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รายงานผลการวิจัย

สัญญาณนอชท์ในการแปรสภาพเป็นเซลล์ไขมันและเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดเม
เซนไคม์ที่แยกได้จาก โคลนเซลล์เดี่ยวของเนื้อเยื่อไขมันของมนุษย์

โดย

ธนภูมิ โอสถานนท์ และ ประสิทธิ์ ภวสันต์

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Project Title   Notch signaling in adipogenic differentiation of single-clone-derived mesenchymal stem cells isolated from human adipose tissue

Name of Investigators   Thanaphum Osathanon and Prasit Pavasant

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Abstract

Stem cells can be isolated from various tissues, including bone marrow, dental pulp, as well as adipose tissues. Due to the non-invasive isolation procedure, the adipose-derived mesenchymal stem cells (ADSCs) are introduced as an alternative stem cell source for regenerative medicine. In addition, it has been shown that Notch signaling participates in the control of ADSCs' behavior. However, those studies were performed in the heterogeneous population of ADSCs. In the present study, human adipose tissue derived single-cell clones were isolated using a cloning ring technique and characterized for their stem cell characteristics. In addition, the participation of Notch signaling on the differentiation toward osteogenic and adipogenic lineage of these adipose derived single-cell-clones was investigated. Eighty-five single cell clones were able to isolate from adipose tissues culture. However, only 14 clones were able to proliferate for further characterization. The results showed that all fourteen clones expressed embryonic and mesenchymal stem cell marker genes. All 14 clones were able to differentiate to both osteogenic and adipogenic lineages. Low adipogenic clones had higher Notch 2, 3, and 4, Jagged1, as well as Delta1 mRNA expression compared to high adipogenic clones. On the contrary, the Notch signaling component mRNA expression was not different between low and high osteogenic clones. Notch receptor mRNA expression decreased with the adipogenic differentiation of both low and high adipogenic clones. Inhibition of Notch signaling using γ-secretase inhibitor enhanced of adipogenic differentiation. Correspondingly, the initiation of Notch signaling using JAGGED1 bound surface resulted in a decrease of intracellular lipid accumulation. These results indicate that Notch signaling inhibited the adipogenic differentiation of single-cell-clone adipose derived mesenchymal stem cells.

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ชื่อโครงการวิจัย สัญญาณนอชท์ในการแปรสภาพเป็นเซลล์ไขมันและเซลล์สร้างกระดูกของเซลล์ต้น
กำเนิดเมเซนไคม์ที่แยกได้จาก โคลนเซลล์เดี่ยวของเนื้อเยื่อไขมันของมนุษย์
ชื่อผู้วิจัย ธนภูมิ โอสถานนท์ และ ประสิทธิ์ ภวสันต์
เดือนและปีที่ทำวิจัยเสร็จ สิงหาคม 2556

