การแสดงออกของเอ็มอาร์เอ็นเอของน็อทช์ในเซลล์โพรงประสาทฟันของมนุษย์ที่ได้รับแรงกด

Expression of *NOTCH* mRNA in human dental pulp cells subjected to mechanical compressive force

โดย

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หทัยชนก เจริญพงศ์ 2566: การแสดงออกของเอ็มอาร์เอ็นเอของน็อทช์ในเซลล์โพรงประสาทฟันของมนุษย์ที่ได้รับแรงกด วิทยาลัยทันตแพทยศาสตร์ มหาวิทยาลัยรังสิต 53 หน้า

ในหลายๆครั้ง เซลล์โพรงประสาทฟันมนุษย์สามารถถูกแรงกดได้ ซึ่งแรงกดสามารถทำให้เกิดผลต่างๆต่อเซลล์โพรงประสาทฟันมนุษย์ได้ แต่กลไกการตอบสนองของเซลล์โพรงประสาทฟันมนุษย์ต่อแรงกดนั้นยังไม่ทราบชัดเจน การศึกษานี้จึงทำขึ้นเพื่อศึกษาการแสดงออกของเอ็มอาร์เนเอของยีนส์เป้าหมายของนอทช์ไดแก่ *HES1* และ *HEY1* รวมถึงการแสดงออกของเอ็มอาร์เนเอของตัวรับของนอทช์ไดแก่ *NOTCH1*, *NOTCH2*, *NOTCH3* and *NOTCH4* ในเซลล์โพรงประสาทฟันมนุษย์ที่ได้รับแรงกด โดยใช้วิธีการให้แรงกดโดยตรงและการให้แรงกดผ่านแรงดันน้ำ

ผลการศึกษาพบว่าเอ็มอาร์เนเอของ *HES1* เพิ่มขึ้นในเซลล์โพรงประสาทฟันมนุษย์ที่ได้รับแรงกดทั้งสองแบบหลังให้แรงไป 2 ชั่วโมง โดยการผ่านแรงดันน้ำสามารถพบการเพิ่มขึ้นของทั้ง HES1 และ *HEY1* ที่ 6 ชั่วโมงด้วย แต่เมื่อให้แรงกดต่อไปจนถึง 24 ชั่วโมงปรากฏว่าไม่พบการเพิ่มขึ้นของยีนส์เป้าหมายทั้งสองของนอทช์แล้ว สำหรับตัวรับของนอทช์นั้นพบว่ามีเฉพาะ *NOTCH2* ที่เพิ่มขึ้น ซึ่งพบการเพิ่มขึ้นที่ 6 ชั่วโมงหลังให้แรงกดด้วยแรงดันน้ำ โดยสรุปแล้ว แรงกดสามารถเพิ่มแสดงออกของเอ็มอาร์เนเอของของยีนส์เป้าหมายของนอทช์ในเซลล์โพรงประสาทฟันมนุษย์ โดยการกดผ่านแรงดันน้ำเพิ่มการแสดงออกของ *HEY1* และ *NOTCH2* ด้วย อย่างไรก็ตามการเพิ่มขึ้นของยีนส์เป้าหมายของนอทช์นี้พบเพียงระยะหนึ่งเท่านั้น ถึงแม้จะให้แรงกดต่อเนื่อง

คำสำคัญ: โพรงประสาทฟัน; แรงทางกล; แรงกด; สัญญาณนอทช์

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Dental pulp cells encounter compressive force in various situations. Mechanical force can produce various effects on dental pulp cells but the mechanisms underlying dental pulp response to mechanical force are still unclear. In this study, we investigated mRNA expression of Notch target genes, *HES1* and *HEY1*, as well as Notch receptors, *NOTCH1*, *NOTCH2*, *NOTCH3* and *NOTCH4*, in human dental pulp cells (HDPCs) subjected to mechanical compressive force. We utilized two in vitro compressive force application models, direct compression and hydrostatic compression.

The results showed that there was an upregulation of Notch target gene, *HES1*, in HDPCs subjected to compressive force generated by both models for 2 hours. Hydrostatic compression also upregulated *HES1* and *HEY1* mRNA expression following 6 hours of force application. However, at 24 of force application, no upregulation of Notch target was observed. *NOTCH2* was the only Notch receptor found to be upregulated in HDPCs following compressive force application in which the upregulation was observed at 6 hours after hydrostatic compression. In conclusion, compressive force can upregulate the mRNA expression of Notch target gene, *HES1*, in HDPCs. Hydrostatic compression also upregulated *HEY1* and *NOTCH2*. However, upregulation of mRNA of Notch targets was transient, although prolong compressive force was delivered to HDPCs.

Key words: Dental pulp cells; mechanical force; compressive force; Notch signaling

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Chapter 1

Introduction

Rationale and background

Dental pulp cells can be subjected to mechanical force in many situations. Since dental pulp is a soft tissue surrounded by hard tissue of the teeth, when the teeth are displaced such as during mastication or orthodontic treatment, mechanical force can be generated to dental pulp cells due to the compression of dentin wall onto pulp tissue. Fluid movement in dental pulp following tooth movement can also produce mechanical shear force to dental pulp cells (Koolstra, 2002; Su et al., 2014). Furthermore, dental pulp cells can subject mechanical force during pulpal inflammation. Because of physical constraint of dental pulp, it was reported that intrapulpal pressure of inflamed pulp is approximately 3 times higher that of normal pulp (Heyeraas & Berggreen, 1999). Tooth preparation, orthodontic force, dental caries, trauma and parafunctional habits can induce pulpal inflammation, thus, can generate mechanical stress to dental pulp cells.

Mechanical stress was reported to have various effect on dental pulp cells including cell proliferation, osteogenic and odontogenic differentiation (Marrelli et al., 2018). It was also reported that mechanical force reduced survival and adhesion of dental pulp cells (Marrelli et al., 2018; Yu et al., 2009). In addition, some studies have demonstrated the possible relationship between mechanical stress-induced dental pulp cells and subsequent stimulation of osteoclasts (Charoenpong & Ritprajak, 2021; Wang et al., 2017) which might be the mechanisms underlying root resorption.

Notch signaling is the evolutionarily conserved pathway that involve in various biological processes during prenatal and postnatal development as well as in adult tissues. Notch receptors, Notch ligands and Notch target genes were expressed in dental pulp and were upregulated following pulpal injuries (Lovschall et al., 2005; Mitsiadis et al., 1999). Notch signaling was also reported to invlove in proliferation and osteogenic/odontoblasitc differentiation of dental pulp cells (Hansamuit et al., 2020; He et al., 2009; Sun et al., 2010; Wang et al., 2011).

The association between Notch signaling and mechanical force was reported in many previous studies. It has been reported that shear stress can induce expression of various Notch signaling components in endothelial cells and limbal epithelial stem cells (Jahnsen et al., 2015; Kang et al., 2014; Mack et al., 2017; Masumura et al., 2009). Mechanical strain was reported to affect notch signaling components in vascular smooth muscle cells and umbilical vein endothelial cells (Loerakker et al., 2018; Morrow et al., 2007; Morrow et al., 2005). Compressive force was found to up-regulate *NOTCH1* mRNA expression in human deciduous dental pulp cells (Peetiakarawach et al., 2015). However, in adult dental pulp cells, the relationship between notch signaling and mechanical force is still lacking. Therefore, this study aimed to investigate expression of Notch signaling components in human dental pulp cells in response to mechanical force.

Objectives

This study aimed to investigate the effect of mechanical compressive force on mRNA expression of Notch target genes and Notch receptors in human dental pulp cells.

Research question

1. Does compressive affect Notch target genes mRNA expression of in human dental pulp cells?
2. Does compressive affect Notch receptors mRNA expression of in human dental pulp cells?

Research hypothesis

1. H_0: Mean fold change in mRNA expression of *HES1* in human dental pulp cells receiving mechanical compressive force is not different from that of control cell without force application.

   H_1: Mean fold change in mRNA expression of *HES1* in human dental pulp cells receiving mechanical compressive force is different from that of control cell without force application.

2. H_0: Mean fold change in mRNA expression of *HEY1* in human dental pulp cells receiving mechanical compressive force is not different from that of control cell without force application.

   H_1: Mean fold change in mRNA expression of *HEY1* in human dental pulp cells receiving mechanical compressive force is different from that of control cell without force application.

3. H_0: Mean fold change in mRNA expression of *NOTCH1* in human dental pulp cells receiving mechanical compressive force is not different from that of control cell without force application.

   H_1: Mean fold change in mRNA expression of *NOTCH1* in human dental pulp cells receiving mechanical compressive force is different from that of control cell without force application.

4. H_0: Mean fold change in mRNA expression of *NOTCH2* in human dental pulp cells receiving mechanical compressive force is not different from that of control cell without force application.

   H_1: Mean fold change in mRNA expression of *NOTCH2* in human dental pulp cells receiving mechanical compressive force is different from that of control cell without force application.

5. H_0: Mean fold change in mRNA expression of *NOTCH3* in human dental pulp cells receiving mechanical compressive force is not different from that of control cell without force application.

H_1: Mean fold change in mRNA expression of *NOTCH3* in human dental pulp cells receiving mechanical compressive force is different from that of control cell without force application.

6. H_0: Mean fold change in mRNA expression of *NOTCH4* in human dental pulp cells receiving mechanical compressive force is not different from that of control cell without force application.

   H_1: Mean fold change in mRNA expression of *NOTCH4* in human dental pulp cells receiving mechanical compressive force is different from that of control cell without force application.

Chapter 2

Review literatures

Dental pulp tissue

Dental pulp is a soft tissue residing within pulp cavity of the tooth (Figure 1). Dental pulp derives from mesenchymal origin. It is a highly vascularized tissue with high sensory innervation. Dental pulp is composed of collagenous and argyrophilic fibers, gelatinous ground substance, blood vessels, nerve and cellular components (Mjor & Heyeraas, 2008).

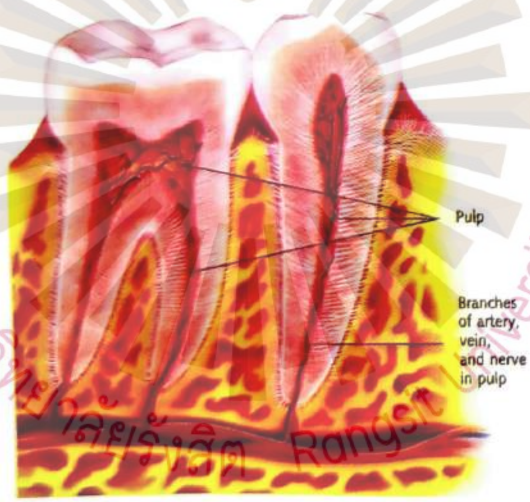

Figure 1 Dental pulp tissue (Picture from Mjor & Heyeraas, 2008)

Various cell types can be found in dental pulp including odontoblasts, the dentin forming cells. Odontoblasts are tall columnar cells lying adjacent to dentin wall (Figure 2) (Arana-Chavez & Massa, 2004; Dabas & Dabas, 2007; Kawashima & Okiji, 2016; Mjor & Heyeraas, 2008; Yu & Abbott, 2007). Beside from odontoblasts, the majority of the cells in dental pulp are pulpal fibroblasts and undifferentiated cells (Dabas & Dabas, 2007; Mjor & Heyeraas, 2008; Yu & Abbott, 2007).

Occasionally, macrophage, dendritic cells and mast cells also present in dental pulp (Dabas & Dabas, 2007; Mjor & Heyeraas, 2008).

