimuli and mechanically-induced signals. The activation and inhibition of P2X7R regulated osteogenic genes and bone formation. The studies in P2X7 receptors of osteoblasts were summarized in Table 2.3

Table 2.3 The studies in P2X7 receptors of osteoblasts (modified from Grol, Panupinthu et al. 2009)

Cell type	Species	Signaling	Proposed function	Reference
SaOs-2, Te85 and MG63 cell lines	Human	Pore formation Membrane blebbing	Enhanced apoptosis	Nakamura 2000 Gartland 2001
Primary cells	Human	Pore formation Membrane blebbing	Enhanced apoptosis	Gartland 2001
Primary cells	Rat	Pore formation Membrane blebbing LPA synthesis/release PGE2 synthesis/release	Enhanced apoptosis Increased bone formation	Orriss 2006 Panupinthu 2007 Panupinthu 2008
MC3T3-E1 and MLO-Y4 cell lines	Mouse	Pore formation PGE2 synthesis/release	Mechanotransduction	Li 2005
Primary cells	Mouse	Pore formation Membrane blebbing LPA synthesis/release PGE2 synthesis/release ERK ½ activation	Enhanced apoptosis Increased bone formation Mechanotransduction	Ke 2003 Li 2005 Panupinthu 2007 Panupinthu 2008 Liu 2008
Primary trabecular-derived cells	Human	Reduced proliferation Reduced ALP activity Reduced in vitro mineralization Partially reduced mechanically-induced calcaium propagation	Inhibited bone formation Involved in mechanically-induced signaling	Agrawal 2017

WNT signaling pathway

WNT signaling occurs via canonical and non-canonical pathways (Tompkins 2011). The canonical WNT or WNT/β-catenin signaling pathway plays an important role in osteoblast differentiation (Baron and Kneissel 2013). When WNT/β-catenin signaling pathway is activated, the level of β-catenin in the cytoplasm is increased and subsequently translocated to the nucleus. In nuclease, β-catenin will bind to T cell factor/lymphoid enhancer factor (TCF). β-Catenin/TCF complex induces expression of genes involved in osteogenic differentiation (Behrens, von Kries et al. 1996, Minear, Leucht et al. 2010). These osteogenic genes include Runx2 and Osx, which eventually results in increased bone formation (Minear, Leucht et al. 2010).

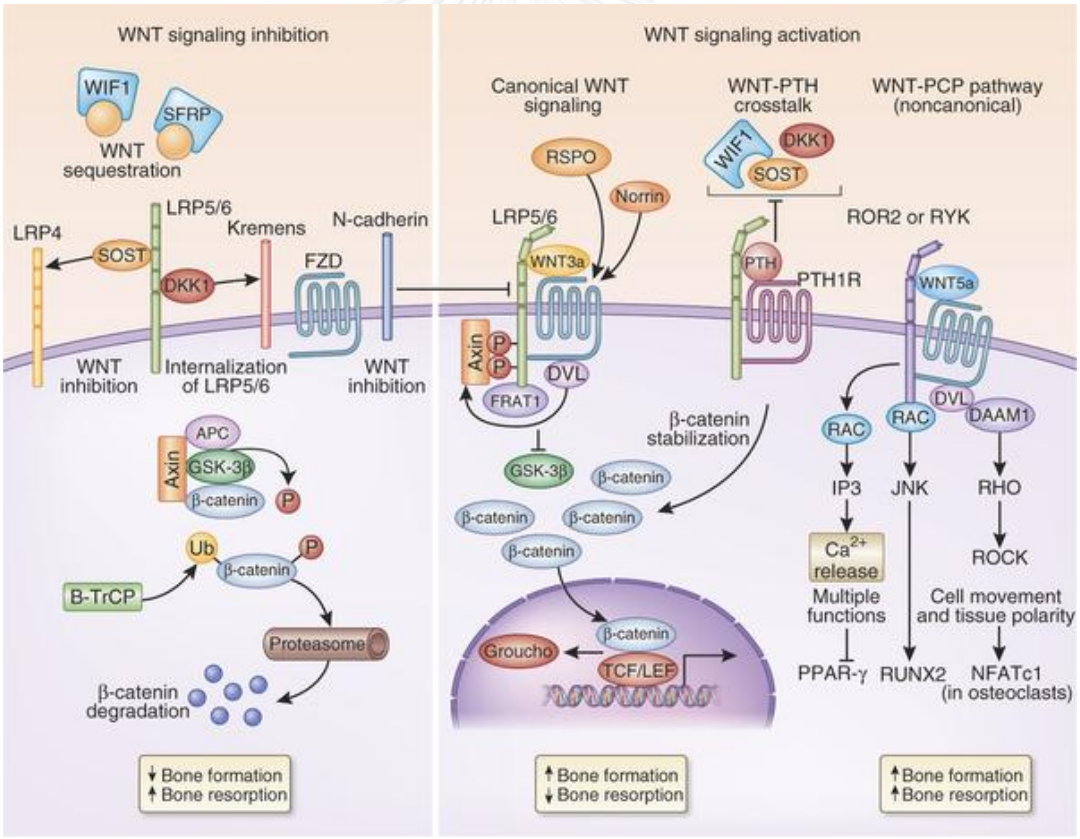


Figure 2.4 The inhibition and activation of WNT signaling (modified from Baron and Kneissel 2013)

Among WNT molecules which are associated with WNT/β-catenin signaling, WNT3a protein is found in osteoblasts and plays a role in the osteoblast differentiation (Hu, Hilton et al. 2005). Wnt3a protein had diverse effects on osteogenic differentiation based on the developmental stage of osteoblasts. In in differentiated prenatal/neonatal mouse calvarial cells, Wnt3a inhibited *in vitro* osteogenic differentiation (Quarto, Behr et al. 2010). Nevertheless, Wnt3a stimulated bone regeneration in mouse tibial injury study (Minear, Leucht et al. 2010) and increased the degree of osseointegration of the implants (Popelut, Rooker et al. 2010).

WNT/β-catenin signaling activation requires 2 co-receptors which are Frizzled (FZD), a seven-pass transmembrane protein family, and the low-density lipoprotein receptor-related protein (LRP) family, LRP 5 or LRP6. DICKKOPF (DKK) is an extracellular inhibitor for WNT/β-catenin signaling. DKK can form a complex with LRP5/6 and the transmembrane protein Kremen. This complex leads to the internalization of LRP5/6 and turn off WNT/β-catenin signaling (Tompkins 2011).

The association of mechanical force and WNT/β-catenin signaling

WNT/β-catenin signaling can be activated by mechanical loading (Lin, Jiang et al. 2009, Tu, Rhee et al. 2012). Fluid shear stress or tensile strain activated β-catenin in osteoblast and osteocytes (Sen, Styner et al. 2009, Case, Sen et al. 2011). These studies suggested the enhancement effect of mechanical focre on bone formation via WNT/β-catenin signaling. The study in mesenchymal stem cells demonstrated that 3600 cycles/day 2% strain could upregulate WNT/β-catenin signaling via glycogen synthase kinase3β (GSK3β) (Sen, Styner et al. 2009).

Recently, the association of P2X7R activation and WNT signaling was investigated in MC3T3-E1 (Grol, Brooks et al. 2016). This study demonstrated that the responses to Wnt3a in calvarial cells isolated from P2X7R KO mice were significantly decreased compared to the cells from wild type. performed the They also experiments in MC3T3-E 1cells and found that P2X7R activation by BzATP prolonged and potentiated WNT/β-catenin signaling. The authors suggested that these processes may be resulted from the phosphorylation inhibition of GSK3β.

CHAPTER III

RESEARCH METHODOLOGY

Patient selection and sample collection

Human MOBs were obtained as previously described (Khonsuphap, Pavasant et al. 2017). The protocol was approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University, Thailand (#027/2016). After obtaining informed consent, mandibular alveolar bone was collected at the Department of Surgery, Chulalongkorn University. Only alveolar bone of healthy adult patients aged 25-35 years from the surgical areas that had no infection and inflammation was collected for this study.

The alveolar bone was kept in a sterile tube containing growth medium (GM), which was composed of 1000 mg/L glucose Dulbecco's Modified Eagle's Medium (Gibco, BRL, Carlsbad, CA) ,110 mg/L sodium pyruvate, 15% (v/v) fetal bovine serum (HyClone, Thermo Scientific, Logan, UT), 2 mM L-glutamine (Gibco), 100 units/ml Penicillin (Gibco), 100 μg/ml Streptomycin (Gibco), and 5 μg/ml Amphotericin B (Gibco).

Cell culture

Within4 h after sample collection, alveolar bone was washed with phosphate buffered saline supplemented with 200 Units/ml Penicillin (Gibco) and 200 μg/ml Streptomycin (Gibco). All soft tissues attached to alveolar bone was removed by surgical blade. The alveolar bone was cut into 5x5x3 mm and placed on the 35mm culture dish (Corning, New York, NY, USA). The explants were cultured with GM. The cultured explant was incubated at 37°c in a humidified atmosphere of 5% CO2 until hMOBs reach their 80% confluence. The hMOBs was detached with 0.2% trypsin and 0.02% EDTA (Gibco) and subcultured to first passage in 60-mm culture dishes. After 80% confluence of each passage, hMOBs was subcultured in 1:3 ratio

and maintained in GM. Only hMOBs from passage 3-8 were used in the further experiment. The hMOBs derived from different individuals were cultured in each separate cell line. At least 3 different cell lines were used as biological replication.

Osteoblast differentiation

The hMOBs at density of 7x104 cells/well were cultured in GM in 24-well plates (Corning) overnight. On Day 0, GM was replaced by osteogenic medium (OM), which wasGM supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich Chemical, St Louis, MO), 100 nM dexamethasone (Sigma-Aldrich Chemical), and 10 mM β-glycerophosphate (Sigma-Aldrich Chemical). The hMOBs, which were maintained in GM throughout the experiment, were served as control.