บทคัดย่อ

เซลล์ต้นกำเนิดสามารถแยกได้จากเนื้อเยื่อหลายชนิด เช่น ไขกระดูก เนื้อเยื่อในฟัน และเนื้อเยื่อ
ไขมัน เป็นต้น เซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันได้ถูกนำเสนอเป็นแหล่งเซลล์ต้นกำเนิดทางเลือกสำหรับ
การศึกษาที่เกี่ยวข้องกับการเจริญงอกใหม่เนื่องจากวิธีการได้มาของเนื้อเยื่อที่ไม่รุกรานร่างกาย มี
การศึกษาพบว่าสัญญาณนอชท์มีความเกี่ยวข้องในการควบคุมพฤติกรรมของเซลล์ต้นกำเนิดจากเนื้อเยื่อ
ไขมัน แต่การศึกษาเหล่านั้นมักจะศึกษาในลักษณะประชากรผสมของเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมัน ใน
การศึกษานี้ได้ทำการแยกโคลนเซลล์เดี่ยวของเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันของมนุษย์ด้วยเทคนิค
โคลนนิ่งริง เพื่อศึกษาคุณสมบัติของเซลล์ต้นกำเนิด นอกจากนี้ยังศึกษาบทบาทของสัญญาณนอชท์ในการ
แปรสภาพไปเป็นเซลล์สร้างกระดูกและเซลล์สร้างไขมันด้วย ในการศึกษานี้ได้ทำการแยกโคลนเซลล์เดี่ยว
จำนวน 85 โคลนจากเนื้อเยื่อไขมัน แต่พบว่ามีเพียง 14 โคลนที่สามารถเพิ่มจำนวนได้มากพอเพื่อ
ทำการศึกษาคุณสมบัติต่อได้ เซลล์ทั้ง 14 โคลนมีการแสดงออกของยีนบ่งชี้เซลล์ต้นกำเนิดจากตัวอ่อน
และเซลล์ต้นกำเนิดเมเซนไคม์ นอกจากนี้ยังสามารถแปรสภาพไปเป็นเซลล์สร้างกระดูกและเซลล์สร้าง
ไขมันได้ โคลนที่มีความสามารถในการแปรสภาพไปเป็นเซลล์สร้างไขมันได้ต่ำจะมีการแสดงออกของอาร์
เอ็นเอนำรหัสของ นอชท์ 2 นอชท์3 นอชท์4 เจ็กเก็ต1 และเดลต้า1 สูงกว่าโคลนที่มีความสามารถในการ
แปรสภาพไปเป็นเซลล์สร้างไขมันได้สูง ในขณะที่ไม่พบความแตกต่างในการแสดงออกของอาร์เอ็นเอนำ
รหัสของสัญญาณนอชท์ระหว่างโคลนที่มีความสามารถในการแปรสภาพไปเป็นเซลล์สร้างกระดูกต่ำและ
สูง การแสดงออกของอาร์เอ็นเอนำรหัสของนอชท์รีเซบเตอร์ลดลงเมื่อมีการแปรสภาพไปเป็นเซลล์สร้าง
ไขมันทั้งในโคลนที่มีความสามารถในการแปรสภาพไปเป็นเซลล์สร้างไขมันได้ต่ำและสูง นอกจากนี้ยัง
พบว่าเมื่อทำการยับยั้งสัญญาณนอชท์ด้วยสารยับยั้งแกมม่า ซีครีเตส จะเพิ่มความสามารถในการแปร
สภาพไปเป็นเซลล์สร้างไขมันได้มากขึ้น ซึ่งสอดคล้องกับเมื่อกระตุ้นสัญญาณนอชท์ด้วยพื้นผิวที่ตรึงด้วย
เจ็กเก็ต1 จะลดประสิทธิภาพในการสะสมไขมันในเซลล์ ผลการศึกษาเหล่านี้แสดงให้เห็นว่าสัญญาณ
นอชท์สามารถยับยั้งการแปรสภาพไปเป็นเซลล์สร้างไขมันในโคลนเซลล์เดี่ยวของเซลล์ต้นกำเนิดจาก
เนื้อเยื่อไขมัน

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Chapter 1

Introduction

Adipose tissues compose of several types of cells for example; mature adipocytes, preadipocytes, stromal fibroblast cells, macrophage and lymphocytes (Brown et al., 2010; Wilson et al., 2010). In addition, it has been shown that adipose tissues contains subpopulation of cells known as the stromal vascular fraction, which is mainly composed of mesenchymal stem cells (Brown et al., 2010; Wilson et al., 2010).

Adipose derived mesenchymal stem cells (ADSCs) have special characteristics that suggest their potential for use in regenerative medicine, such as the ability to be proliferative after transplantation, multipotential differentiation capacity, and the ability to release angiogenic growth factor(Miranville et al., 2004; Moon et al., 2006; Planat-Benard et al., 2004).Moreover, ADSCs can be stimulated to become specific cell types (Cheng et al., 2012; Choi et al., 2010; Choi et al., 2012; Kim et al., 2012; Lu et al., 2011; Marino et al., 2012; Park et al., 2011; Scholz et al., 2011; Taha and Hedayati, 2010).Thus, ADSCs are a candidate stem cell source for various regenerative therapeutic applications.