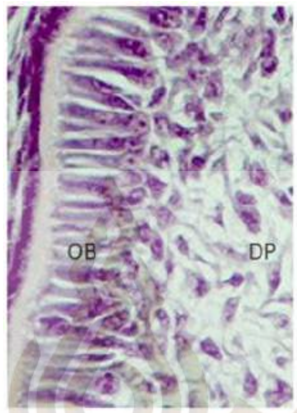

Figure 2 Odontoblasts, the dentin forming cells. (Picture from Kawashima & Okiji, 2016)

Pulpal fibroblast and undifferentiated cells are stellate shape with large nuclei and little cytoplasm (Figure 3). Because of the similarity in morphology, pulpal fibroblast and undifferentiated cells are difficult the distinguish from each other (Dabas & Dabas, 2007). However, the undifferentiated cells have the ability to develop into many cell types including odontoblasts, fibroblasts, adipocytes and neural-like cells (Dabas & Dabas, 2007; Gronthos et al., 2002; Nakashima et al., 2013).

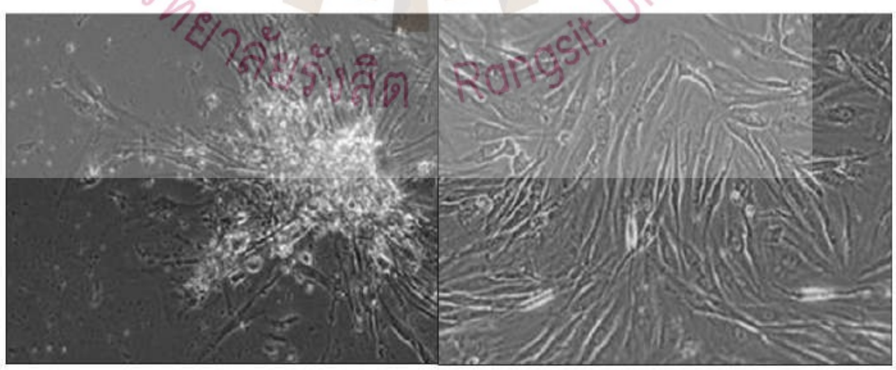

Figure 3 Mesenchymal cells of dental pulp. Left: explant stage, right: confluence stage. (Picture from Shekar & Ranganathan, 2012)

In vitro isolation of dental pulp cells by explant method gives rise to the mixed population of dental pulp stem cells. These cells are highly proliferative. They exhibit self-renewal potential and have the ability to differentiate various cell types (Nakashima et al., 2013).

Histomorphologically, dental pulp can be divided into 4 zones (Dabas & Dabas, 2007; Mjor & Heyeraas, 2008) (Figure 4). The first zone adjacent to dentin is odontoblastic zone which contains the single layer of odontoblasts. Nerve fiber and some dendritic cells may also present within odontoblastic layer. Adjacent to odontoblastic layer into pulpal side is the cell poor zone which is a narrow zone with approximately 40 um width. This zone is relatively cell-free and may not be apparent in some pulps. Cell rich zone locates pulpally to cell free zone. It is composed of mainly of pulpal fibroblasts although some immune cells can also be found. The innermost of the pulp is pulp proper. This zone has large blood vessels and nerves supply and also contains many cells (Dabas & Dabas, 2007; Mjor & Heyeraas, 2008) (Figure 4).

Figure 4 Four zones of dental pulp. O=odontoblasts, CF=cell-free zone, CR=cell rich zone, BV=blood vessel (picture from Dabas & Dabas, 2007)

Notch signaling

Notch signaling pathway is a highly conserved pathway first discovered a century ago (Borggrefe & Oswald, 2009). Notch signaling provide a simple cell-cell communication that plays important roles during development and throughout life to maintain tissue homeostasis (Bi & Kuang, 2015). Notch signaling regulates cellular response in highly context specific (Bi & Kuang, 2015; Henrique & Schweisguth, 2019). It was reported to involve in a wide range of biological response including cell fate determination, cell proliferation, cell survival, cell differentiation and maintaining stemness of cells (Bi & Kuang, 2015; Henrique & Schweisguth, 2019).

Notch signaling mainly consists of Notch receptors, Notch ligands and intracellular proteins that convey the signal into nucleus (Bi & Kuang, 2015). In mammals, four Notch receptors and five Notch ligands have been identified. NOTCH1, NOTCH2, NOTCH3 and NOTCH4 are the known Notch receptors. They are single-pass transmembrane proteins composed of Notch extracellular domain (NECD), transmembrane domain (TM), and Notch intracellular domain (NICD) (Figure 5). Five Notch ligands identified in human are also single pass transmembrane proteins that are in DSL (Delta/Serrate/LAG-2) family proteins. The know Notch ligands are Delta-like (DLL) 1, DLL3, DLL4 and Jagged (JAG) 1, JAG2.

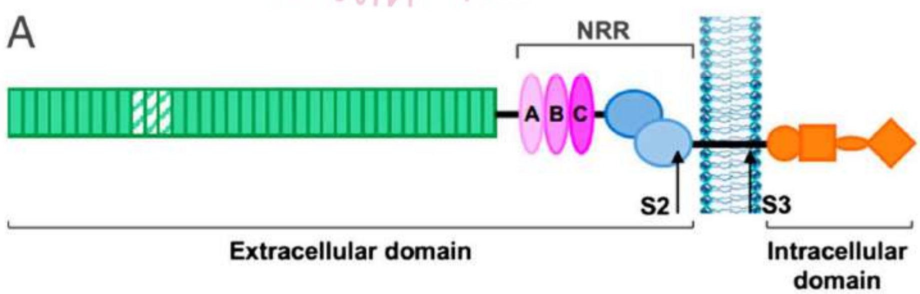

Figure 5 Diagram illustrating structure of Notch receptor (picture from Stephenson & Avis, 2012)

Notch signaling is activated in juxtacrine manner upon binding of Notch ligand to its receptor on neighboring cell. Binding of Notch ligand to its receptor leads to conformation changes of Notch receptor unfolding the juxtamembrane negative control region (NRR) (Kopan, 2012) (Figure 5). This allows ADAM10, an ADAM family metalloprotease, to cleave Notch receptor at S2 cleavage site releasing Notch extracellular domain (Bi & Kuang, 2015; Henrique & Schweisguth, 2019; Kopan, 2012) (Figure 5 and 6). Following S2 cleavage by ADAM10, γ-secretase then cleaves NOTCH at S3 cleavage site within its transmembrane domain resulting in the release of Notch intracellular domain (Henrique & Schweisguth, 2019; Kopan, 2012) (Figure 5 and 6). Notch intracellular domain then translocates into nucleus where it binds to DNA binding protein, RBPJ (recombination signal binding protein for immunoglobulin kappa j region, also known as CSL in mammals), and recruits other factors to form a transcriptional complex that activate transcription of Notch targets genes including Hairy/enhancer-of-split (HES) and Hes related with YRPW motif protein (HEY) family genes (Bi & Kuang, 2015; Henrique & Schweisguth, 2019; Wang et al., 2015) (Figure 6).

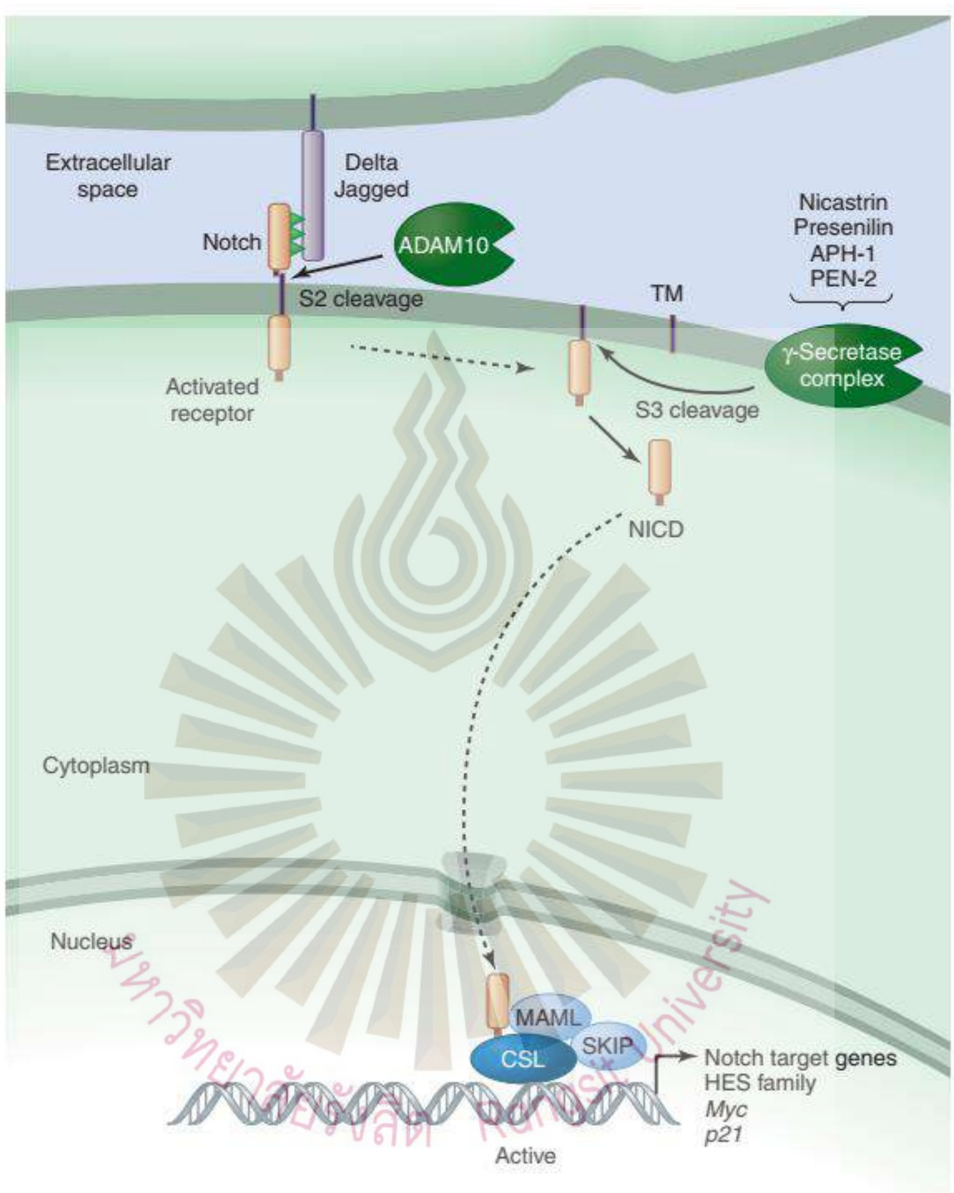

Figure 6 Notch signaling pathway (picture from Kopan, 2012)

Notch signaling in dental pulp

Mitsiadis et al. (1999) studied in 12-month-old male Wistar rats and reported that Notch1, Notch2 and Notch3 cannot be detected in normal pulp. However, when there is an injury, Notch expression was upregulated. Expression of Notch1 expression was weak and restricted to few pulpal cells close to injury site while Notch2 and Notch3 expressed more intensely in injured dental pulp. Expression of Notch2 after injury was found in mesenchymal cells of the pulp close to and also at a distance from injury sites. Notch3 expression was observed mainly along with vascular structures. While all of Notch receptors investigated were not observed in odontoblastic cells, expression of Notch ligand, Delta1, was found to upregulate in odontoblasts of injured teeth and also in vascular structures.