To investigate the effect of ATP and BzATP on osteoblast differentiation, hMOBs was cultured for 4 and 14 days in OM in addition of 0.1, 1 , or 10 μM ATP (Sigma-Aldrich Chemical) or 0.5, 2.5 , or 5 μM BzATP (Trocris Bioscience, United Kingdom). GM and OM were replaced every 2 days. Osteogenic marker gene expression, ALP activity and mineral deposition were investigated.

To determine the influence of WNT/β-catenin signaling, 50 or 100 ng/ml rhWNT3A (R&D Systems, Minneapolis, MA) and 100 ng/ml rhDICKOFF 1 (rhDKK1) (R&D Systems), a WNT/β-catenin antagonist, were used. The osteogenic and WNT signaling gene expression was investigated after 4 days of culture. *In vitro* mineralization was determined at day 14.

Intermittent compressive force application

The mechanical loading apparatus (Thai Patent ID: 1401006767) used in this study was designed and manufactured by the Faculty of Engineering, Thai-Nichi Institute of Technology. The compressive force can be set as static or intermittent. Magnitude and frequency of ICF can be adjusted by using Compressive force generator V2.5 software. The magnitude of load was set at 1.5 g/cm^2. The loading

cycle was set to press for 1 s and to unpress for 2 s to yield a loading cycle approximately 1/3 Hertz.

The hMOBs was cultured in 6-well plate (Corning) at a density of $3x10^5$ cells/well for 1 day before experiments. On experimental day (Day 0), hMOBs was starved with serum-free culture medium (SFM) for 2 h before ICF was applied. Then SFM was replaced by fresh SFM immediately before ICF was applied for 20 h. The mRNA expression of osteogenic markers and WNT signaling was assessedby qRT - PCRs.

To investigate the effect of ICF on *in vitro* mineralization, after 20-h application of ICF, hMOBs were further cultured in OM for 7 days, Alizarin Res S staining was performed to determine *in vitro* mineralized nodules.

To clarify the role of ICF-induced ATP, Apyrase (0. 5unit/ml), an enzyme for hydrolysis ATP, was added30 min prior to ICF application. Then ICF was applied for 20 h. The mRNA expression of osteogenic markers and WNT signaling was assessed by qRT-PCRs.

ATP luminescence assay

ATP efflux of each time point was measured immediately after ICF was removed using ENLITEN® ATP assay system Bioluminescence Detection kit (Promega, Madison, WI, USA). Fifty µl of reagent was added in 50 µl of culture medium collected from control and experimental groups. Light output was detected at of an absorbance 560 nm by microplate reader (Synergy H1, Biotek multi-mode reader, Winooski, VT, USA). This assay uses recombinant luciferase to catalyze the following reaction:

$$ATP + D\text{-}Luciferin + O2 \rightarrow Oxyluciferin + AMP + PPi + CO2 + Light\ (560nm)$$

***In vitro* mineralization assay**

The hMOBs were cultured in OM for 14 days, then fixed in -20ºC methanol, and stained with 1% Alizarin Red S solution (Sigma-Aldrich Chemical). The amount of calcium deposition was quantified by eluting the staining with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich Chemical) in 10 mM sodium phosphate (Sigma-Aldrich Chemical). The absorbance of 570 nm was measured using a microplate reader (ELx800, Biotek, Winooski, VT).

Alkaline phosphatase activity assay

The hMOBs were lysed in a pH 8 lysis buffer. The cell lysates were incubated at 37ºC in a solution containing 2 mg/ml p-nitrophenol phosphate (Life Technologies Corp, Frederick, MD), 2 mM MgCl2 (Life Technologies Corp) and 0.1 M 2-amino-2methyl-1-propanol (Life Technologies Corp). After 15 minutes, 50 mM NaOH (Emsure, Merck, Darmstadt, Germany) was added to stop the reaction. The presence of p-nitrophenol was measured at an absorbance of 410 nm using a microplate reader (ELx800, Biotek). Total cellular protein was determined by a BCA assay (Thermo Scientific Rockford, IL, USA). The enzyme activity was expressed per total cellular protein.

MTT assay

The effect of ICF and BzATP on cell viability and proliferation was investigated. An MTT assay was performed at 1, 3, and 7 days. The hMOB proliferation/viability was analyzed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (USB Corp., Cleveland, OH). The formazan crystals were dissolved, and quantified using microplate reader (ELx800, Biotek) at an absorbance of 540 nm.

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Human MOBs at density of 3x105 cells were cultured in 6-well plate. Total RNA was extracted using 1 ml of RiboEx™ lysis reagent (Geneall Biotechnology Co., Ltd, Seoul, Korea) according to the manufacturer's instructions. One µg of mRNA from each sample was reverse transcribed to cDNA using Improm-II™ (Promega, Madison, WI). Subsequently, qRT-PCR was performed using a miniOpticon Real-Time PCR Detection System (Bio-Rad, Singapore) with a FastStart Essential DNA Green Master kit (Roche Diagnostics, Indianapolis, IN). The oligonucleotidesequences used in this study were shown in Table 3.1. The PCR protocol was: denaturation at 94°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10s for 45 cycles. Gene expression was normalized to the 18S ribosome expression and further normalized to the control of each experiment. Bio-Rad CFC Manager3.1 (Bio-Rad) was used to determine relative gene expression.

Small interfering RNA (siRNA) transfection

To investigate the role of P2X7R on osteoblast differentiation, hMOBs were cultured in antibiotic-free growth medium for 24 h. Then cells were treated with a solution of 4 µl of P2X7R siRNA) siP2X7R oligonucleotide (1:250; Santa Cruz Biotechnology, Santa Cruz, CA) and 6 µl of transfection reagent (LipofectamineTM2000 Reagent, Invitrogen, Carlsbad, CA) for 24 h before the experiments. The hMOBs treated with 4 µl of control siRNA (siC) (1:250; Santa Cruz Biotechnology) and 6 µl of transfection reagent were served as control.

Immunofluorescence staining for β-catenin

The cells were fixed with cold methanol and incubated with 10% (v/v) horse serum in PBS for 1 h to prevent nonspecific antibody binding. The cells were incubated with the primary antibody, Rabbit anti-human β-catenin polygonal antibody (EMD Millipore, Temecula, CA) at a 1:500 dilution for 3 h at 37°C followed by a

1:500 dilution of biotinylated-secondary antibody (Abcam, Cambridge, MA) for 45 min. β-catenin detection was perform using streptavidin-FITC (Sigma-Aldrich Chemical). Cell nuclei were stained with DAPI. The fluorescence was evaluated by fluorescence microscope (Axio Oserver.Z1, Zeiss, Jena, Germany). For a negative control, the primary antibody was omitted from the staining method.

Statistical analysis

The experiments were performed in triplicate, with the data represented as mean ± standard deviation (SD). The data were analyzed by one-way analysis of variance using statistical software (SPSS Version 22, Chicago, IL). Scheffé's test was used for post hoc analysis.

Table 3.1 Oligonucleotide sequences

Gene	Accession no.	Primer sequence	Size (bp)
COL I	NM000088.3	Forward: 5' GTGCTAAAGGTGCCAATGGT 3' Reverse: 5' ACCAGGTTCACCGCTGTTAC 3'	128
BSP	NM004967.3	Forward: 5' ATGGCCTGTGCTTTCTCAATG 3' Reverse: 5' AGGATAAAAGTAGGCATGCTTG 3'	125
RUNX2	NM001024630.3	Forward: 5' ATGATGACACTGCCACCTCTGA 3' Reverse: 5' GGCTGGATAGTGCATTCGTG 3'	167
OSX	NM001300837.1	Forward: 5' GCCAGAAGCTGTGAAACCTC 3' Reverse: 5' GCTGCAAGCTCTGCATAACC3'	161
WNT3A	NM033131.3	Forward: 5' CTGTTGGGCCACAGTATTCC 3' Reverse: 5' GGGCATGATCTCCACGTAGT 3'	113
WNT5A	NM003392.4	Forward: 5' TCAGGCACCATTAAACCAGA 3' Reverse: 5' AATTCACAGAGGTGTTGCAGC 3'	159
P2X1R	NM002558.3	Forward: 5' GCTACGTGGTGCAAGAGTCA 3' Reverse: 5' GTAGTTGGTCCCGTTCTCCA 3'	215
P2X2R	NM174872.2	Forward: 5' GCTCCTTTCCATCTCACTGG 3' Reverse: 5' GGAAGTGAGCAGCCCTGTA 3'	237
P2X3R	NM002559.3	Forward: 5' ACAGCCAGGGACATGAAGAG 3' Reverse: 5'AGCCGGGTGAAGGAGTATTT 3'	209
P2X4R	NM001261397.1	Forward: 5' GAGATTCCAGATGCGGACC 3' Reverse: 5' GACTTGAGGTAAGTAGTGG 3'	215
P2X5R	NM002561.3	Forward: 5' CTGGTCGTATGGGTGTTCCT 3' Reverse: 5' CTGGGCTGGAATGACGTAGT 3'	159
P2X6R	NM005446.3	Forward: 5' ACTCTGTGTGGAGGGAGCTG 3' Reverse: 5' GGCAAGTGGGTGTCAGAACT 3'	151
P2X7R	NM002562.5	Forward: 5' AAGCTGTACCAGCGGAAAGA 3' Reverse: 5' GCTCTTGGCCTTCTGTTTTG 3'	202
P2Y1R	NM002563.4	Forward: 5' AAAACTAGCCCCCTGCAACT 3' Reverse: 5' GATCTGATGCCGGATGAACT 3'	153
18S	NR003286.2	Forward: 5' GGCGTCCCCCAACTTCTTA 3' Reverse: 5' GGGCATCACAGACCTGTTATT 3'	76

CHAPTER IV

RESULTS

OBJECTIVE 1: To investigate an effect of ICF on ATP efflux and osteoblast differentiation in hMOBs

Characterization of hMOBs

The isolated cells expressed *COL I*, *BSP*, *RUNX2*, and *OSX* mRNA. After cultured in OM for 4 days, the upregulation of *COL I*, *BSP*, *RUNX2*, and *OSX* mRNA levels and ALP enzymatic activity were observed. Alizarin Red S staining showed that hMOBs formed mineralized nodules after 14 days cultured in OM. These data indicated that the isolated cells exhibit osteoblast characteristics (Fig. 4.1).