Notch signaling plays an important role in development and differentiation, and mediates its functions through direct cell-cell contact. The expression of Notch signaling components in adipose derived mesenchymal stem cells has been reported (Andersen et al., 2008; Bray, 2006). Notch signaling alteration effected the ability of adipose derived mesenchymal stem cells to differentiate into various cell types including the adipogenic, osteogenic and neurogenic lineages (Cornell and Eisen, 2005; Huang et al., 2010; Jing et

al., 2010; Joseph et al., 2004; Kingham et al., 2009; Taylor et al., 2007; Wrage et al., 2008). However, these experiments were conducted using **heterogeneous** populations of cells isolated from adipose tissue.

Despite the evidences confirming crucial function of Notch signaling in adipose stem cells, the biological analysis of its function in single cell-derived adipose stem cell clone, which has **homogeneous** cell population, is indeed lacking. The investigation of differentiation ability of single-cell-clones derived from human adipose tissue and the potential role of Notch signaling on the differentiation capacity of these adipose derived single-cell-clones will further knowledge on basic stem cell biology toward clinical application.

The present study aims to (1) establish single-cell-derived adipose mesenchymal stem cell clones and investigate their stem cell characteristics as well as osteogenic and adipogenic differentiation capacity, (2) investigate the expression pattern of Notch signaling component in undifferentiated state and upon osteogenic as well as adipogenic differentiation of single-cell-clone-derived adipose mesenchymal stem cells, and (3) examine the potential role of Notch signaling in the control of osteogenic and adipogenic differentiation of adipose derived mesenchymal stem cells.

Chapter 2

Literature Review

Adiposed-derieved mesenchymal stem cells (ADSCs)

A stem cell is the cell that has ability to self renew and to differentiate with multilineage potential. The ideal stem cell for regenerative medicine application should be abundant, easy to harvest, able to control and reproduce the multiple lineage differentiation, safe and effective for transplantation(Gimble, 2003). Stem cells can be isolated from wide variety of tissues i.e. bone marrow stem cells, pulp dental stem cells, adipose stem cells and etc.

Adipose tissue consists of mature adipocytes, preadipocytes, connective tissue matrix, which is established by collagen, elastin, stromal cell neurovascular structure, resident monocytes, macrophages, lymphocytes(Brown et al., 2010; Wilson et al., 2010). In addition, adipose tissue also contains a group of cell called stromal vascular fraction (Wilson et al., 2010). Cells in this fraction are composed of mesenchymal stem cells and other cell types such as circulating blood cells, fibroblasts, pericytes, endothelial cells, preadipocyte or adipocyte progenitors cell (Brown et al., 2010). The niche of adipose-derived mesenchymal stem cells (ADSCs) has not yet been known. Some researcher hypothesize that these cells are found at surrounding the blood vessels, therefore, it can show that ADSCs are subpopulation of fibroblast in adipose tissue(Bianco et al., 2008; Crisan et al., 2008; da Silva Meirelles et al., 2008; Dellavalle et al., 2007).

ADSCs have the special characteristic that make them have potential benefits for grafting, such as the ability to perpetually proliferative after transplantation, the multipotential differentiation capacity and the ability to release angiogenic growth factor (Miranville et al., 2004; Moon et al., 2006; Planat-Benard et al., 2004). Moreover, ADSCs can be stimulated to become other specific cells, such as adipocyte, cardiomyocyte, chondrocyte, endothelial, myocyte, neuronal-like and osteoblast(Brown et al., 2010; Zuk et al., 2002).

When cultured ADSCs with adipogenic medium, the cells express the genes including lipoprotein lipase, PPARgamma2, leptin, Glut4. Moreover, the accumulation of intracellular lipid droplets can be also used for adipogenic character (Sen et al., 2001; Wickham et al., 2003; Zuk et al., 2001; Zuk et al., 2002). Upon osteogenic induction of ADSCs, the expression of genes and proteins are observed to associate with an osteoblasts phenotype, including type I collagen, osteopontin, osteonectin, osteocalcin, bone sialoprotein, RunX-1, BMP-2, BMP-4, BMP receptor I and II, PTH-receptor and also alkaline phosphatase(Halvorsen et al., 2001; Zuk et al., 2002). Finally, the differentiation of ADSCs along the neuronal lineages has the exhibition of neural markers including nestin, neuron-specific enolase (NSE) and neuron specific protein (NeuN). The expression of trkA and voltage gated potassium channels have also detected (Ashjian et al., 2003; Safford et al., 2002; Zuk et al., 2002).