Lovschall et al. (2005) studied Notch signaling components in dental pulp of adult rat molars after pulp capping with calcium hydroxide. Expression of Notch1 increased at the area close to injury site, in sub-odontoblast zone and in a few perivascular cells. Notch2 expression increased in pulpal stroma inside coronal odontoblasts and in the whole root pulp. Increase expression of Notch3 was found mainly in perivascular cell groups and some expression was observed in the stroma adjacent to injury site. All Notch receptors were rarely detected in odontoblast layer along the dentin wall. Expression of Notch ligands, Delta1 and Jagged1 was very low in dental pulp. Slightly increase in expression of these Notch ligands was observed following pulpal injury. Notch target gene, Hes1, was expressed close to the lesion and along the adjacent dentin walls. Hes5 was not observed in dental pulp. Expression of all the Notch receptors, ligands and target genes, Hes1, were increased on day 1 after pulp injury and tended to decrease on day 3.

Sun et al. (2010) found that primary rat dental pulp cells expressed Notch receptors, Notch1, Notch2 and Notch3 but not Notch 4. Notch target genes, Hes1 and Hey1 were also found to express in rat dental pulp cells. When inducing these cells to differentiate into odontoblasts, it was found that Hey1 was significantly down-regulated during odontoblastic differentiation indicating that Hey1 may be the negative regulator in odontoblastic differentiation of dental pulp cells.

X. Wang et al. (2011) showed that Delta1 was expressed in human dental pulp cells. This study also investigated the effect of Delta1 on proliferation and differentiation of human dental pulp cells by knocking down Delta1 using lentivirus-mediated Delta1-RNAi. The results showed that cell proliferation was significantly suppressed in Delta1 knocked-down dental pulp cells. Odontoblastic differentiation, on the other hand, was enhanced in Delta1 knocked-down dental pulp cells.

Hansamuit et al. (2020) reported that human dental pulp cells significantly upregulated Notch target genes, HES1 and HEY1 when seeded on Jagged1 immobilization surface. Dental pulp cells seeded on Jagged1 immobilization surface and maintained in osteogenic induction medium for 14 days showed an increase in mineral deposition comparing to dental pulp cells seeded on hFc immobilized control surface. These results indicated that Jagged 1 increase notch signaling and promote osteogenic differentiation of human dental pulp cells.

He et al. (2009) found that Notch receptor, NOTCH1 and NOTCH2 as Notch ligand, Delta1, were expressed in human dental pulp cells throughout the process of cellular proliferation and differentiation. Expression of NOTCH1, NOTCH2 and Delta1 were located in cell membrane and/or in nucleus. By overexpression of Delta1, it was found that Delta1 could significantly enhance proliferation and odontoblastic differentiation of human dental pulp cells. It was also found that overexpression of Delta1 led to an increase in Notch target gene, HES1, expression but not Deltex.

Notch signaling and mechanical force

Many studies reported the association between Notch signaling and cellular response to mechanical force. Obi et al. (2009) found that endothelial progenitor cells (EPCs) derived from human peripheral blood upregulated *NOTCH1/3*, *HEY1/2* mRNA expression when exposing to shear stress produced by flow-loading device for 6 and 24 hours.

Jahnsen et al. (2015) studied using the lamina flow to create shear stress to human abdominal aortic endothelial cells (HAAEC). It was found that low level of shear stress induced expression of NOTCH1 while high level of shear stress (more than 10 dynes/cm2) did not. Low level of shear stress also induced expression of DLL1, DLL4, JAG1 and HEY1 but not HEY2 and NOTCH4 in these cells. Furthermore, by knocking down of NOTCH1, the results indicated that flow-induced expression of DLL4 and HEY1 as well as Nrp1 and EphB4, the arterial and venous marker genes, depended on NOTCH1.

The study by Kang et al. (2014) in limbal epithelial stem cells (LESCs) subjected to flow-induced shear stress found that there was a transient upregulation of Notch-1 (and p63) gene expression in response to intermittent flow. Steady flow, however, did not produce changes in expression of this gene. The upregulation of Notch-1 in response to intermittent flow was transient; the significant upregulation of the gene was observed shortly (2 days) after application of shear force but decreased thereafter (4 days later).

Mack et al. (2017) studied in human aortic endothelial cells (HAECs) and reported that *NOTCH1* transcript increased significantly after 12 hours exposing to shear stress. Notch1 target genes, HES1, NRARP, and FABP4 transcript level also increased in response to shear stress. Blocking NOTCH1 blocked the increase in NOTCH1 and its target genes. NOTCH1 protein expression was also significantly increased after 24 hours exposing to shear stress. Furthermore, it was found that NOTCH1 is essential for endothelial cells response to shear stress which affect cell–cell junctions, cell polarity, and cell proliferation.

The study in murine embryonic stem cells (ES) cell–derived VEGFR2+ cells by Masumura et al. (2009) found that Notch receptors, Notch1 and Notch4, as well as Notch ligand, Dll4, Jagged1 and Jagged 2, increased in response to mechanical shear stress (10 dynes/cm2) in both mRNA and protein level. Nuclear translocation of Notch intracellular domain also increased in time-dependent manner when these cells were exposed to shear stress. This activation of notch signaling was required to induce expression of arterial cells marker gene, ephrineB2, in response to shear stress.

Peetiakarawach et al. (2015) studied using computerized cell compressive force loading apparatus to apply compressive force to human deciduous dental pulp cells. *NOTCH*1 mRNA expression was significantly upregulated in human deciduous dental pulp cells exposing to mechanical compressive for of 2 g/cm2 for 2 hours compared to control without force application.

Loerakker et al. (2018) investigated Notch receptors, Notch ligand and Notch target genes mRNA expression in vascular smooth muscle cells (VSMCs) exposing to mechanical strain produced by cyclically stretching on flexible membranes for 24 hours. This study found that among Notch receptors and Notch ligands investigated, Notch3 and Jagged 1 mRNA expression was down-regulated with the increasing degree of mechanical strain. For notch target genes, it was found that all Notch target genes investigated which were HES, HEY1, and HEY2 were down-regulated in response to mechanical strain.

Morrow et al. (2005) studied in rat vascular smooth muscle cells (SMCs) and found that Notch receptor, Notch1 and Notch3, Notch ligands, Jagged 1, and Notch target genes, *hrt-1, hrt-2, hrt-3, hes-1, and hes-5* mRNA expression decreased with cyclin strain applied. Cyclic strain also caused suppression of proliferation and stimulation of cell apoptosis which can be reversed by overexpression of Notch 3 receptor suggesting that Notch signaling involve in regulation of mechanical strain-induced changes in cell proliferation and apoptosis.

Morrow et al. (2007) studied in human umbilical vein endothelial cells (HUVECs) subjected to cyclic mechanical strain. This study found that there was a maximal increase in mRNA expression of *NOTCH1*, Notch4 and Notch target gene, HRT at 4 hours after receiving mechanical strain then mRNA expression of these genes declined. NOTCH1 and NOTCH4 intracellular protein level was significantly increased at 2 and 4 hours respectively after cells exposing to mechanical strain and declined thereafter. In addition, by knocking down NOTCH1 and NOTCH4 using siRNA and blocking Notch mediated CBF-1/RBP-Jk regulated gene expression, it was found that notch signaling can regulate angiogenesis in response to mechanical strain.

Conceptual framework

Figure 7 illustrates conceptual framework of this study

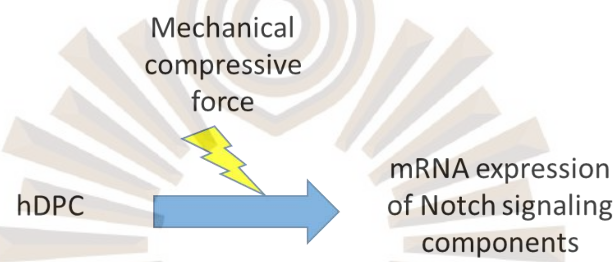

Figure 7 Conceptual framework

Chapter 3

Materials and Method

RNA samples

Stock RNA samples from previous study will be used in this study. The procedure to obtain mRNA sample was as followed.

1. HDPCs were obtained from caries-free lower third molars using explant method. The cells were expanded until the 3rd to the 6th passage before used for compressive force application experiments.
2. Mechanical compressive force was generated by two compressive force application models.
    a. Hydrostatic compression model. This model utilized a computerized compressive force application apparatus as shown in Figure 8. This force application apparatus generates the determined level of compressive force onto 6-well cell culture plate. This compressive force application model was described in detail in previous study of Manokawinchoke et al. (2015).
    b. Direct compression model. This model used a metal weight containing-plastic cylinders that were fit into the wells of 6-well plate to deliver compressive force onto the cells (Figure 9). This model of compressive force application was used in many previous studies (Charoenpong & Ritprajak, 2021; Govitvattana et al., 2013; Satrawaha et al., 2011).
3. HDPCs were plated in 6-well plates at the density of 300,000 cells/well overnight. Two hours prior to force application cells were starved in serum-free media. The media were changed again prior to force application. The cells were then subjected to mechanical force of either 1 or 2 g/cm^2 for 2, 6 or 24 hours as indicated in result section. HDPCs culture in the same manner

without force application were served as control. The experimental design is illustrated in Figure 10

4. Immediately after force application, mRNA sample were extracted using TRIzol™ (Invitrogen, ThermoFisher Scientific) reagent. The amount of RNA obtained was measured using a spectrophotometer (NanoDrop2000, Thermo Scientific). RNA samples were kept in -80°C and will be used in this study. RNA samples of at 3 lines of HDPCs will be used.

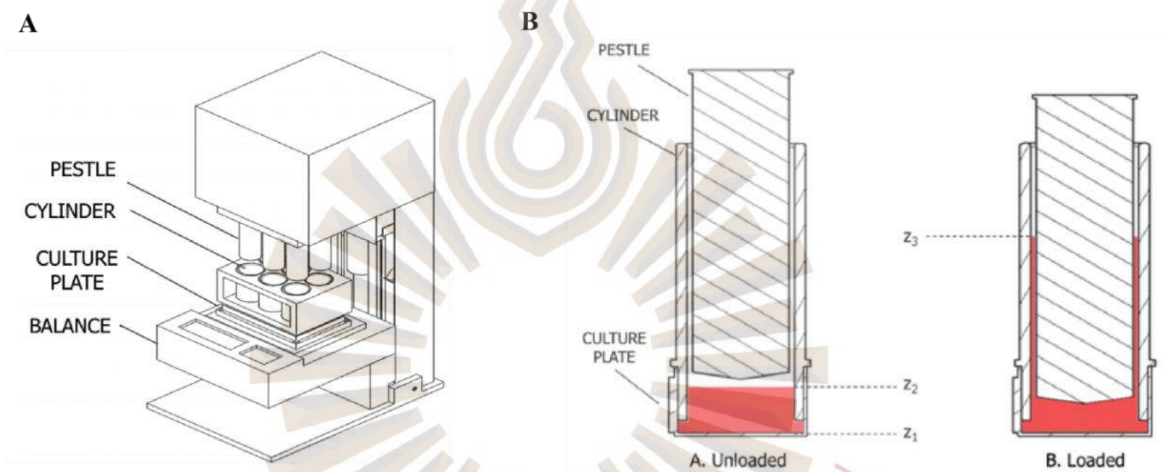

Figure 8 Hydrostatic compression model (A) The compressive force apparatus with 6-well plate. (B) The unloaded and loaded pestle and cylinder. (Picture from Manokawinchoke et al. (2015)