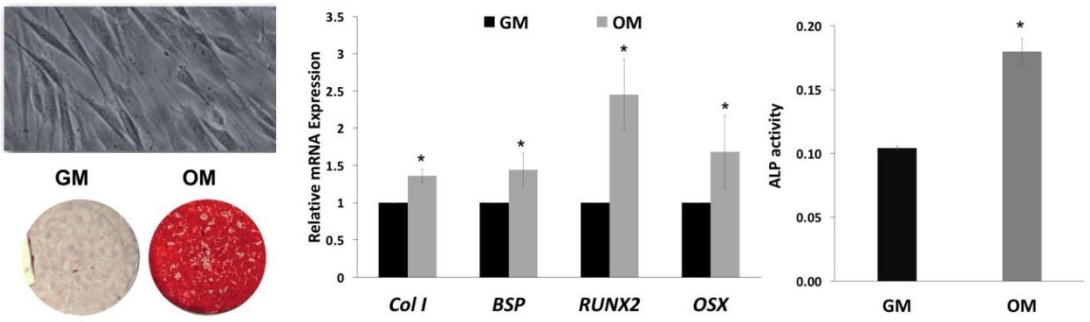


Figure 4.1 Characteristics of hMOBs. Cells were cultured in growth medium (GM) or osteogenic medium (OM). The light micrographs showed cell morphology of isolated cells. At day 14, *in vitro* mineralization was investigated using Alizarin Red S staining. Osteogenic marker genes were determined by qRT-PCR and ALP activity was evaluated at day 4. Data are shown as mean ± SD, n=3, *= $p<0.05$ versus GM.

ICF induced ATP efflux in hMOBs.

To investigate the effect of ICF to ATP efflux in hMOBs, ATP efflux was measured immediately after ICF was removed. ICF significantly induced ATP efflux by 1.79±0.14, 1.45±0.16 and 2.36±0.32-fold compared to control at 2, 4 and 20 h of ICF application respectively (Fig. 4.2). Although, ATP efflux at 8 h was increased by ICF when compared to control but there was no statistical significance. Because the highest ATP efflux was induced, 20-h application of ICF was used in further experiments.

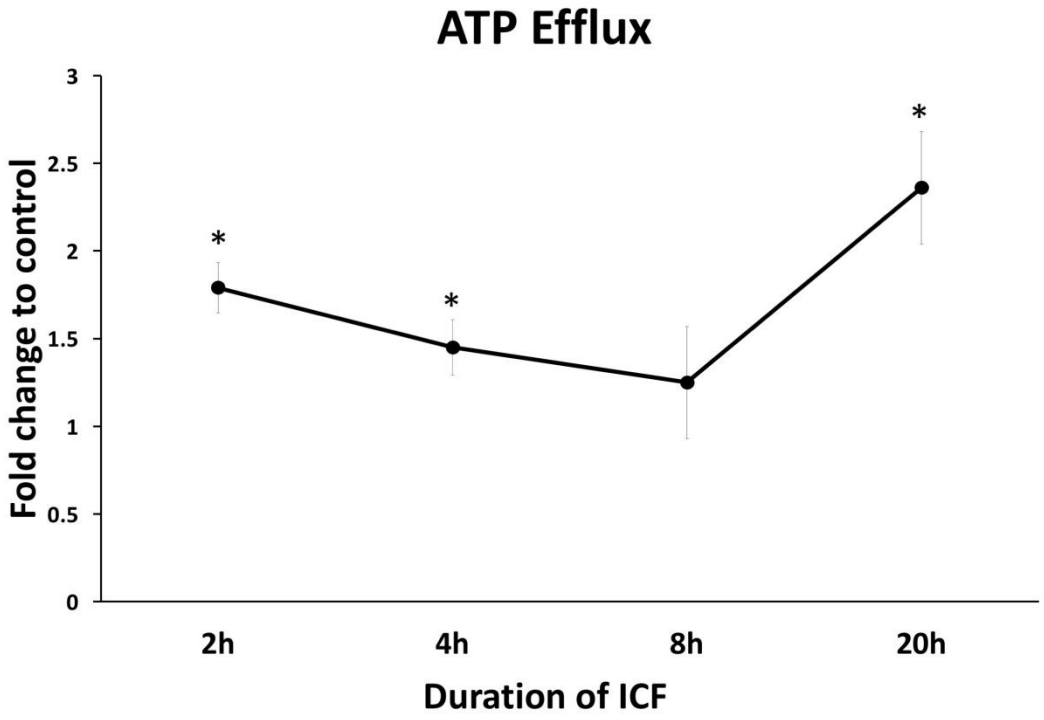


Figure 4.2 The effect of ICF on ATP efflux in hMOBs at different time points. ATP efflux was measured immediately after ICF was removed. The hMOBs with no ICF applicationat each time point were served as control. Data are shown as mean of fold change compared to control ± SD, n=3, *= $p<0.05$ versus control.

ICF-induced ATP increased level of *OSX* and *ALP* mRNA expression an *in vitro* mineralization.

To investigate the role of ICF-induced ATP on osteogenic mRNA expression, Apyrase (0.5 unit/ml) was used. MTT assay showed that ICF and Apyrase had no effect on cell viability. However, Apyrase reduced extracellular ATP to 52% (Fig. 4.3).

When ICF was applied on hMOBs for 20 h, *OSX* and *ALP* mRNA expression was significantly increased. However, *COL I* and *RUNX2* mRNA expression was not significantly changed. The induction of *OSX* and *ALP* mRNA expression by ICF was attenuated by Apyrase. These results suggested a role of ICF- inducedATP on the mRNAexpression of *OSX* and *ALP* (Fig. 4.4).

To clarify whether ICF enhance *in vitro* mineralization hMOB were cultured in OM for ICF was applied 20 hr. The hMOBs were further cultured in OM for 7 days. Alizarin red S staining showed that ICF significantly enhanced *in vitro* minralization in hMOBs (Fig. 4.5)

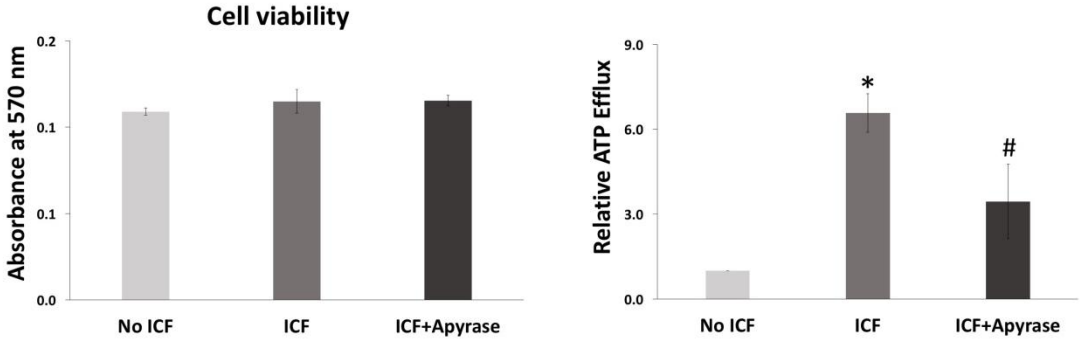


Figure 4.3 The role of ICF and Apyrase on cell viability and ATP efflux. The hMOBs were cultured in SFM and ICF was applied for 20 h. Apyrase (0.5 unit/ml) ,an enzyme for ATP hydrolysis, was added 30 min prior to ICF application. MTT assay showed that ICF and Apyrase had no effect on cell viability. Apyrase significantly reduced ICF-induced ATP efflux.The hMOBs with no ICF application were served as control . Data are shown as mean ± SD, n=3, *= $p<0.05$ versus No ICF, #= $p<0.05$ versus ICF.

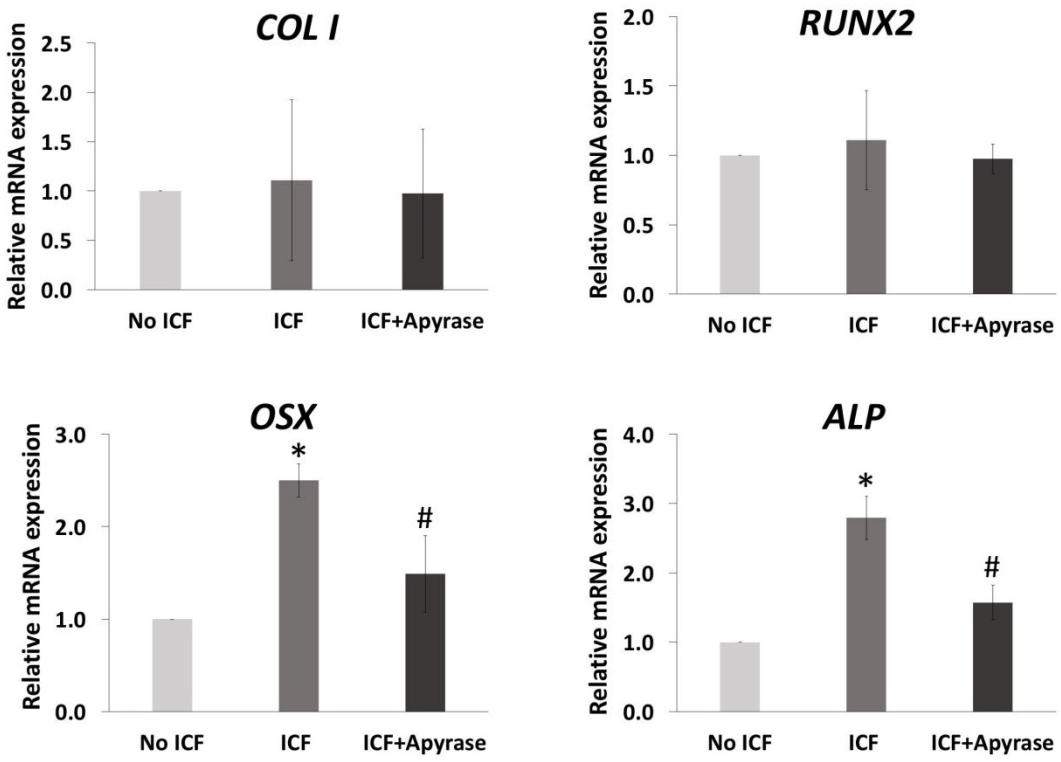


Figure 4.4 The role of ICF and ICF-induced ATP on osteoblast differentiation. The hMOBs were cultured in SFM and ICF was applied for 20h. Apyrase was added to determine the role of ICF-induced the expression of osteogenic gene. ICF significantly induced the expression of *OSX* and *ALP*, but not *COL I* and *RUNX2*. The induction of *OSX* and *ALP* by ICF was attenuated by Apyrase. Data are shown as mean ± SD, n=3, *= $p<0.05$ versus No ICF, #= $p<0.05$ versus ICF.