In our laboratory, we were able to isolate the heterogeneous population of human adipose derived stem cells(Prasitsumrit, 2011). The isolated cells exhibited mesenchymal-like morphology and the expression of STRO-1 protein was also noted

(Fig. 1A and B). These cells were expressed both embryonic (*OCT4* and *NANOG*), neural crest cell (*NESTIN*) and mesenchymal stem cell (*CD44, CD73* and *CD105*) markers (Fig. 1C). In addition, this heterogeneous population was able to differentiate into adipogenic lineage as determined by intracellular lipid accumulation (Fig. 2). Upon exposed cells with osteogenic medium, the upregulation of alkaline phosphatase enzymatic activity was observed (Fig. 3A). In addition, the mineral deposition was noted upon cultured heterogeneous adipose derived mesenchymal stem cells in osteogenic induction medium (Fig. 3B). The increase of both alkaline phosphatase enzymatic activity and mineralization was occurred in time dependent manner. Finally, these cells were able to form neurospheres in neurogenic condition (Fig. 4), corresponding with the mRNA upregulation of neurogenic markers (Fig. 4E). Together, the data in our laboratory confirm previously published evidences of **heterogeneous** adipose derived mesenchymal stem cell's characteristics and lead us to further investigate and establish single cell derived clone for exploring basic adipose stem cell biology and potential therapeutic application.

In vivo study, ADSCs in combination of biodegradable genipin crosslinked gelatin annexed with tricalcium phosphate were utilized for peripheral nerve regeneration in rat. The results showed that morphology and pattern of regenerated nerve fibers were similar to those of the autologous grafts, suggesting the potential clinical application in neuro-regenerative treatment (Liu et al., 2012). Further, the potential utilization of ADSCs in clinical treatment has been investigated in various applications for both soft and hard tissue regeneration for example, grafting for mastectomy defects (Perez-Cano et al.,

2012), facial tissue defects (Sterodimas et al., 2011), skin defects (Kim et al., 2011), and cranial bone defects(Thesleff et al., 2011). Together, these data suggest that ADSCs are a candidate stem cell source for various regenerative therapeutic applications.

Notch signaling

Notch signaling plays an important role in cell development, cell cycle, and cell differentiation. Notch signaling contains of Notch receptor and ligand. Notch receptor is a transmembrane protein containing 3 subunits; 1) large extracellular portion, 2) transmembrane portion and 3) small intracellular portion (Blaumueller et al., 1997; Brou et al., 2000)(Logeat et al., 1998). The extracellular portion is composed of epidermal growth factor (EGF)-like repeated unit and plays a role in interacting with the domain of notch ligand (Rebay et al., 1991).

Four types of Notch receptor are indentified in mammalian, namely, Notch-1, Notch-2, Notch-3 and Notch-4 (Zhang et al., 2008). For the ligand, it is also a transmembrane protein. Notch ligand is classified into 2 groups, Delta and Jagged. The ligands found in mammalian are Delta1, Delta3, Delta4, Jagged1 and Jagged2 (Chiba, 2006). Notch signaling critically mediates cellular function through direct cell-cell-contact. When notch receptor interacts with its ligand, the signaling cascade is initiated and further regulates cell fate decisions to differentiate, proliferation or apoptosis (Engin and Lee, 2010; He et al., 2009; Lovschall et al., 2005).

In the process of Notch protein synthesis, Notch proteins are transported to the golgi network and the first cleavage is occurred by a furin-like enzyme to form a two non-covalently attached subunits and then transferred to cell surface (Engin and Lee,

2010). Upon notch receptor interacts with its ligand, the second cleavage occurs, resulting in a split of the extracellular domain away from transmembrane portion. This second cleavage is performed using metalloproteinase called tumor necrosis factor-alpha converting enzyme (TACE). Subsequently, notch receptor is cleaved by the gamma-secretase complex and further release Notch intracellular domain (NICD) from the transmembrane domain(Edbauer et al., 2003; Lovschall et al., 2005). NICD then activates various target genes to determine and control cellular behavior. The common target genes are Hes and Hey family.