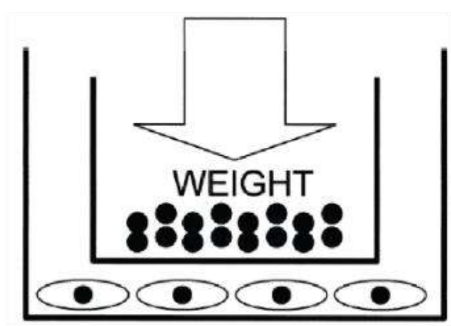

Figure 9 Direct compression model

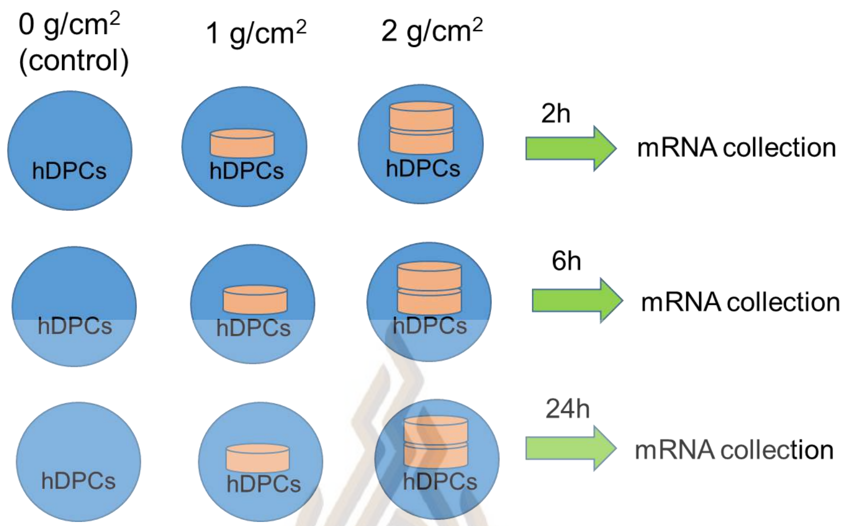

Figure 10 The design of compressive force application experiments

Synthesis of complementary DNA (cDNA)

Instruments and materials

1. iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-RAD, CA, USA)
2. Nuclease free water
3. PCR tubes
4. Pipettes
5. Pipette tips
6. Autoclave chamber
7. PCR tube centrifuge
8. Thermocycler (PCR GeneAmp 9700; Applied Biosystems)

Methods

1. Add varying amount of RNA samples that contain 1 µg into PCR tube
2. Add nuclease free water to make up the volume of 16 µl

3. Add 4 µl of iScript RT Supermix which will make the total reaction mix of 20 µl
4. Mix thoroughly and briefly centrifuge to collect all the components
5. Incubate reaction mix in thermocycler with the temperature setting at 25°C for 5 minutes, 46°C for 20 minutes and 95°C for 1 minutes.
6. Keep cDNA samples obtained at -20°C until used

Quantification of mRNA by real-time polymerase chain reaction (PCR)

Instruments and materials

1. PCR plate
2. PCR cabinet
3. PCR cooling block
4. Pipettes
5. Pipette tips
6. Centrifuge tubes
7. Autoclave chamber
8. iTaq™ Universal SYBR® Green Supermix
9. Nuclease free water
10. Gene primers
11. Microplate spinner
12. Real-time PCR machine, LightCycler® 480 II (Roche, Basel, Switzerland)

Methods

The primers sequences of are prepared as reported from GenBank which are listed in Table 1. mRNA expression of Notch receptors: *NOTCH1*, *NOTCH2, NOTHC3* and *NOTCH4* will be investigated together with Notch target genes *HES1* and *HEY1*. *GAPDH* gene was used as internal control to normalize target gene expression

Table 1 The list of primers used in this study

Gene name	**Primer Sequences (5' to 3')**	**Accession Number**
GAPDH	Forward- CACTGCCAACGTGTCAGTGGTG	NM_002046.6
	Reverse- GTAGCCCAGGATGCCCTTGAG-R	
NOTCH1	Forward -GCCGCCTTTGTGCTTCTGTTC	NM_017617.5
	Reverse-CCGGTGGTCTGTCTGGTCGTC	
NOTCH2	Forward -CCAGAATGGAGGTTCCTGTA	NM_024408.4
	Reverse-GTACCCAGGCCATCAACACA	
NOTCH3	Forward -TCTTGCTGCTGGTCATTCTC	NM_000435.3
	Reverse-TGCCTCATCCTCTTCAGTTG	
NOTCH4	Forward -AGCCGATAAAGATGCCCA	NM_004557.4
	Reverse-ACCACAGTCAAGTTGAGG	
HES1	Forward -AGGCGGACATTCTGGAAATG	NM_005524.4
	Reverse-CGGTACTTCCCCAGCACACTT	
HEY1	Forward -CTGCAGATGACCGTGGATCA	NM_012258.4
	Reverse-CCAAACTCCGATAGTCCATAGCA	

1. Prepare each primer at the concentration of 100 µM and stock at -20°C
2. Dilute the primer to 10 µM for use
3. Prepare mastermix for PCR reaction for all reactions in which each reaction contains

a. 5 µl of iTaq™ Universal SYBR® Green Supermix

b. 0.5 µl of 10 µM forward primer

c. 0.5 µl of 10 µM reverse primer

d. Water to make up the volume of 8 µl

4. Add 8 µl of PCR mastermix into each wells of PCR plate
5. Add 2 µl of diluted cDNA which will make up the total volume of 10 µl
6. Briefly spin the PCR plate to collect all content
7. Each reaction volume will be prepared with 5 µl of iTaq™ Universal SYBR® Green Supermix, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer and 4 µl of cDNA in water to make up the total volume of 10 µl.
8. Place PCR plate in real-time PCR machine in which the cycling temperature is set at 95°C for 5 min for pre-incubation, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 30 s.

Statistical analysis

Level of mRNA expression of each gene was calculated as fold change compared to mRNA expression of that gene in control cells from the same donor. Statistically analyses were performed using IBM SPSS Statistic 21. Normality test was performed with Shapiro-Wilk test. ANOVA was performed to compare mean fold change of mRNA among 3 groups of data. The least significance difference (LSD) *post hoc* test was performed following any significance indicated by ANOVA. Independent t-test and Mann-Whitney U test were used to compared mean fold change of mRNA between two groups of data with normal and non-normal distribution respectively.

Chapter 4

Results

Effect of force magnitude on Notch target genes activation

To determine the effect of compressive force magnitude on Notch target genes activation, compressive force of 1 g/cm^2 and 2 g/cm^2 was applied to HDPCs for 2 hours and mRNA expression of Notch target genes was investigated. After applying hydrostatic compressive force of 1 g/cm^2 and 2 g/cm^2 to HDPCs for 2 hours, HES1 mRNA expression was increased to 1.74+-0.19 and1.37+-0.21. fold respectively compared to control cells not receiving force (Figure 11). Statistical analysis (ANOVA followed by LSD post hoc test) revealed a significant increase in HES1 mRNA expression between control cells and cells received hydrostatic compressive force of 1 g/cm^2 and 2 g/cm^2 ($P<0.01$ and $P<0.05$ respectively). However, HEY1 mRNA expression were 1.12+-0.55. and 1.43+-1.36 fold following application of hydrostatic force of 1 g/cm^2 and 2 g/cm^2 respectively in which no statistical significance was observe (Figure 11).

Direct compression also showed an increase in HES1 mRNA expression with mean fold change of HEY1 mRNA expression 2.72+- and 0.96 fold in cells receiving force of 1 g/cm^2 and 2 g/cm^2 respectively (Figure 12). Statistical analysis (ANOVA followed by LSD post hoc test) revealed also indicated a significant increase in HES1 mRNA expression between control cells and cells received direct compressive force of 1 g/cm^2 and 2 g/cm^2 ($P<0.05$). HEY1 mRNA expression change were 0.73+-0.30 and 0.94+-0.32 fold following application of direct compressive force of 1 g/cm^2 and 2 g/cm^2 which was not statistically significant. (Figure 12)

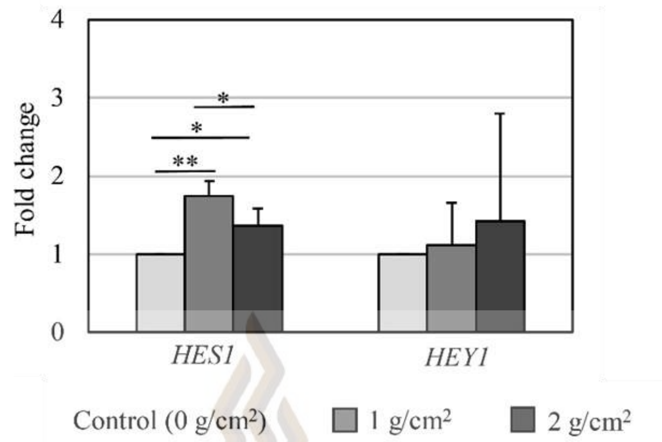

Figure 11: Fold changes of *Notch target genes, HES1 and HEY1* mRNA expression following hydrostatic compressive force application in HDPCs for 2 hours. Data are presented in mean±SD (n=3), **P* < 0.05, ***P* < 0.01

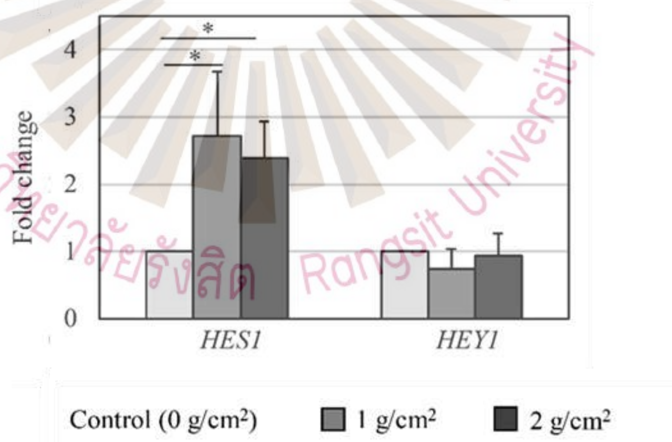

Figure 12: Fold changes of *Notch target genes, HES1 and HEY1* mRNA expression following direction compressive force application in HDPCs for 2 hours. Data are presented in mean±SD (n=3), **P* < 0.05.

Effect of force duration on Notch target genes activation

The lightest force that can trigger Notch target gene expression which was 1 g/cm^2 were chosen for further experiments to determine the effect of force duration on mRNA expression of Notch target genes. The effect of force duration on mRNA expression of Notch target genes was investigated by applying compressive force of 1 g/cm^2 to HDPCs for varying durations and determined the mRNA expression of Notch target genes.

It was found that *HES1* mRNA expression in HDPCs subjected to hydrostatic compressive force for 2, 6 and 24 hours were 1.74+0.19 , 1.84+0.07 and 1.14+0.08 respectively (Figure 13). Student t-test and Mann-Whitney U test revealed statistically significant change in HES1 mRNA expression at 2 and 6 hours respectively (P<0.05). At 24 hours of hydrostatic compressive force application, no significant difference was observed in HES1 mRNA expression between control cells and cells receiving force indicated by Student t-test (Figure 13). The change in HEY1 mRNA expression following application of hydrostatic force were 1.16+0.55, 1.24+0.09 and 1.43+1.36 fold after 2, 6 and 24 hours of force application respectively (Figure 13). Student-t-test indicated the statistically significant upregulation of HEY1 mRNA expression only at 6 hours of hydrostatic force application (P<0.01).