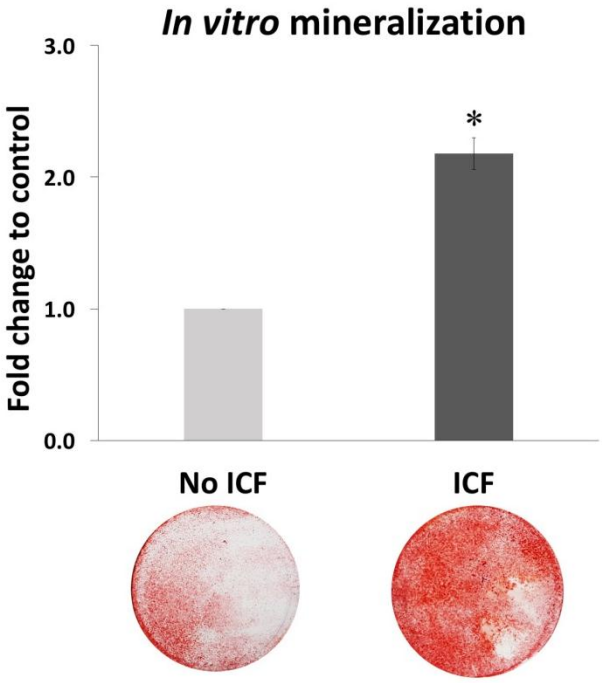


Figure 4.5 ICF increased *in vitro* mineralized nodules in hMOBs. The hMOBs were cultured in OM and ICF was applied for 20 h. Then hMOBs were further cultured in OM. At day 7, *in vitro* mineralized nodules were stained with Alizarin Red S. The hMOBs without ICF application were served as control. ICF significantly increased *in vitro* mineralization in hMOBs. Data are shown as the mean ± SD, n=3, *=p<0.05 versus No ICF.

ICF-induced ATP increased *WNT3A*, but not *WNT5A* mRNA expression.

To determine the effect of ICF on WNT signaling mRNA expression, ICF was applied on hMOBs cultured in SFMfor 20 h. The data showed that *WNT3A* mRNA expression was significantly increasedin ICF group . The ICF-induced *WNT3A* was inhibited by Apyrase. However, ICF have no effect on *WNT5A*. These results suggested the effect of ICF and ICF -induced ATP on WNT/β-catenin signaling (Fig. 4.6).

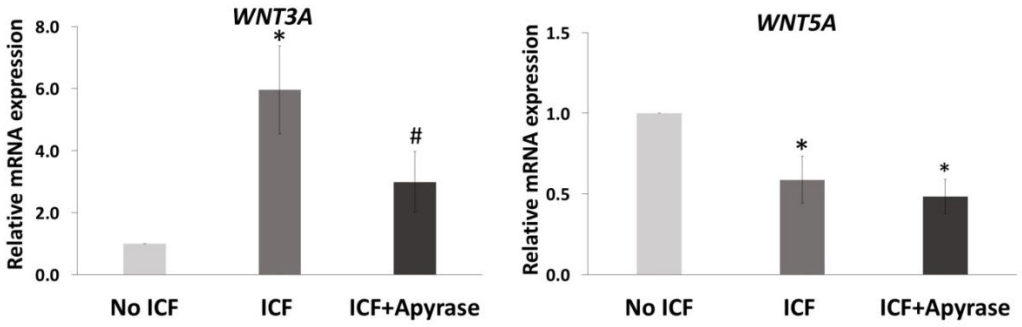


Figure 4.6 The role of ICF and ICF-induced ATP on WNT signaling genes. The hMOBs were cultured in SFM and ICF was applied for 20h. Apyrase was added to determine the role of ICF-induced ATP. The hMOBs without ICF application were served as control. ICF significantly induced the expression of *WNT3A*, but not *WNT5A*. The induction of *WNT3A* by ICF was inhibited by Apyrase. Data are shown as mean ± SD, n=3, *= $p<0.05$ versus No ICF, #= $p<0.05$ versus ICF.

Exogenous ATP induced *WNT3A*, but not *WNT5A* mRNA expression in dose-dependent manner.

To confirm the effect of ATP on *WNT3A* mRNA expression, hMOBs cultured in SFM was treated with ATP (0.1, 1, or 10 μM) for 1 day. MTT assay showed that all doses of ATP used in this study had no effect on cell viability (Fig. 4.7A). The results demonstrated that ATP significantly induced *WNT3A* mRNA expression in dose-dependent manner. While 1 μM ATP significantly induced *WNT5A* mRNA expression, the expression of *WNT5A* was reduced by 10 μM of ATP (Fig. 4.7).

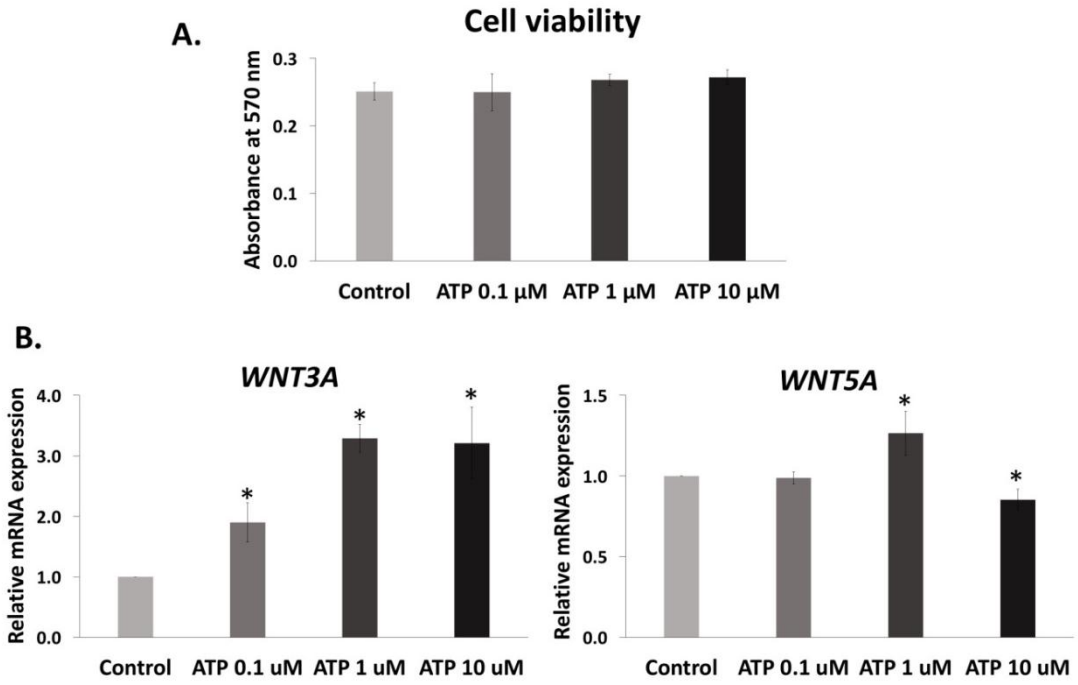


Figure 4.7 The role of ATP on cell viability and WNT signaling genes. The hMOBs were cultured in SFM. ATP (0.1, 1, and 10 µM) was added for 1 day. The hMOBs cultured in SFM without ATP were served as control. A)MTT assay showed that all doses of ATP had no effect on cell viability. B) While ATP significantlyinduced *WNT3A* mRNA expression in dose-dependent manner, *WNT5A* expression was significantlyreduced by 10 µM ofATP. Data are shown as mean ± SD, n=3, *= $p<0.05$ versus control.

ICF-induced ATP enhanced nuclear translocation of β-catenin signaling which could be inhibited by Apyrase.

To confirm the role of ICF-induced ATP on WNT/β-catenin signaling, nuclear translocation of β-catenin was investigated. Immunofluorescence staining for β-catenin showed that ICF induced translocation of β-catenin into nucleus (Figure 4.8). In addition, β-catenin nuclear translocation was inhibited by Apyrase. These results indicated that ICF-induced ATP could regulate WNT/β-catenin signaling.