Compressive force from direct compression models induced an increase in *HES1* mRNA expression only at 2 hours of force application in which the expression was 2.72+0.96 fold of control (Figure 14). This increase was statistically significance (P<0.05) indicated by Student t-test (P<0.05). At 6 and 24 hours of direct compressive force application, *HES1* mRNA expression were decreased to 0.64+0.54 and 0.58+0.42 fold of control respectively but no statistical significance was observed as indicated by student t-test (Figure 14). *HEY1* mRNA expression was downregulated to 0.73+0.30, 0.56+0.28 and 0.52+0.21 fold after applying direct compressive force to HDPCs for 2, 6 and 24 hours respectively (Figure 14). However, Student t-test indicated only significant reduction in mRNA expression of *HEY1* only at 24 hours of direct compressive force application ($P < 0.05$).

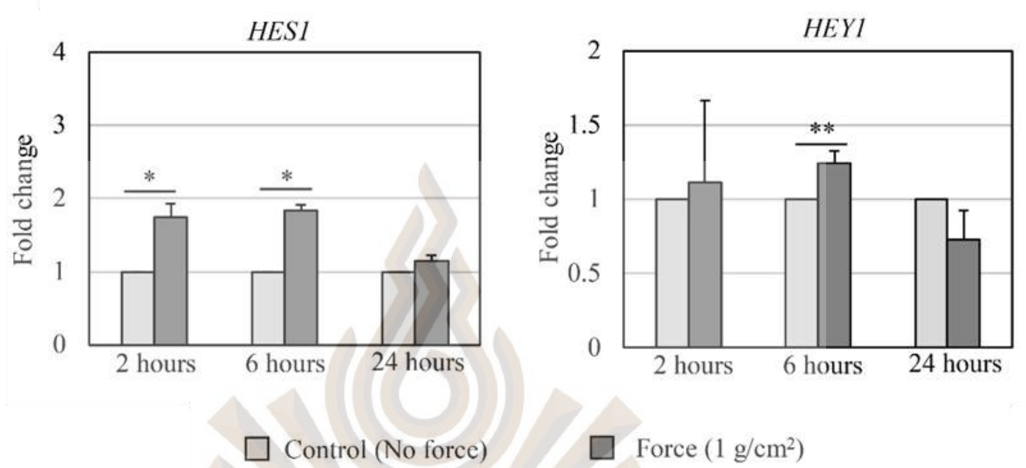

Figure 13: Fold changes of *HES1* and *HEY1* mRNA expression following hydrostatic compressive force application in HDPCs for 2, 6 and 24 hours. Data are presented in mean±SD (n=3), *$P < 0.05$, **$P < 0.01$

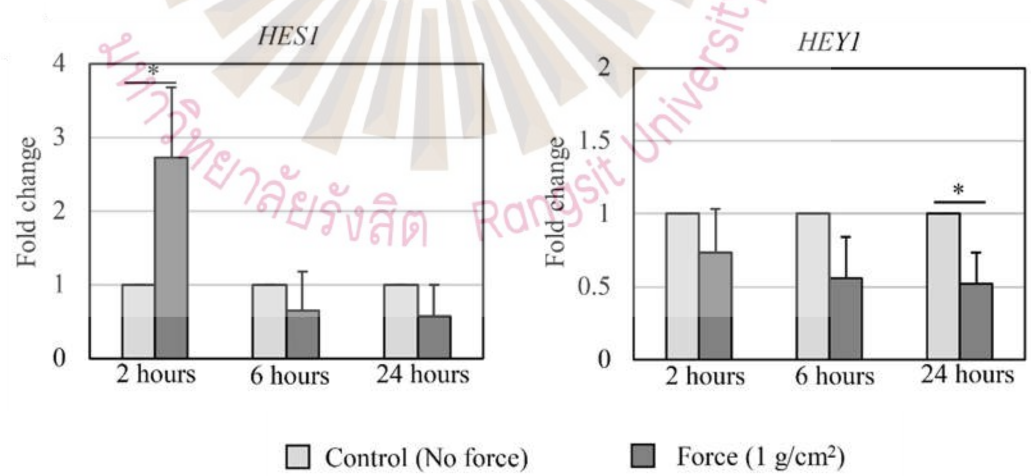

Figure 14: Fold changes of *HES1* and *HEY1* mRNA expression following direct compressive force application in HDPCs for 2, 6 and 24 hours. Data are presented in mean±SD (n=3), *$P <$ 0.05.

Effect of compressive force on mRNA expression of NOTCH receptors

Hydrostatic compression model

Since the results showed that Notch target genes was upregulated at 2 and 6 hour following compressive force application by hydrostatic model and at 2 hours following direct compression, the expression of NOTCH receptors was also carried out corresponding to these durations with the force of 1 g/cm^2.

NOTCH1 mRNA expression in HDPCs receiving hydrostatic compressive force for 2 and 6 hours were 1.03+0.23 and 0.90+0.66 fold compared to control cells without force application respectively (Figure 15). No statistically difference was observed for *NOTCH*1 mRNA expression between HDPCs receiving direct compressive force and control cell not receiving force at both 2 hours and 6 hours following force application indicated by Student t-test and Mann-Whitney U test respectively.

NOTCH2 mRNA expression was 0.96+0.21 and 1.42+0.18 fold in hDPCs subjected to hydrostatic compressive force compared to control for 2 and 6 hours respectively (Figure 15). Student T-test showed significant difference in NOTCH2 expression at following 6 hours of force application (P<0.05) while at 2 hours of force application no significant difference was observed.

mRNA expression of *NOTCH3* in HDPCs receiving mechanical compressive force was 0.93+0.08 and 1.12+0.42 compared to control cells after 2 and 6 h of force application respectively (Figure 15). Student t-test analysis revealed no significant difference in *NOTCH3* mRNA expression in hDPCs subjected to hydrostatic and compressive force compared to in both 2 and 6 hours of force application.

NOTCH4 mRNA expression at 2 and 6 of hydrostatic compressive force application were 0.87+0.37 and 0.94+0.20 fold in HDPCs subjected to mechanical force compared to control (Figure 15). There was no difference in *NOTCH4* expression between HDPCs subjected to hydrostatic compressive force and control HDPCs in both 2 and 6 hours of force application indicated by Student t-test.

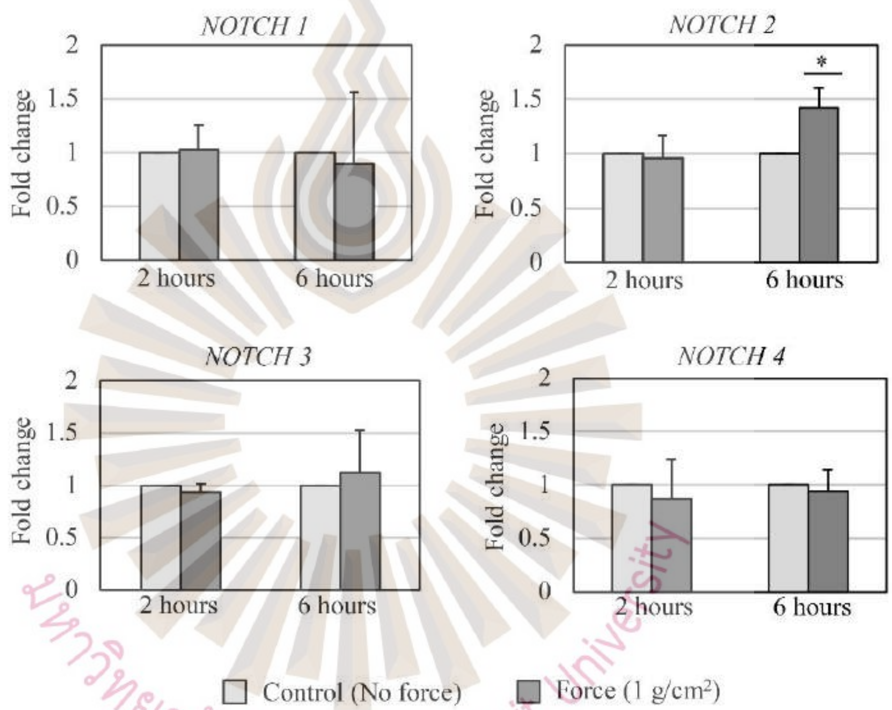

Figure 15: Fold changes of mRNA expression of Notch receptors following compressive force application by hydrostatic compression model for 2 and 6 hours. Data are presented in mean±SD (n=3), *$P < 0.05$

<u>*Direct compression model*</u>

For direct compression, we investigated mRNA expression of Notch receptors at 2 hours of force application which corresponded to the time point that we observed the upregulation of *HES1*. mRNA expression of *NOTCH1*, *NOTCH2*, *NOTCH3* and *NOTCH4* were 0.97+0.47, 0.79+0.30, 0.55+0.50 and 0.67+0.51 fold of control respectively (Figure 16). Student T-test indicated that there was no significant change in mRNA expression of all Notch receptors in HDPCs receiving direct compressive force compared to control.

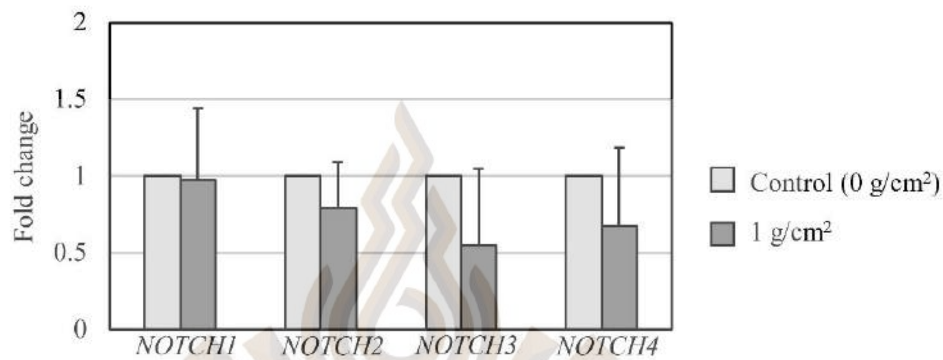

Figure 16: Fold changes of mRNA expression of Notch receptors following compressive force application by direct compression model for 2 hours. Data are presented in mean±SD (n=3)

Chapter 5

Discussion

The cells used in this study were human dental pulp cells derived by an explant method. HDPCs derived by this method were previously characterized by flow cytometry and were found to be highly positive for the fibroblast markers, CD44, CD90, and CD105 while negative the immune progenitors (CD34) and leukocytes markers (CD45) (Charoenpong et al., 2019). In this study, human dental pulp cells were subjected to mechanical compressive force from 2-24 hours and mRNA expression of Notch target gene HES1 and HEY1 as well as mRNA of Notch receptors was investigated to clarify the relationship between Notch signaling and dental pulp cell response to mechanical force.

Compressive force was applied to HDPCs by two different models. Both models of compressive force application can trigger an upregulation mRNA of Notch target genes in HDPCs, suggesting that Notch signaling could be one of the targets of compressive force in HDPCs. However, compressive force from direct compression model produced shorter activation of mRNA of Notch target genes compared to hydrostatic compression model. This may be due to some differences in characteristics of force generated by these two models (Natenstedt et al., 2015; Salinas et al., 2018). Hydrostatic compression model produces more harmonious load in which the force comes from all direction throughout the force application period while in direct compression model, the force was generated only from the top so that it can cause more deformation of cell shape (Natenstedt et al., 2015; Salinas et al., 2018).