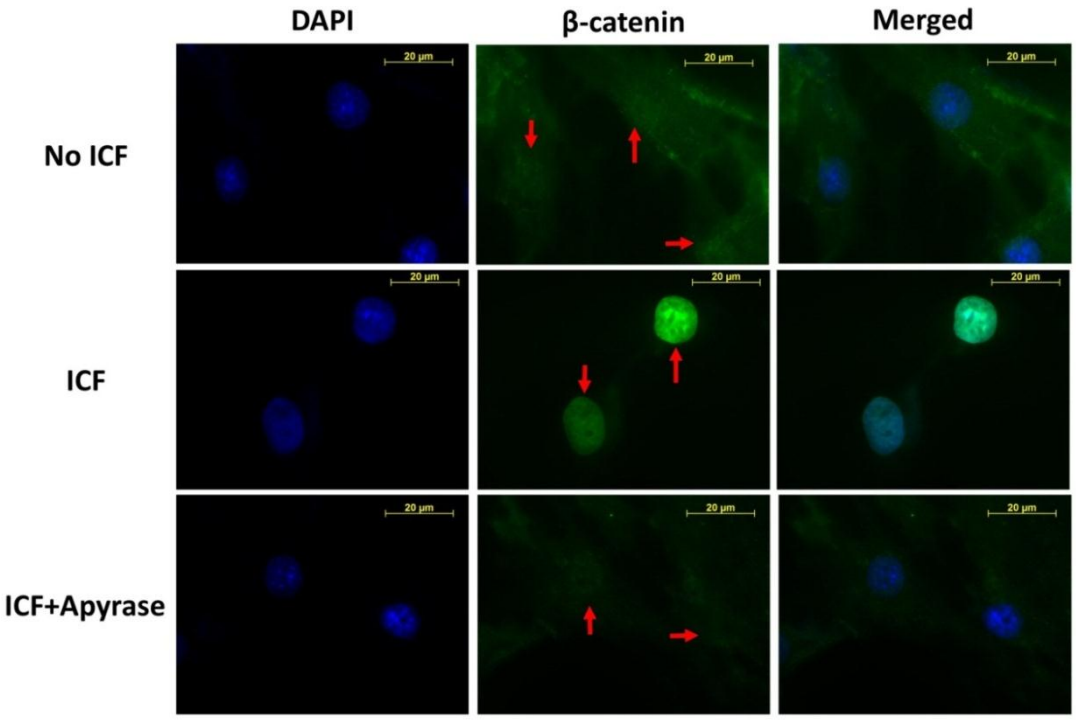


Figure 4.8 ICF-induced ATP enhanced β-catenin accumulation in nucleus. The hMOBs were cultured in SFM and ICF was applied for 20 h. Apyrase was added to investigate the role of ATP on β-catenin nuclear translocation. Immunofluorescence staining for β-catenin showed that ICF induced translocation of β-catenin (indicated by arrows) into nucleus (stained by DAPI). The addition of Apyrase inhibited nuclear translocation of β-catenin.

WNT/β-catenin regulated *RUNX2* and *OSX* mRNA expression in hMOBs.

To investigate the role of WNT signaling in osteoblast differentiation of hMOBs, the expression pattern of WNT signaling-related genes was investigated after 1 and 4 days cultured in GM and OM. The results demonstrated a significantly increased expression of *WNT3A* mRNA expression by hMOBs cultured in OM at both day1 and 4. Under these conditions, *WNT5A* mRNA expression significantly decreased (Fig. 4.9A). These results suggested a role of WNT3A and WNT/β-catenin signaling in osteoblast differentiation of hMOBs.

To clarify whether WNT/β-catenin signaling plays a role in osteogenic gene expression by hMOBs, rhDKK1, a WNT/β-catenin antagonist was used. The results showed a significant reduction of *RUNX2* and *OSX* mRNA expression in hMOBs treated with rhDKK1 at day 4 (Fig. 4.9B). These results demonstrated that WNT/β-catenin signaling was involved in osteoblast differentiation of hMOBs by regulating *RUNX2* and *OSX*.

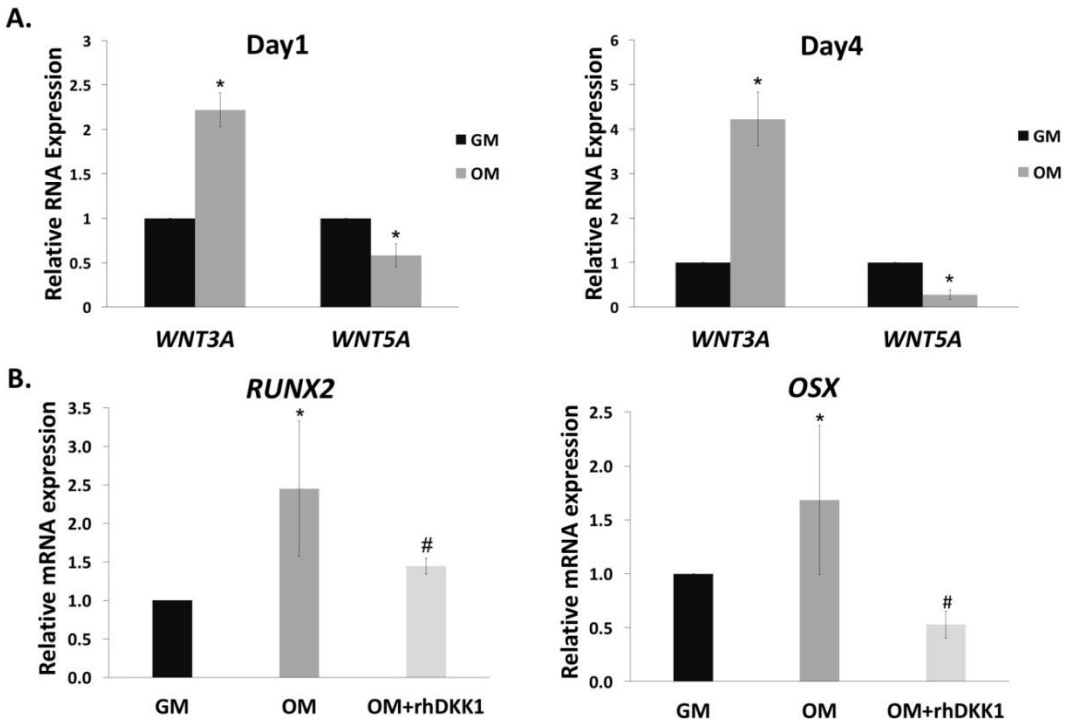


Figure 4.9 WNT/β-catenin regulated *RUNX2* and *OSX* mRNA expression in hMOBs. (A) The mRNA expression of WNT-signaling molecules was determined by qRT-PCR in hMOBs cultured in GM and OM at 1 and 4 days. *WNT3A* mRNA expression significantly increased in OM, however *WNT5A* mRNA expression significantly decreased. (B) The rhDKK1, a WNT/β-catenin antagonist, significantly inhibited OM- induced *RUNX2* and *OSX* mRNA expression. Data are shown as mean± SD, n=3, *= $p<0.05$ versus GM, #= $p<0.05$ versus OM.

OBJECTIVE 2: To investigate molecular mechanism of ATP/P2X7R activation in hMOBs in term of osteoblast differentiation regulation

BzATP had no effect on cell proliferation but decreased *in vitro* mineralization.

To determine the effect of ATP/P2X7R activation on osteoblast differentiation, hMOBs were cultured in OM with BzATP, a selective agonist of P2X7R. MTT assay showed that none of the concentrations of BzATP used in this study had an effect on proliferation or viability of hMOBs as assessed on day 1, 3 and 7 (Fig. 4.10A).

Next, we analyzed the effect of ATP/P2X7R activation on *in vitro* mineralization using Alizarin Red S staining. The results showed that BzATP inhibited *in vitro* mineralization in a dose-dependent manner (Fig. 4.10B and C). This result corresponded with the inhibitory effect of ATP in ATP-treated groups.

Because all concentrations of BzATP used in this experiment inhibited *in vitro* mineralization and none of them had an effect on cell proliferation, the highest concentration, 5 μM of BzATP was selected for further experiments.

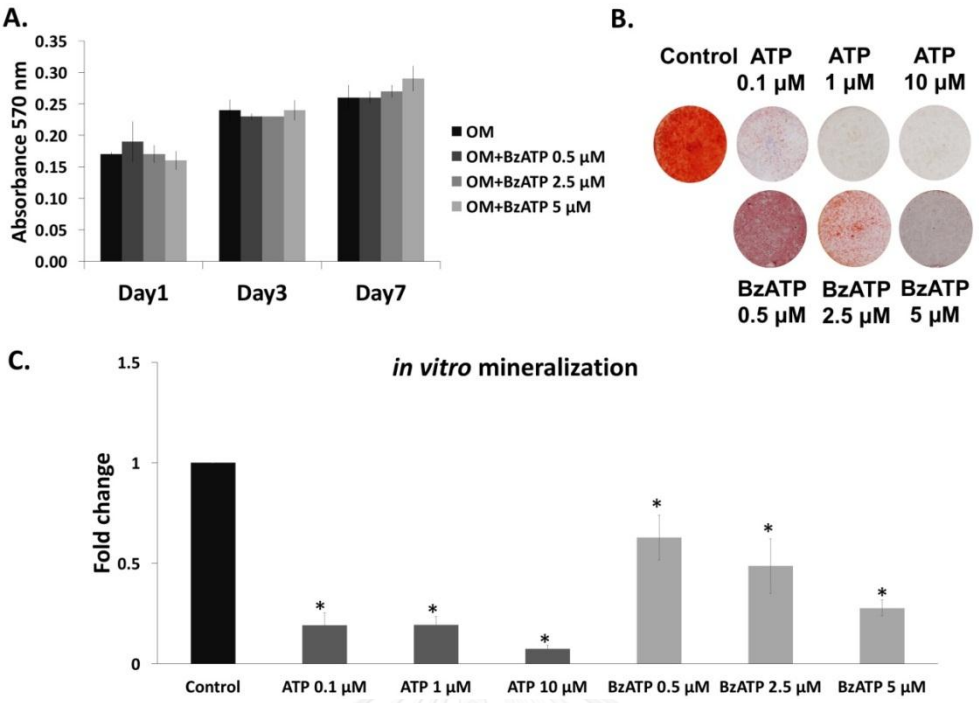


Figure 4.10 ATP/P2X7R activation regulated hMOB differentiation. The hMOBs were cultured in OM and treated with ATP (0.1, 1, or 10 µM), or BzATP (0.5, 2.5, or 5 µM). The hMOBs cultured in OM without ATP andBzATP served as control. (A) To investigate cell proliferation, MTT assay was performed at 1, 3, and 7 days. BzATP had no effect on cell proliferation. Data are shown as mean± SD, n=3, *=p<0.05 versus OM. (B) At day 14, *in vitro* mineralized nodules were stained with Alizarin Red S. (C) *In vitro* mineralization was quantified. ATP and BzATP inhibited *in vitro* mineralization in a dose-dependent manner. Data are shown as the mean± SD, n=3, *=p<0.05 versus OM.

BzATP decreased *WNT3A, RUNX2* and *OSX* mRNA expression.

The hMOBs were cultured in OM for 4 days in the presence of BzATP and the mRNA expression of *WNT3A, WNT5A, RUNX2* and *OSX* was assessed. BzATP significantly decreased the expression of these genes with the exception of *WNT5A* (Fig. 4.11).

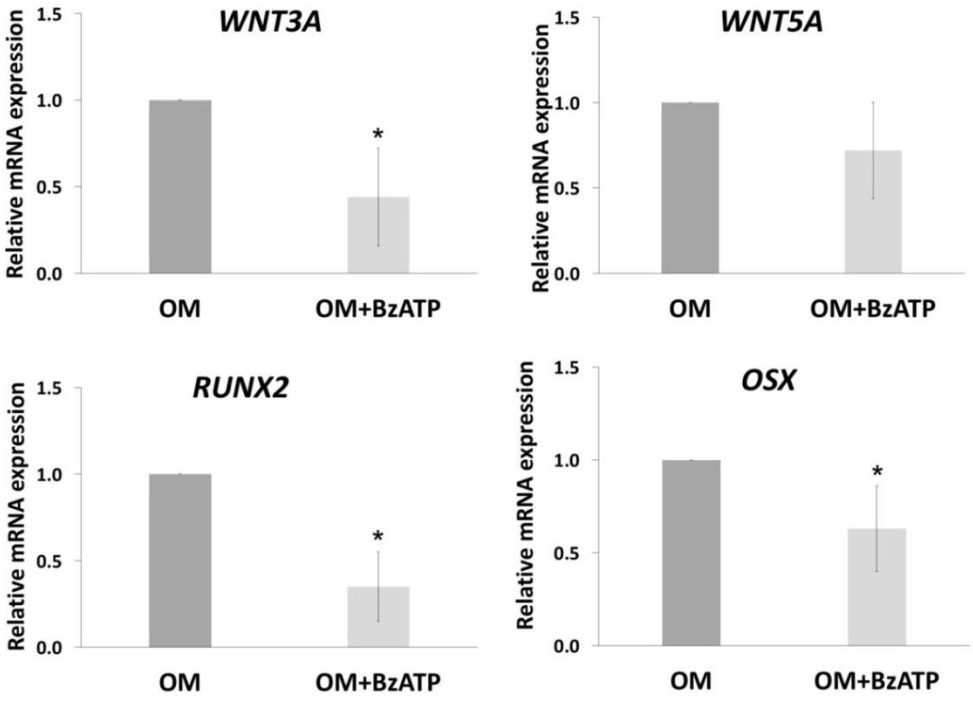


Figure 4.11 Effect of BzATP on *WNT3A, WNT5A, RUNX2,* and *OSX* mRNA expression. The hMOBs were cultured in OM with or without 5 μM of BzATP. The mRNA expression was assessed by qRT-PCR at day 4. *WNT3A, RUNX2,* and *OSX* expression in the BzATP-treated group significantly decreased. Data are shown as mean± SD, n=3, *=p<0.05 versus OM.

ATP/P2X7 activation regulated *WNT3A, RUNX2* and *OSX* mRNA expression.

To further assess the role of P2X7R activation in the reduction of expression of *WNT3A, WNT5A, RUNX2,* and *OSX,* siP2X7R was used to block synthesis of the P2X7R. The results demonstrated that siP2X7R reduced the level of P2X7R by 70%

(Fig. 4.12). While BzATP significantly increased the expression of *WNT3A*, *RUNX2* and *OSX* in hMOBs cultured in the presence of siP2X7R, there was no significant change in *WNT5A* expression.

To determine whether siP2X7R affects other P2 subtypes, the mRNA expression of *P2X1R* and *P2Y1R* investigated. The results demonstrated that the levels of *P2X1R* and *P2Y1R* mRNA expression was significantly increased in siP2X7R group (Fig. 4.13).

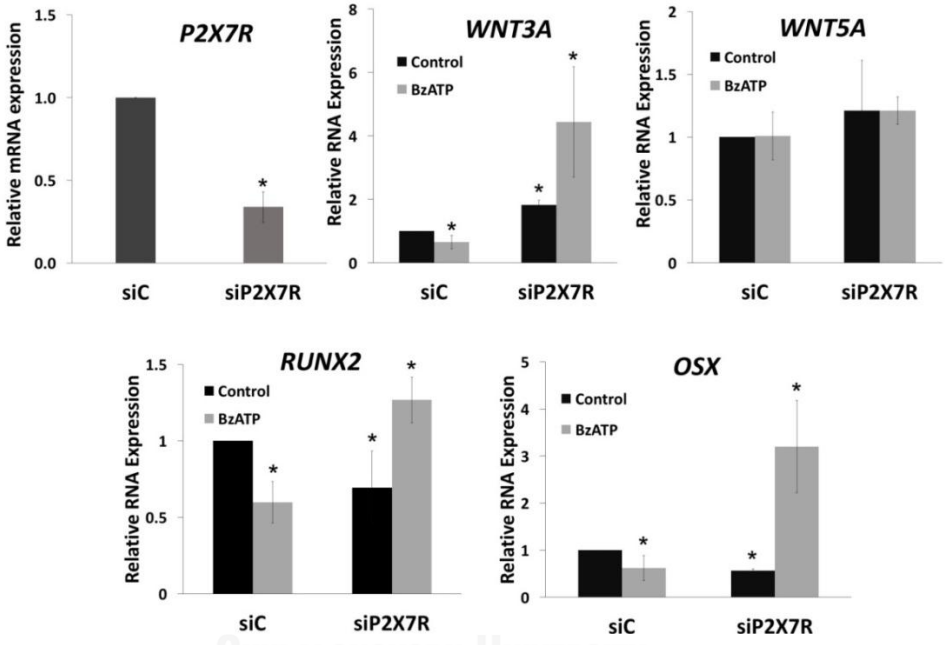


Figure 4.12 P2X7R synthesis was blocked using siP2X7R. The siP2X7R reduced the level of *P2X7R* by 70%. Although BzATP significantly increased *WNT3A, RUNX2*, and *OSX* mRNA expression in the siP2X7R-treated hMOBs, there was no significant change in *WNT5A* mRNA expression. Data are shown as mean± SD, n=3, *=$p<0.05$ versus siC in OM.

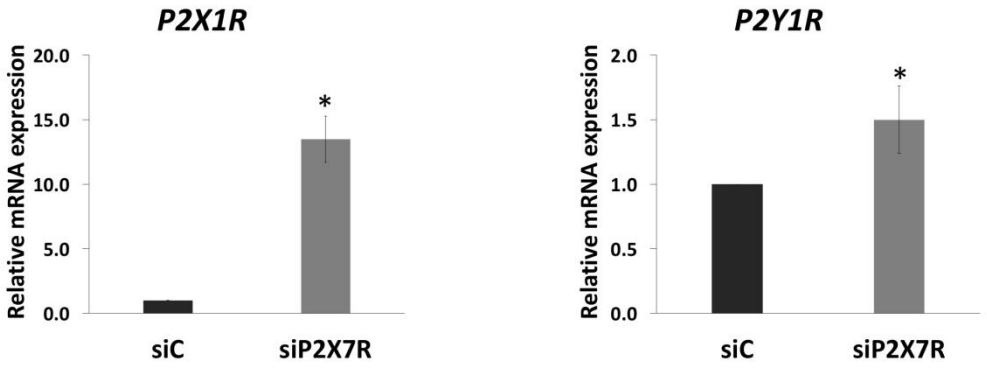


Figure 4.13 The effect of siP2X7R on *P2X1R* and *P2Y1R* mRNA expression. The siP2X7R significantly increased the level of *P2X1R* and *P2Y1R*. Data are shown as the mean± SD, n=3, *=p<0.05 versus siC.

BzATP prevented nuclear translocation of β-catenin.

The WNT/β-catenin signaling pathway can be activated by WNT3A. Under these conditions β-catenin is translocated from the cytoplasm to the nucleus. To investigate whether this also occurs with our hMOBs when cultured in OM, the presence of β-catenin was evaluated by immunofluorescence staining. Immune-localization showed that in hMOBs treated with BzATP, the level of nuclear β-catenin was strongly decreased (Fig. 4.14). BzATP prevented the nuclear translocation induced by OM. A similar effect was found in rhDKK1-treated hMOBs. These results demonstrated that BzATP inhibited the activation of WNT/β-catenin signaling pathway.

Exogenous rhWNT3A rescued the reduction of *RUNX2* and *OSX* by BzATP.

To investigate the role of WNT3A on the effect of BzATP, hMOBs were cultured in OM and then incubated with or without exogenous BzATP and rhWNT3A. The results demonstrated that rhWNT3A strongly stimulated the expression of *RUNX2* and *OSX*. The decreased expression of *RUNX2* and *OSX* induced by BzATP was entirely counteracted by rhWNT3A (Fig. 4.15A).

In addition to the increased expression of the osteogenic related genes, we found an increased *in vitro* mineralization in the BzATP-rhWNT3A-treated groups (Fig. 4.15 B and C).