The association between Notch signaling and mechanical force has also been demonstrated in previous studies in endothelial cells, in which an increase in expression of some Notch target genes, as well as Notch receptors, Notch 1, Notch 3 and Notch 4, was observed in relation to shear force application (Jahnsen et al., 2015; Mack et al., 2017; Masumura et al., 2009). Mechanical compressive force was also reported to significantly upregulate *NOTCH1* mRNA expression in human deciduous dental pulp cells (Peetiakarawach et al., 2015). In contrast, the decrease in Notch target genes, Notch1 and Notch3 expression following application of cyclic strain was observed in vascular smooth muscle cells (Loerakker et al., 2018; Morrow et al., 2005). In this study, we observed the upregulation of mRNA of notch target genes and *NOTCH2* in HDPCs receiving mechanical compressive force. Our results together with the results from previous studies suggest that Notch signaling associates with cellular response to mechanical force in various cell types including HDPCs. However, the differences in response could be observed depending on cell types and type of mechanical force applied.

The results from our study were in concordant with the results from previous in vivo studies, that found Notch receptors, ligands and target genes were upregulated following pulpal injuries (Lovschall et al., 2005; Mitsiadis et al., 1999; Mitsiadis et al., 2003). The upregulation of *NOTCH2*, among all Notch receptors, observed in our study also corresponds with previous report in rats and human teeth, that found Notch2 being the most prominent Notch receptors upregulated following pulpal injuries (Mitsiadis et al., 1999; Mitsiadis et al., 2003). Upregulation of Notch2 following pulpal injury was previously observed mainly in undifferentiated cells that were likely to differentiate into odontoblasts (Cai et al., 2011; Mitsiadis et al., 1999; Mitsiadis et al., 2003). In vitro study also reported that, Notch 2 involved in osteogenic differentiation of dental pulp and PDL cells (Manokawinchoke et al., 2017; Manokawinchoke et al., 2020). In addition, it was also reported that, compressive force enhanced odontogenic differentiation of dental pulp dells (Miyashita et al., 2017; Rad et al., 2021; Yu et al., 2009). Therefore, it is possible that upregulation of *NOTCH2* and Notch target genes in HDPCs following mechanical compressive force application in our study, may be related to odontogenic differentiation these cells. However, further investigation is required to confirm this speculation.

Activation of Notch signaling was reported to be a mechanosensitive process. Mechanical force generated from ligand endocytosis after binding of Notch ligand to its receptor, exposes S2 cleavage site on negative regulatory region (NRR) of Notch (DuFort et al., 2011; Langridge & Struhl, 2017; Meloty-Kapella et al., 2012). This allows Notch cleavage and releasing of NICD to activate Notch target genes. Previous studies reported that external mechanical force applied to ligand-bound Notch receptor or Notch receptor alone, can increase the cleavage at this S2 site (Gordon et al., 2015; Stephenson & Avis, 2012). Therefore, it is possible that mechanical force given to HDPCs in this study enhanced the mechanical force required to trigger Notch cleavage. This may explain our results that found an upregulation of Notch target gene, *HES1*, as early as 2 hours of compressive force application although no upregulation of Notch receptors was observed at this time point. In addition, since Notch signaling is activated in juxtacrine manner, it was found that the amount of Notch signaling correlated with contact area between cells (Sestan et al., 1999; Shaya et al., 2017). Extrinsic mechanical force can affect cell shape and orientation which affect cell-cell contact area (Matamoro-Vidal & Levayer, 2019; Sumi et al., 2018). Therefore, it is also possible that compressive force given to HDPCs increased cellular contact so that Notch signaling was enhanced. On the contrary, mechanical strain which stretches the cells, was found to decrease Notch target genes in vascular smooth muscle cells (Loerakker et al., 2018; Morrow et al., 2005).

Although we observed the upregulation of mRNA of notch target gene, *HES1*, as early as 2 hours after compressive force application by both models, upregulation of *HEY1* can be observed only at 6 hours following hydrostatic compression. The upregulation of *HEY1* was concomitant with the upregulation of *NOTCH2*. This implicated an association between *NOTCH2* and *HEY1* in HDPCs which was agreed with previous study that found a significant downregulation of *HEY1* mRNA expression in shNOTCH2-HDPCs while *HES1* expression was not affected in these cells (Manokawinchoke et al., 2017). The association between Notch2 and Hey1 was also reported in previous study in chick heart, that found Notch2 can specifically induce Hey1 but not Hey2 (Rutenberg et al., 2006).

The increase in Notch signaling, observed in our study, was transient. Upregulation of Notch target genes in HDPCs receiving mechanical compressive force was observed up to 2 and 6 hours in direct and hydrostatic compression model respectively. At longer duration, there was a tendency towards reduction in Notch signaling. The transient activation of Notch signaling, observed in our study, could result from the fact that Notch signaling is direct without signal amplification, and one Notch receptor can be used only once per one signaling activation, since it is targeted cleavage and degradation shortly thereafter (Bi & Kuang, 2015; Henrique & Schweisguth, 2019). In direct compression, we did not observe any upregulation of Notch receptors. Therefore, it is possible that after initial activation at 2 hours, mechanical compressive force given to HDPCs could not further activate Notch signaling due to the lack of Notch receptors to replace those used. For hydrostatic compression, more prolonged activation of Notch signaling may result from an upregulation of *NOTCH2* at 6 hours which increase the availability of Notch receptors for longer activation of Notch signaling.

The temporal upregulation Notch receptors and Notch target genes following mechanical force application was also observed in previous study in human umbilical vein endothelial cells, in which Notch 1, Notch 4 and Notch target gene hrt-1 were upregulated at 4-8 h following application cyclic mechanical strain, then the expression level declined (Morrow et al., 2007). Kang et al., 2014 also observed temporal increase in Notch1 expression at day 2 after application of intermittent flow in limbal epithelial stem cells which decreased 4 days later. In dental pulp, Lovschall et al., 2005 studied Notch signaling components in adult rat molars after pulp capping with calcium hydroxide and found that expression of all the Notch receptors, ligands and target genes, Hes1, were increased in dental pulp on day 1 after injury and tended to decrease on day 3.

Since dental pulp cells can be subjected to other types of force such as shear force, the effect of other types of mechanical force should be further investigated. Mechanism underlying mechanical force enhancing Notch signaling in HDPC and the protein level should also be investigated.

Conclusion

Both hydrostatic compressive force and direct compressive force application induced mRNA expression of Notch target genes. Hydrostatic compression triggered more prolonged activation of Notch target genes up to 6 hours of force application. Increase in mRNA expression of Notch receptors was observed for only *NOTCH2* following 6 hours of hydrostatic force application. Notch activation in HDPCs by mechanical compressive force was transient since no upregulation in target genes was observed following 24 hours of force application.

References

Arana-Chavez, V. E., & Massa, L. F. (2004). Odontoblasts: the cells forming and maintaining dentine. *International Journal of Biochemistry and Cell Biology*, *36*(8), 1367-1373. https://doi.org/10.1016/j.biocel.2004.01.006

Bi, P., & Kuang, S. (2015). Notch signaling as a novel regulator of metabolism. *Trends in Endocrinology and Metabolism*, *26*(5), 248-255. https://doi.org/10.1016/j.tem.2015.02.006

Borggrefe, T., & Oswald, F. (2009). The Notch signaling pathway: transcriptional regulation at Notch target genes. *Cellular and Molecular Life Sciences*, *66*(10), 1631-1646. https://doi.org/10.1007/s00018-009-8668-7

Cai, X., Gong, P., Huang, Y., & Lin, Y. (2011). Notch signalling pathway in tooth development and adult dental cells. *Cell Proliferation*, *44*(6), 495-507. https://doi.org/10.1111/j.1365-2184.2011.00780.x

Charoenpong, H., Osathanon, T., Pavasant, P., Limjeerajarus, N., Keawprachum, B., Limjeerajarus, C. N., . . . Ritprajak, P. (2019). Mechanical stress induced S100A7 expression in human dental pulp cells to augment osteoclast differentiation. *Oral Diseases*, *25*(3), 812-821. https://doi.org/10.1111/odi.13033

Charoenpong, H., & Ritprajak, P. (2021). Effect of metal ions released from orthodontic mini-implants on osteoclastogenesis. *Dent Med Probl*, *58*(3), 327-333. https://doi.org/10.17219/dmp/133891

Dabas, U., & Dabas, V. K. (2007). *Textbook of Endodontics* (First ed.). AITBS.

DuFort, C. C., Paszek, M. J., & Weaver, V. M. (2011). Balancing forces: architectural control of mechanotransduction. *Nature Reviews: Molecular Cell Biology*, *12*(5), 308-319. https://doi.org/10.1038/nrm3112

Gordon, W. R., Zimmerman, B., He, L., Miles, L. J., Huang, J., Tiyanont, K., . . . Blacklow, S. C. (2015). Mechanical Allostery: Evidence for a Force Requirement in the Proteolytic Activation of Notch. *Developmental Cell*, *33*(6), 729-736. https://doi.org/10.1016/j.devcel.2015.05.004

Govitvattana, N., Osathanon, T., Taebunpakul, S., & Pavasant, P. (2013). IL-6 regulated stress-induced Rex-1 expression in stem cells from human exfoliated deciduous teeth. *Oral Diseases*, *19*(7), 673-682. https://doi.org/10.1111/odi.12052

Gronthos, S., Brahim, J., Li, W., Fisher, L. W., Cherman, N., Boyde, A., . . . Shi, S. (2002). Stem cell properties of human dental pulp stem cells. *Journal of Dental Research*, *81*(8), 531-535. https://doi.org/10.1177/154405910208100806

Hansamuit, K., Osathanon, T., & Suwanwela, J. (2020). Effect of Jagged1 on the expression of genes in regulation of osteoblast differentiation and bone mineralization ontology in human dental pulp and periodontal ligament cells. *J Oral Biol Craniofac Res*, *10*(2), 233-237. https://doi.org/10.1016/j.jobcr.2019.12.003

He, F., Yang, Z., Tan, Y., Yu, N., Wang, X., Yao, N., & Zhao, J. (2009). Effects of Notch ligand Delta1 on the proliferation and differentiation of human dental pulp stem cells in vitro. *Archives of Oral Biology*, *54*(3), 216-222. https://doi.org/10.1016/j.archoralbio.2008.10.003

Henrique, D., & Schweisguth, F. (2019). Mechanisms of Notch signaling: a simple logic deployed in time and space. *Development*, *146*(3). https://doi.org/10.1242/dev.172148

Heyeraas, K. J., & Berggreen, E. (1999). Interstitial fluid pressure in normal and inflamed pulp. *Critical Reviews in Oral Biology and Medicine*, *10*(3), 328-336. http://www.ncbi.nlm.nih.gov/pubmed/10759412

Jahnsen, E. D., Trindade, A., Zaun, H. C., Lehoux, S., Duarte, A., & Jones, E. A. (2015). Notch1 is pan-endothelial at the onset of flow and regulated by flow. *PloS One*, *10*(4), e0122622. https://doi.org/10.1371/journal.pone.0122622

Kang, Y. G., Shin, J. W., Park, S. H., Oh, M. J., Park, H. S., Shin, J. W., & Kim, S. H. (2014). Effects of flow-induced shear stress on limbal epithelial stem cell growth and enrichment. *PloS One*, *9*(3), e93023. https://doi.org/10.1371/journal.pone.0093023

Kawashima, N., & Okiji, T. (2016). Odontoblasts: Specialized hard-tissue-forming cells in the dentin-pulp complex. *Congenit Anom (Kyoto)*, *56*(4), 144-153. https://doi.org/10.1111/cga.12169

Koolstra, J. H. (2002). Dynamics of the human masticatory system. *Critical Reviews in Oral Biology and Medicine*, *13*(4), 366-376. https://doi.org/10.1177/154411130201300406

Kopan, R. (2012). Notch signaling. *Cold Spring Harbor Perspectives in Biology*, *4*(10). https://doi.org/10.1101/cshperspect.a011213

Langridge, P. D., & Struhl, G. (2017). Epsin-Dependent Ligand Endocytosis Activates Notch by Force. *Cell*, *171*(6), 1383-1396 e1312. https://doi.org/10.1016/j.cell.2017.10.048

Loerakker, S., Stassen, O., Ter Huurne, F. M., Boareto, M., Bouten, C. V. C., & Sahlgren, C. M. (2018). Mechanosensitivity of Jagged-Notch signaling can induce a switch-type behavior in vascular homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(16), E3682-E3691. https://doi.org/10.1073/pnas.1715277115

Lovschall, H., Tummers, M., Thesleff, I., Fuchtbauer, E. M., & Poulsen, K. (2005). Activation of the Notch signaling pathway in response to pulp capping of rat molars. *European Journal of Oral Sciences*, *113*(4), 312-317. https://doi.org/10.1111/j.1600-0722.2005.00221.x

Mack, J. J., Mosqueiro, T. S., Archer, B. J., Jones, W. M., Sunshine, H., Faas, G. C., . . . Iruela-Arispe, M. L. (2017). NOTCH1 is a mechanosensor in adult arteries. *Nat Commun*, *8*(1), 1620. https://doi.org/10.1038/s41467-017-01741-8

Manokawinchoke, J., Limjeerajarus, N., Limjeerajarus, C., Sastravaha, P., Everts, V., & Pavasant, P. (2015). Mechanical Force-induced TGFB1 Increases Expression of SOST/POSTN by hPDL Cells. *Journal of Dental Research*, *94*(7), 983-989. https://doi.org/10.1177/0022034515581372

Manokawinchoke, J., Nattasit, P., Thongngam, T., Pavasant, P., Tompkins, K. A., Egusa, H., & Osathanon, T. (2017). Indirect immobilized Jagged1 suppresses cell cycle progression and induces odonto/osteogenic differentiation in human dental pulp cells. *Scientific Reports*, *7*(1), 10124. https://doi.org/10.1038/s41598-017-10638-x

Manokawinchoke, J., Sumrejkanchanakij, P., Boonprakong, L., Pavasant, P., Egusa, H., & Osathanon, T. (2020). NOTCH2 participates in Jagged1-induced osteogenic differentiation in human periodontal ligament cells. *Scientific Reports*, *10*(1), 13329. https://doi.org/10.1038/s41598-020-70277-7

Marrelli, M., Codispoti, B., Shelton, R. M., Scheven, B. A., Cooper, P. R., Tatullo, M., & Paduano, F. (2018). Dental Pulp Stem Cell Mechanoresponsiveness: Effects of Mechanical Stimuli on Dental Pulp Stem Cell Behavior. *Frontiers in Physiology*, *9*, 1685. https://doi.org/10.3389/fphys.2018.01685

Masumura, T., Yamamoto, K., Shimizu, N., Obi, S., & Ando, J. (2009). Shear stress increases expression of the arterial endothelial marker ephrinB2 in murine ES cells via the VEGF-Notch signaling pathways. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *29*(12), 2125-2131. https://doi.org/10.1161/ATVBAHA.109.193185

Matamoro-Vidal, A., & Levayer, R. (2019). Multiple Influences of Mechanical Forces on Cell Competition. *Current Biology*, *29*(15), R762-R774. https://doi.org/10.1016/j.cub.2019.06.030

Meloty-Kapella, L., Shergill, B., Kuon, J., Botvinick, E., & Weinmaster, G. (2012). Notch ligand endocytosis generates mechanical pulling force dependent on dynamin, epsins, and actin. *Developmental Cell*, *22*(6), 1299-1312. https://doi.org/10.1016/j.devcel.2012.04.005

Mitsiadis, T. A., Fried, K., & Goridis, C. (1999). Reactivation of Delta-Notch signaling after injury: complementary expression patterns of ligand and receptor in dental pulp. *Experimental Cell Research*, *246*(2), 312-318. https://doi.org/10.1006/excr.1998.4285

Mitsiadis, T. A., Romeas, A., Lendahl, U., Sharpe, P. T., & Farges, J. C. (2003). Notch2 protein distribution in human teeth under normal and pathological conditions. *Experimental Cell Research*, *282*(2), 101-109. https://doi.org/10.1016/s0014-4827(02)00012-5

Miyashita, S., Ahmed, N. E., Murakami, M., Iohara, K., Yamamoto, T., Horibe, H., . . . Nakashima, M. (2017). Mechanical forces induce odontoblastic differentiation of mesenchymal stem cells on three-dimensional biomimetic scaffolds. *Journal of Tissue Engineering and Regenerative Medicine*, *11*(2), 434-446. https://doi.org/10.1002/term.1928

Mjor, I. A., & Heyeraas, K. (2008). Pulp-Dentin and Periodontal Anatomy and Physiology. In D. Orstavik & T. P. Ford (Eds.), *Essential Endodontology* (Second ed., pp. 10-40). Blackwell Munksgaard.

Morrow, D., Cullen, J. P., Cahill, P. A., & Redmond, E. M. (2007). Cyclic strain regulates the Notch/CBF-1 signaling pathway in endothelial cells: role in angiogenic activity. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *27*(6), 1289-1296. https://doi.org/10.1161/ATVBAHA.107.142778

Morrow, D., Sweeney, C., Birney, Y. A., Cummins, P. M., Walls, D., Redmond, E. M., & Cahill, P. A. (2005). Cyclic strain inhibits Notch receptor signaling in vascular smooth muscle cells in vitro. *Circulation Research*, *96*(5), 567-575. https://doi.org/10.1161/01.RES.0000159182.98874.43

Nakashima, M., Iohara, K., & Murakami, M. (2013). Dental pulp stem cells and regeneration. *Endodontics Topics*, *28*, 38-50.

Natenstedt, J., Kok, A. C., Dankelman, J., & Tuijthof, G. J. (2015). What quantitative mechanical loading stimulates in vitro cultivation best? *J Exp Orthop*, *2*(1), 15. https://doi.org/10.1186/s40634-015-0029-x

Obi, S., Yamamoto, K., Shimizu, N., Kumagaya, S., Masumura, T., Sokabe, T., . . . Ando, J. (2009). Fluid shear stress induces arterial differentiation of endothelial progenitor cells. *J Appl Physiol (1985)*, *106*(1), 203-211. https://doi.org/10.1152/japplphysiol.00197.2008

Peetiakarawach, K., Osathanon, T., Pavasant, P., Rattanaworawipa, R., & Sukarawan, W. (2015). Compressive Stress Enhances NOTCH1 mRNA Expression in Human Deciduous Dental Pulp Cells in vitro. *CU Dent J*, *38*(Suppl), 13-20.

Rad, R. R., Mohaghegh, S., Kouhestani, F., & Motamedian, S. R. (2021). Effect of Mechanical Forces on the Behavior of Dental Stem Cells: A Scoping

Review of In-Vitro Studies. *Molecular & Cellular Biomechanics*, *18*(2), 51-67. https://doi.org/10.32604/mcb.2021.015136

Rutenberg, J. B., Fischer, A., Jia, H., Gessler, M., Zhong, T. P., & Mercola, M. (2006). Developmental patterning of the cardiac atrioventricular canal by Notch and Hairy-related transcription factors. *Development*, *133*(21), 4381-4390. https://doi.org/10.1242/dev.02607

Salinas, E. Y., Hu, J. C., & Athanasiou, K. (2018). A Guide for Using Mechanical Stimulation to Enhance Tissue-Engineered Articular Cartilage Properties. *Tissue Eng Part B Rev*, *24*(5), 345-358. https://doi.org/10.1089/ten.TEB.2018.0006

Satrawaha, S., Wongkhantee, S., Pavasant, P., & Sumrejkanchanakij, P. (2011). Pressure induces interleukin-6 expression via the P2Y6 receptor in human dental pulp cells. *Archives of Oral Biology*, *56*(11), 1230-1237. https://doi.org/10.1016/j.archoralbio.2011.05.003

Sestan, N., Artavanis-Tsakonas, S., & Rakic, P. (1999). Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. *Science*, *286*(5440), 741-746. https://doi.org/10.1126/science.286.5440.741

Shaya, O., Binshtok, U., Hersch, M., Rivkin, D., Weinreb, S., Amir-Zilberstein, L., . . . Sprinzak, D. (2017). Cell-Cell Contact Area Affects Notch Signaling and Notch-Dependent Patterning. *Developmental Cell*, *40*(5), 505-511 e506. https://doi.org/10.1016/j.devcel.2017.02.009

Shekar, R., & Ranganathan, K. (2012). Phenotypic and growth characterization of human mesenchymal stem cells cultured from permanent and deciduous teeth. *Indian Journal of Dental Research*, *23*(6), 838-839. https://doi.org/10.4103/0970-9290.111281

Stephenson, N. L., & Avis, J. M. (2012). Direct observation of proteolytic cleavage at the S2 site upon forced unfolding of the Notch negative regulatory region. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(41), E2757-2765. https://doi.org/10.1073/pnas.1205788109

Su, K. C., Chuang, S. F., Ng, E. Y., & Chang, C. H. (2014). An investigation of dentinal fluid flow in dental pulp during food mastication: simulation of fluid-structure interaction. *Biomech Model Mechanobiol*, *13*(3), 527-535. https://doi.org/10.1007/s10237-013-0514-z

Sumi, A., Hayes, P., D'Angelo, A., Colombelli, J., Salbreux, G., Dierkes, K., & Solon, J. (2018). Adherens Junction Length during Tissue Contraction Is Controlled by the Mechanosensitive Activity of Actomyosin and Junctional Recycling. *Developmental Cell*, *47*(4), 453-463 e453. https://doi.org/10.1016/j.devcel.2018.10.025

Sun, H., Kawashima, N., Xu, J., Takahashi, S., & Suda, H. (2010). Expression of Notch signalling-related genes in normal and differentiating rat dental pulp cells. *Aust Endod J*, *36*(2), 54-58. https://doi.org/10.1111/j.1747-4477.2009.00188.x

Wang, H., Zang, C., Liu, X. S., & Aster, J. C. (2015). The role of Notch receptors in transcriptional regulation. *Journal of Cellular Physiology*, *230*(5), 982-988. https://doi.org/10.1002/jcp.24872

Wang, L., Zhou, Z., Chen, Y., Yuan, S., Du, Y., Ju, X., . . . Wang, X. (2017). The Alpha 7 Nicotinic Acetylcholine Receptor of Deciduous Dental Pulp Stem Cells Regulates Osteoclastogenesis During Physiological Root Resorption. *Stem Cells Dev*, *26*(16), 1186-1198. https://doi.org/10.1089/scd.2017.0033

Wang, X., He, F., Tan, Y., Tian, W., & Qiu, S. (2011). Inhibition of Delta1 promotes differentiation of odontoblasts and inhibits proliferation of human dental pulp stem cell in vitro. *Archives of Oral Biology*, *56*(9), 837-845. https://doi.org/10.1016/j.archoralbio.2011.02.006

Yu, C., & Abbott, P. V. (2007). An overview of the dental pulp: its functions and responses to injury. *Australian Dental Journal*, *52*(1 Suppl), S4-16. https://doi.org/10.1111/j.1834-7819.2007.tb00525.x

Yu, V., Damek-Poprawa, M., Nicoll, S. B., & Akintoye, S. O. (2009). Dynamic hydrostatic pressure promotes differentiation of human dental pulp stem cells. *Biochemical and Biophysical Research Communications*, *386*(4), 661-665. https://doi.org/10.1016/j.bbrc.2009.06.106

Appendix

Statistical test for Figure 11

<u>HES1</u>

Tests of Normality[a]

	MachineForce2h	Kolmogorov-Smirnov[b] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
FoldChange	1	.361	3	.	.806	3	.130
	2	.185	3	.	.998	3	.925

a. FoldChange is constant when MachineForce2h = 0. It has been omitted.

b. Lilliefors Significance Correction

Test of Homogeneity of Variances

FoldChange

Levene Statistic	df1	df2	Sig.
3.578	2	6	.095

ANOVA

FoldChange

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.831	2	.415	15.752	.004
Within Groups	.158	6	.026		
Total	.989	8			

Multiple Comparisons

Dependent Variable: FoldChange

	(I) MachineForce2h	(J) MachineForce2h	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
LSD	0	1	-.74423*	.13260	.001	-1.0687	-.4198
		2	-.37061*	.13260	.031	-.6951	-.0462
	1	0	.74423*	.13260	.001	.4198	1.0687
		2	.37363*	.13260	.030	.0492	.6981
	2	0	.37061*	.13260	.031	.0462	.6951
		1	-.37363*	.13260	.030	-.6981	-.0492

*. The mean difference is significant at the 0.05 level.