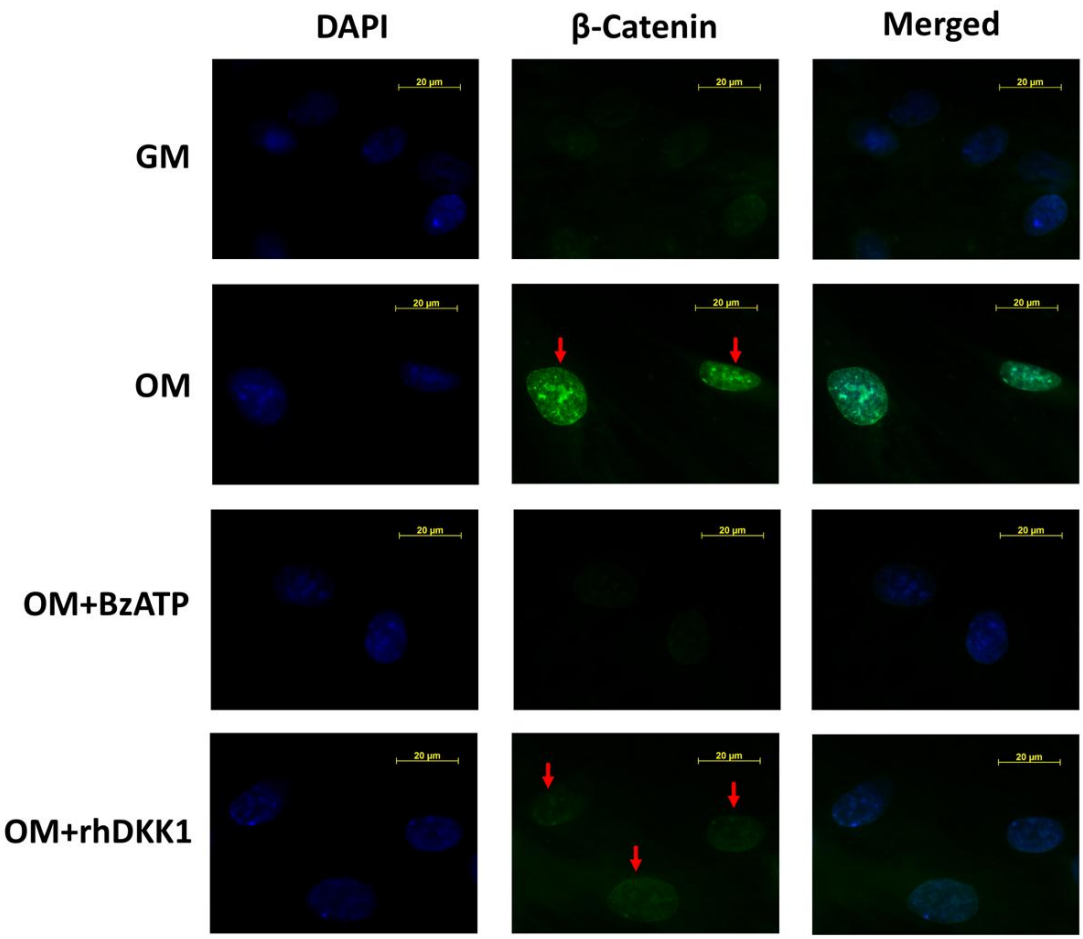


Figure 4.14 BzATP inhibited OM-induced β-catenin accumulation in nucleus. The hMOBs were cultured in GM or OM and incubated with/without BzATP (5 μM) or rhDKK1 (100 ng/ml) for 4 days. Immunofluorescence staining for β-catenin showed that OM induced translocation of β-catenin (indicated by arrows) into nucleus (stained by DAPI). The addition of BzATP and rhDKK1 inhibited nuclear translocation of β-catenin.

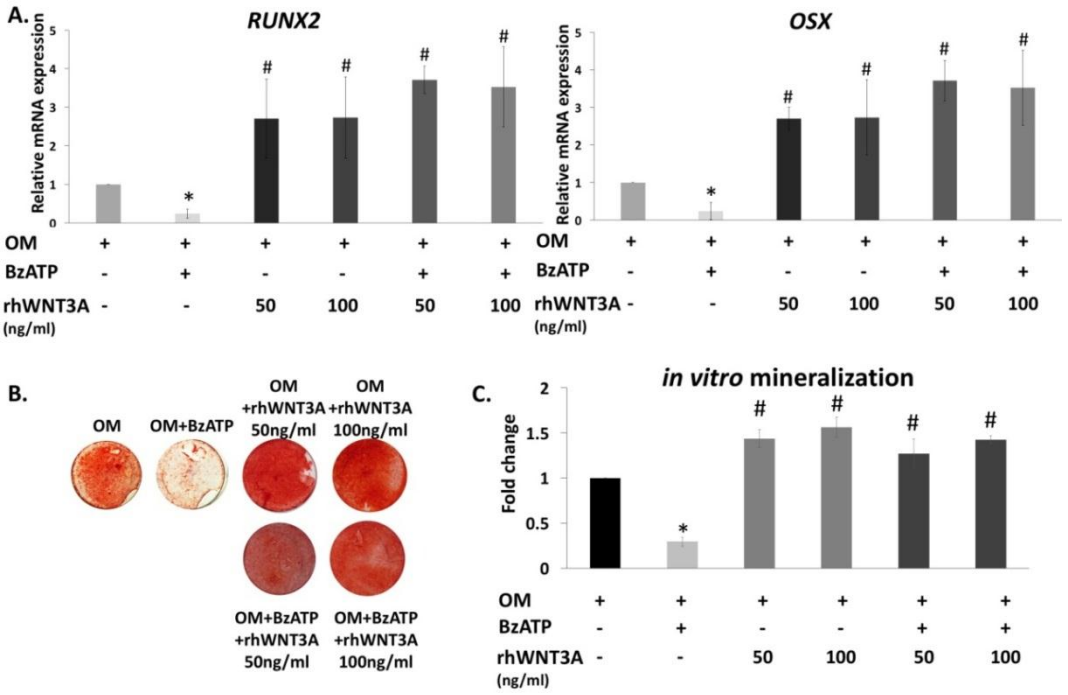


Figure 4.15 The BzATP-inhibited *RUNX2* and *OSX* expression was rescued by rhWNT3A. Human MOBs were cultured in OM and then incubated with5 μMof BzATP with or without exogenous 50 or 100 ng/ml rhWNT3A for 4 days. (A) The rhWNT3A strongly stimulated *RUNX2* and *OSX* expression in the BzATP-treated group. Data are shown as mean± SD, n=3, *=p<0.05 versus OM, #=p<0.05 versus OM with BzATP. (B) At 14 days, rhWNT3A increased *in vitro* mineralization in BzATP-treated hMOBs. (C) *In vitro* mineralization was quantified. The rhWNT3A increased *in vitro* mineralization in BzATP-treated ATP. Data are shown as the mean± SD, n=3, *=p<0.05 versus OM. #=p<0.05 versus OM with BzATP.

ICF altered P2X receptor mRNA expression.

To investigate whether ICF has an effect on all subtype P2X receptors, hMOBs were cultured in SFM and ICF was applied for 20 h. The results showed that while ICF significantly increased the expression of *P2X1R* and *P2X5R*, it reduced *P2X2R*, *P2X3R*, *P2X6R*, and *P2X7R*. These data suggested the effect of ICF on P2X receptor mRNA expression.

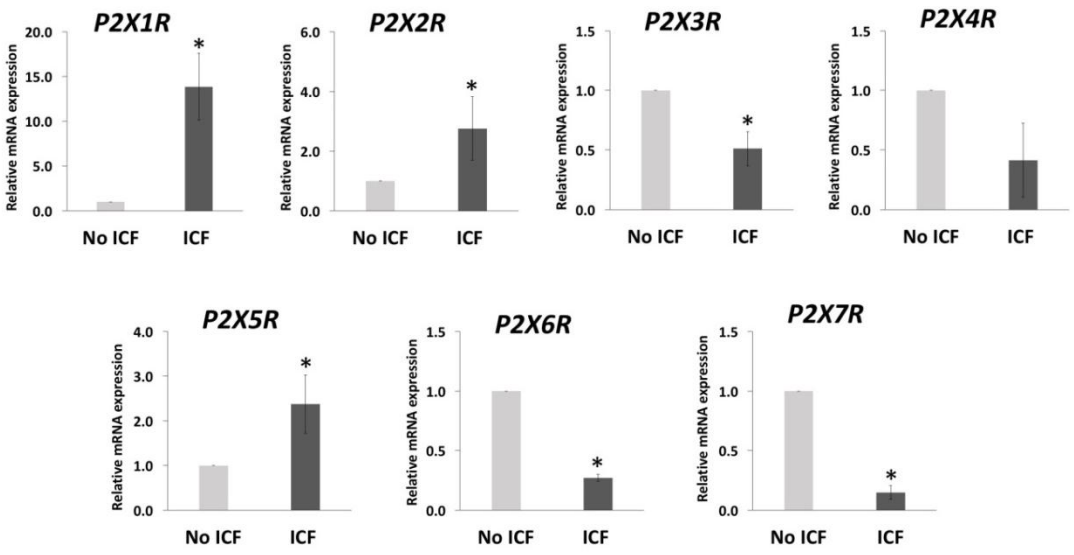


Figure 4.16 The effect of ICF on P2X receptor mRNA expression. The hMOBs were cultured in SFM and ICF were applied for 20h. The hMOBs without ICF were served as control. The mRNA expression of P2X receptors was assessed by qRT-PCR. Data are shown as mean ± Data are shown as the mean± SD, n=3, *=p<0.05 versus control.

CHAPTER V

DISCUSSION AND CONCLUSION

In this study, we demonstrated that ICF enhanced osteoblast differentiation in hMOBs. This enhancement was associated with ATP efflux induced by ICF. However, ATP/P2X7R activation using BzATP inhibited osteoblast differentiation in hMOBs. These results showed, for the first time, that while ICF-induced ATP enhanced WNT/β-catenin signaling, the P2X7R activation in hMOBs inhibited WNT/β-catenin signaling, resulting in reduced *RUNX2* and *OSX* expression, the essential genes in osteoblast differentiation.

Our results demonstrated that ICF significantly induced ATP efflux by hMOBs. Previous study have shown that different type of mechanical force increased ATP efflux by osteoblasts (Romanello, Pani et al. 2001, Hecht, Liedert et al. 2013, Orriss, Key et al. 2013, Kariya, Tanabe et al. 2015). We also demonstrated that 20-h ICF application enhanced *in vitro* mineralization and increased *OSX* and *ALP* mRNA expression. Study in MC3T3-E1 showed that tension force promoted osteogenesis by increasing Runx2 and Osx (Kariya, Tanabe et al. 2015).

Our data suggested that the enhancement of osteoblast differentiation by ICF was associated ATP efflux. This effect of ATP was corresponding with the study by Kariya, Tanabe et al. 2015. However, our data showed that when extracellular ATP (0.1-10 μM) was added without ICF, *in vitro* mineralization was inhibited (Fig. 4.10B and C). Previous studies also reported the inhibitory effect of extracellular ATP (Hoebertz, Mahendran et al. 2002, Orriss, Key et al. 2013). The contradictory results might be resulted from the doses of ATP used in those studies which were 10-100 μM. These dosed were much higher than mechanical force-induced ATP efflux (Romanello, Pani et al. 2001, Kariya, Tanabe et al. 2015). In addition, cultured cells were not subjected to any mechanical forces. Our results demonstrated that ICF could alter the expression of P2X receptors (Fig. 4.16). This alteration may lead to the changes of osteoblast characters and responses to ATP. Taken together, the data suggested the role of the combination of ATP efflux and mechanical force on osteoblast differentiation.

Studies about the enhancement effect of P2X7R on osteoblast differentiation have brought our attention to the role P2X7R in hMOBs (Ke, Qi et al. 2003, Li, Liu et al. 2005, Kariya, Tanabe et al. 2015, Grol, Brooks et al. 2016). Recent study also demonstrated that P2X7R activation prolonged and potentiated WNT/β-catenin signaling (Grol, Brooks et al. 2016). The authors suggested that the cross-talk between P2X7R and WNT/β-catenin signaling may modulate osteoblast activity in response to mechanical loading. Our results demonstrated for the first time that ICF promoted WNT/β-catenin signaling. Therefore, we hypothesized that ICF-induced ATP promoted osteoblast differentiation through P2X7R in hMOBs.

We found an upregulation of *WNT3A* mRNA expression in hMOBs cultured in OM at day 1 and 4 (Fig.4.9A). This increased expression coincided with increased *RUNX2* and *OSX* mRNA expression. The rhDKK1, a canonical WNT-signaling inhibitor, inhibited OM-induced *RUNX2* and *OSX* mRNA expression. These results strongly suggested the role of WNT3A in the upregulation of these two osteogenic genes. In further experiment, BzATP down-regulated *WNT3A* mRNA expression. Immunohistochemistry confirmed that P2X7R activation by BzATP inhibited nuclear translocation of β-catenin, an important canonical WNT signaling mechanism. Our findings suggested that P2X7Ractivation inhibited hMOB differentiation by down-regulating the canonical WNT signaling pathway.

The inhibitory effect of P2X7R activation on mineralization by hMOBs we observed agrees with the results obtained from primary rat and human osteoblasts (Hoebertz, Mahendran et al. 2002, Orriss, Key et al. 2012, Agrawal, Henriksen et al. 2017). BzATP significantly decreased ALP activityin primary rat calvarial osteoblasts at 14 days (Orriss, Key et al. 2012) and reduced mineralized nodule in primary human trabecular osteoblas (Agrawal, Henriksen et al. 2017). Previous study demonstrated that ATP and BzATP can be hydrolyzed by ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), resulting in increased extracellular pyrophosphate (PPi), a potent apatite formation inhibitor (Orriss, Key et al. 2012). Therefore, the inhibition of mineralization by BzATP may also occur via increased PPi. The effects of BzATP and phosphate generating enzymes cannot be excluded. However, the regulation of phosphate metabolism is complex. The participation of

other genes, e.g. progressive ankylosis protein homolog (ANKH), and sodium-dependent Pi symporter 1 (PIT1), could also influence the extracellular phosphate/pyrophosphate ratio, leading to changes in the final molecular and cell response (Orriss, Arnett et al. 2016). Therefore, the interaction of BzATP-P2X7 and BzATP-phosphate generating enzyme pathway in hMOBs requires further investigation.

Using P2X7R siRNA, we determined that the reduced *WNT3A*, *RUNX2*, and *OSX* expression resulted from P2X7R signaling. Interestingly, under these conditions, BzATP increased *WNT3A*, *RUNX2*, and *OSX* expression. This finding suggested, in addition to P2X7R, the existence of an alternative signaling pathway induced by BzATP. BzATP has been reported to partially activate P2X1 (P2X1R) and P2Y1 receptor (P2Y1R) (Zhong, Dunn et al. 1998); however, these receptors differentially affected osteoblast differentiation (Orriss, Syberg et al. 2011, Orriss, Key et al. 2012). Moreover, our results indicated that *P2X1R* and *P2Y1R* mRNA expression significantly increased in cells transfected with siP2X7R (Fig. 4.13). Therefore, we hypothesize that when P2X7R expression is reduced, BzATP can alternatively activate P2X1R or P2Y1R, resulting in increased osteogenic gene expression.

Interestingly, P2X7R activation was recently found to have a stimulating, rather than an inhibiting, effect on *in vitro* mineralization (Rodrigues-Ribeiro, Alvarenga et al. 2015) and WNT/β-catenin signaling (Grol, Brooks et al. 2016). BzATP significantly increased ALP activity at day 7 in a preosteoblastic MC3T3-E1 cell line cultured in OM, and these results were attenuated by AZ11645373, a P2X7R antagonist. (Rodrigues-Ribeiro, Alvarenga et al. 2015). The combination of Wnt3a and BzATP elicited more sustained β-catenin nuclear localization and transcriptional activity than those induced by Wnt3a alone (Grol, Brooks et al. 2016). An important difference between those studies and ours is the use of cells from different species and origin. In previous studies, a mouse cell line was used, while we analyzed primary human osteoblasts. Furthermore, MC3T3-E1 cells are derived from the calvaria, while hMOBs were explanted from the mandible. Given these important differences, man versus mouse and primary cells versus a cell line, we assume that the response of these cells is likely to be different.

Our results are in contrast to previous findings. Gartland et al. and Panupinthu et al. showed that P2X7R activation in osteoblasts generated pore formation and plasma membrane blebbing, resulting in apoptosis. (Gartland, Hipskind et al. 2001, Panupinthu, Zhao et al. 2007). However, an important difference between their studies and the present study is the concentration of BzATP used. We cultured the cells with 0.5–5 μM BzATP, whereas the studies mentioned above used 300–1000 μM BzATP; concentrations up to 200 times higher than those used in our study. We assume that these high concentrations of BzATP negatively affect the cells and induce apoptosis.

Several studies have demonstrated the effects of P2X7R activation on osteoblast differentiation (Panupinthu, Rogers et al. 2008, Orriss, Key et al. 2012, Kariya, Tanabe et al. 2015, Rodrigues-Ribeiro, Alvarenga et al. 2015). The present study demonstrated that BzATP reduced osteogenic marker gene expression and P2X7R knockdown abolished these effects. These results confirm the participation of the BzATP-P2X7R pathway in osteoblast differentiation. In addition, our data are the first to show a relationship between P2X7R activation and canonical WNT-signaling in human osteoblasts. Therefore, we propose a model of the effect of P2X7R activation on hMOB differentiation (Fig. 5.1). When ICF is applied on hMOBs, the expression of *WNT3A* increases, which leads to β-catenin nuclear translocation. Subsequently, *RUNX2* and *OSX* transcription is induced. However, when P2X7R is activated, β-catenin nuclear translocation and *RUNX2* and *OSX* upregulation will be inhibited, thus inhibiting hMOB differentiation.

In conclusion, our findings demonstrate that ICF-induced ATP enhances osteoblast differentiation in hMOBs. This effect suggests the role of purinergic receptors in osteoblast differentiation. However, ATP/P2X7R activation inhibits human osteoblast differentiation. These results demonstrate the influence of ICF and ATP/P2X7R signaling in regulating bone formation, especially in alveolar bone. Further study about the intracellular signaling in human osteoblasts induced by ICF, ATP and purinergic 2 receptors will provide valuable knowledge for alveolar bone regeneration.

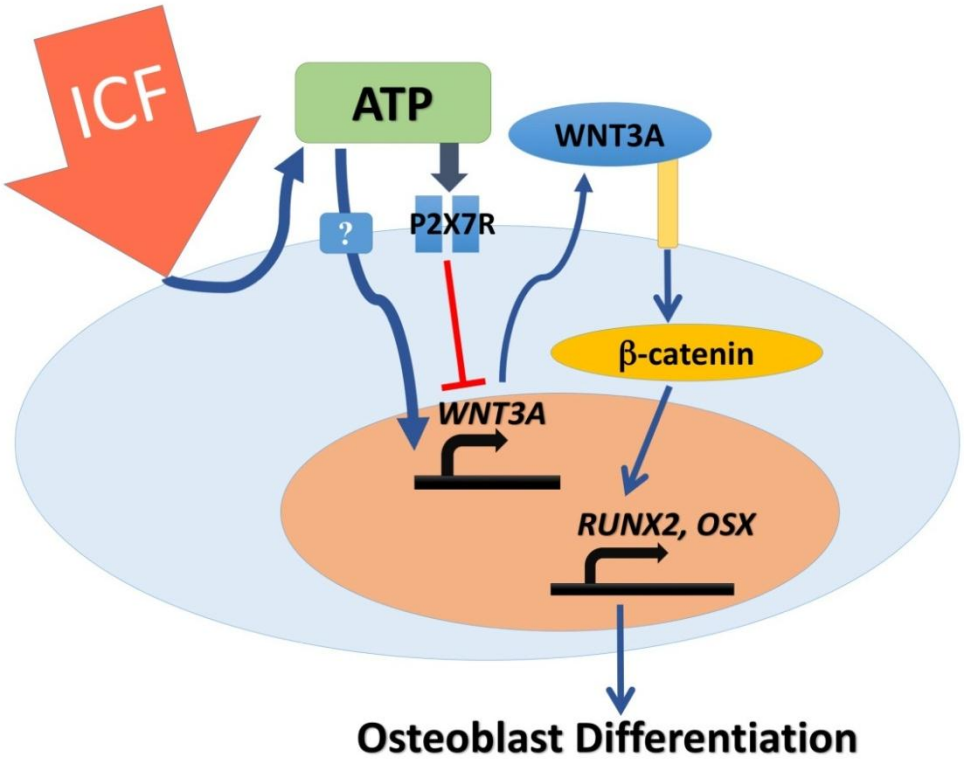


Figure 5.1 Schematic of our proposed mechanism. When hMOBs are subjected to ICF, *WNT3A* expression increases, leading to β-catenin nuclear translocation. Subsequently, *RUNX2* and *OSX* transcription is induced. However, when P2X7R is activated by BzATP a specific P2X7R agonist, this process is inhibited, reducing hMOB differentiation.

APPENDIX

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