HEY1

Tests of Normality[a]

	MachineForce2h	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	1	.289	3	.	.928	3	.480
	2	.280	3	.	.937	3	.517

a. FoldChange is constant when MachineForce2h = 0. It has been omitted.

b. Lilliefors Significance Correction

ANOVA

FoldChange

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.297	2	.148	.206	.820
Within Groups	4.326	6	.721		
Total	4.623	8			

Statistical test for Figure 12

HES1

Tests of Normality[a]

	MachineForce2h	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	1	.361	3	.	.806	3	.130
	2	.177	3	.	1.000	3	.971

a. FoldChange is constant when MachineForce2h = 0. It has been omitted.

b. Lilliefors Significance Correction

Test of Homogeneity of Variances

FoldChange

Levene Statistic	df1	df2	Sig.
2.937	2	6	.129

ANOVA

FoldChange

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.917	2	.458	6.871	.028
Within Groups	.400	6	.067		
Total	1.317	8			

Multiple Comparisons

Dependent Variable: FoldChange

	(I) MachineForce2h	(J) MachineForce2h	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	0	1	-.74423*	.21087	.012	-1.2602	-.2282
		2	-.16502	.21087	.464	-.6810	.3510
	1	0	.74423*	.21087	.012	.2282	1.2602
		2	.57921*	.21087	.033	.0632	1.0952
	2	0	.16502	.21087	.464	-.3510	.6810
		1	-.57921*	.21087	.033	-1.0952	-.0632

*. The mean difference is significant at the 0.05 level.

<u>HEY1</u>

Tests of Normality[a]

	MachineForce2h	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	1	.289	3	.	.928	3	.480
	2	.280	3	.	.937	3	.517

a. FoldChange is constant when MachineForce2h = 0. It has been omitted.

b. Lilliefors Significance Correction

ANOVA

FoldChange

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.297	2	.148	.206	.820
Within Groups	4.326	6	.721		
Total	4.623	8			

Statistic test for Figure 13

HES1

Tests of Normality[a]

	MachineTime1g	Kolmogorov-Smirnov[b] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
FoldChange	2	.361	3	.	.806	3	.130
	6	.385	3	.	.750	3	.000
	24	.286	3	.	.930	3	.490

a. FoldChange is constant when MachineTime1g = 0. It has been omitted.

b. Lilliefors Significance Correction

2h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	15.360	.017	-6.813	4	.002	-.74423	.10924	-1.04753	-.44094
	Equal variances not assumed			-6.813	2.000	.021	-.74423	.10924	-1.21425	-.27422

6h

Test Statistics[a]

	FoldChange
Mann-Whitney U	.000
Wilcoxon W	6.000
Z	-2.121
Asymp. Sig. (2-tailed)	.034
Exact Sig. [2*(1-tailed Sig.)]	.100[b]

a. Grouping Variable: MachineTime1g

b. Not corrected for ties.

24h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	9.878	.035	-2.999	4	.040	-.14009	.04671	-.26979	-.01039
	Equal variances not assumed			-2.999	2.000	.096	-.14009	.04671	-.34108	.06091

<u>*HEY1*</u>

Tests of Normality[a]

	MachineTime1g	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	2	.289	3	.	.928	3	.480
	6	.177	3	.	1.000	3	.962
	24	.277	3	.	.941	3	.530

a. FoldChange is constant when MachineTime1g = 0. It has been omitted.

b. Lilliefors Significance Correction

2h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	10.044	.034	-.367	4	.732	-.11625	.31634	-.99456	.76206
	Equal variances not assumed			-.367	2.000	.749	-.11625	.31634	-1.47737	1.24487

6h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	4.287	.107	-4.867	4	.008	-.24169	.04966	-.37958	-.10381
	Equal variances not assumed			-4.867	2.000	.040	-.24169	.04966	-.45537	-.02802

24h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
FoldChange	Equal variances assumed	9.252	.038	2.401	4	.074	.27129	.11299	-.04243	.58502
	Equal variances not assumed			2.401	2.000	.138	.27129	.11299	-.21488	.75747

Statistic test for Figure 14

<u>HES1</u>

Tests of Normality[a]

	MachineTime1g	Kolmogorov-Smirnov[b] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
FoldChange	2	.182	3	.	.999	3	.935
	6	.175	3	.	1.000	3	.996
	24	.351	3	.	.827	3	.182

a. FoldChange is constant when MachineTime1g = 0. It has been omitted.

b. Lilliefors Significance Correction

2h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
FoldChange	Equal variances assumed	4.501	.101	-3.105	4	.036	-1.71705	.55291	-3.25218	-.18193
	Equal variances not assumed			-3.105	2.000	.090	-1.71705	.55291	-4.09603	.66193

6h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
FoldChange	Equal variances assumed	4.030	.115	1.146	4	.316	.35517	.30994	-.50537	1.21571
	Equal variances not assumed			1.146	2.000	.370	.35517	.30994	-.97841	1.68875

24h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	14.784	.018	1.747	4	.155	.42094	.24090	-.24790	1.08979
	Equal variances not assumed			1.747	2.000	.223	.42094	.24090	-.61557	1.45745

<u>*HEY1*</u>

Tests of Normality[a]

	MachineTime1g	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	2	.367	3	.	.794	3	.100
	6	.330	3	.	.867	3	.286
	24	.212	3	.	.990	3	.813

a. FoldChange is constant when MachineTime1g = 0. It has been omitted.

b. Lilliefors Significance Correction

2h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	15.617	.017	1.548	4	.196	.26892	.17367	-.21326	.75110
	Equal variances not assumed			1.548	2.000	.262	.26892	.17367	-.47832	1.01615

6h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	13.285	.022	2.688	4	.055	.44177	.16432	-.01446	.89800
	Equal variances not assumed			2.688	2.000	.115	.44177	.16432	-.26525	1.14879

24h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	5.614	.077	3.908	4	.017	.48273	.12352	.13979	.82566
	Equal variances not assumed			3.908	2.000	.060	.48273	.12352	-.04872	1.01418

Statistic test for Figure 15

<u>NOTCH1</u>

Tests of Normality[a]

	MachineTime1g	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	2	.340	3	.	.848	3	.236
	6	.379	3	.	.764	3	.031

a. FoldChange is constant when MachineTime1g = 0. It has been omitted.

b. Lilliefors Significance Correction

2h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	14.046	.020	-.186	4	.861	-.02506	.13468	-.39898	.34887
	Equal variances not assumed			-.186	2.000	.870	-.02506	.13468	-.60453	.55441

6h

Test Statistics[a]

	FoldChange
Mann-Whitney U	3.000
Wilcoxon W	9.000
Z	-.696
Asymp. Sig. (2-tailed)	.487
Exact Sig. [2*(1-tailed Sig.)]	.700[b]

a. Grouping Variable: MachineTime1g

b. Not corrected for ties.

NOTCH2

Tests of Normality[a]

	MachineTIME1G	Kolmogorov-Smirnov[b] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
FoldChange	2	.211	3	.	.991	3	.816
	6	.317	3	.	.889	3	.350

a. FoldChange is constant when MachineTIME1G = 0. It has been omitted.

b. Lilliefors Significance Correction

2h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	5.583	.077	.356	4	.740	.04349	.12204	-.29534	.38232
	Equal variances not assumed			.356	2.000	.756	.04349	.12204	-.48160	.56857

6h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	12.218	.025	-4.173	4	.014	-.42449	.10171	-.70689	-.14208
	Equal variances not assumed			-4.173	2.000	.053	-.42449	.10171	-.86213	.01315

NOTCH3

Tests of Normality[a]

	MachineTime1g	Kolmogorov-Smirnov[b] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
FoldChange	2	.331	3	.	.864	3	.280
	6	.278	3	.	.940	3	.526

a. FoldChange is constant when MachineTime1g = 0. It has been omitted.

b. Lilliefors Significance Correction

2h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	13.383	.022	1.440	4	.223	.06626	.04600	-.06147	.19399
	Equal variances not assumed			1.440	2.000	.286	.06626	.04600	-.13168	.26420

6h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	9.308	.038	-.491	4	.649	-.11796	.24011	-.78462	.54871
	Equal variances not assumed			-.491	2.000	.672	-.11796	.24011	-1.15108	.91517

<u>NOTCH 4</u>

Tests of Normality[a]

	MachineTime1g	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	2	.314	3	.	.894	3	.365
	6	.345	3	.	.840	3	.213

a. FoldChange is constant when MachineTime1g = 0. It has been omitted.

b. Lilliefors Significance Correction

2h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	11.966	.026	.620	4	.569	.13358	.21533	-.46428	.73145
	Equal variances not assumed			.620	2.000	.598	.13358	.21533	-.79293	1.06010

6h

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	14.380	.019	.492	4	.648	.05618	.11413	-.26070	.37305
	Equal variances not assumed			.492	2.000	.671	.05618	.11413	-.43489	.54724

Statistic test for Figure 16

NOTCH1

Tests of Normality[a]

	CoinForce2h	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	1	.327	3	.	.872	3	.302

a. FoldChange is constant when CoinForce2h = 0. It has been omitted.

b. Lilliefors Significance Correction

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	13.028	.023	.136	4	.898	.02812	.20605	-.54396	.60021
	Equal variances not assumed			.136	2.000	.904	.02812	.20605	-.85843	.91468

NOTCH2

Tests of Normality[a]

	CoinForce2h	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	1	.236	3	.	.977	3	.709

a. FoldChange is constant when CoinForce2h = 0. It has been omitted.

b. Lilliefors Significance Correction

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	6.768	.060	1.208	4	.294	.20848	.17255	-.27060	.68756
	Equal variances not assumed			1.208	2.000	.350	.20848	.17255	-.53395	.95091

NOTCH3

Tests of Normality[a]

	CoinForce2h	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	1	.257	3	.	.961	3	.622

a. FoldChange is constant when CoinForce2h = 0. It has been omitted.

b. Lilliefors Significance Correction

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	7.898	.048	1.557	4	.195	.44654	.28684	-.34987	1.24295
	Equal variances not assumed			1.557	2.000	.260	.44654	.28684	-.78765	1.68073

NOTCH4

Tests of Normality[a]

	CoinForce2h	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FoldChange	1	.255	3	.	.962	3	.627

a. FoldChange is constant when CoinForce2h = 0. It has been omitted.

b. Lilliefors Significance Correction

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
FoldChange	Equal variances assumed	7.820	.049	1.098	4	.334	.32543	.29646	-.49766	1.14852
	Equal variances not assumed			1.098	2.000	.387	.32543	.29646	-.95012	1.60097