Hes family is the basic helix-loop-helix transcription factors, which is the target gene of canonical Notch signaling (except Hes2 and 3) (Zanotti and Canalis, 2010). Hes1, 3, and 5 regulate the maintaining of precursor cells in undifferentiated state (Zanotti and Canalis, 2010). Another target gene for Notch signaling is Hey family. Hey proteins share significant resemblance. Hey1 and 2 combination knockout mice exhibited similar phenotype as those occurred in Notch1 knockout mice (Zanotti and Canalis, 2010). Yet, CSL can also bind to other promoters, which implies other potential target genes in Notch signaling (Zanotti and Canalis, 2010).

<u>Notch signaling in heterogeneous adipose-derived mesenchymal stem cells (ADSCs)</u>

It has been found that subpopulation of stromal vascular fraction of adipose tissues expresses Notch1, Delta1, Delta4, Jagged1, Nump and Hes1 (Andersen et al., 2008; Bray, 2006). Using DAPT (gamma-secretaseinhibitior) to block notch signaling, Notch signaling have had no effect on in neurothophic and myelination ability (Kingham et al., 2009) and neuro-gial proliferation and expression of neuro-gila marker (Wrage et al.,

2008)of ADSCS. However, Notch signaling has altered ADSCs cells growth, morphology or marker expression in osteogenesis, adipogenesis, neurogenesis, Schwann cell development. Jing *et al.* have found that notch signaling induce the proliferation and reduced the osteogenesis in ADSCs(Jing et al., 2010). In adipogenesis, it has been found that DAPT have inhibited Notch2-Hes1 signaling pathway and have promoted transcription of PPAR-gamma (marker of adipocyte differentiation) and depressed the transcription of DLK-1/Pref-1 (marker of undifferentiated pre-adipocytes). These results suggest that blocking notch signaling induced adipogenesis of ADSCs by mean of PPAR-gamma promotion and DLK-1/Pref-1 depression through inhibition of Notch2-Hes1 (Huang et al., 2010). Moreover, Notch1 signaling suppresses neurogenesis and promotes development of Schwann cell (Cornell and Eisen, 2005; Joseph et al., 2004; Taylor et al., 2007).

In our previous study, the heterogeneous population of adipose-derivedmesenchymal stem cell had the alteration in the Notch target gene mRNA expression after differentiation toward osteogenic, adipogenic and neurogenic lineages (Fig. 5).In this regard, the downregulation of *HES1* was noted upon cells differentiated into adipocyte and neuron while the *HES1* mRNA levels was not changed in osteoblast differentiation process. In the regard of another Notch target gene, *HEY1*, the mRNA levels was increased upon differentiation in all three lineages. These results suggest the preferential role of Notch signaling in particular differentiation process of adipose-derived mesencymal stem cells.

Significance of the investigation in homogeneous population.

Using standard isolation method, heterogeneous population of stem cell was usually obtained. However, upon exposing heterogenous population of stem cells in differentiation medium, only subpopulation of cells was undergone differentiation into desired lineage. For example, it has been indicated that mesenchymal stem cells differentiating into cardiomyocytes was less than thirty percents (Wei et al., 2011). In addition, selected subpopulation of mesenchymal stem cells, which had high cardiomyogenic differentiation capacity, had low adipogenic, osteogenic and chondrogenic differentiation potency (Wei et al., 2011). In adipose tissue derived mesenchymal stem cells, it has been demonstrated that selected cell subpopulation isolated from adipose tissues had different differentiation potency(Paredes et al., 2011; Rada et al., 2011). Thus, to investigate the role of specific signaling on stem cell's behaviors, the homogeneous population is indeed required. In this proposal, the isolation of single cell clone will be employed to obtain homogeneous population of adipose stem cells.

Chapter 3

Research Methodology

Cell isolation and characterization

The protocol for the isolation of human adipose derived stem cells (hADSCs) was approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Adipose tissue was initially acquired fromresecting hip subcutaneous tissues during iliac bone harvesting procedure for craniofacial reconstructive surgery. The adipose tissue was washed with sterile PBS, cut into small pieces and digested with collagenase (Sigma, USA)and maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA), containing 10% fetal bovine serum (FBS; Gibco, USA), 2mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5μg/mL amphotericin B (GIBCO, USA) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After the cells reached confluence, they were sub-cultured at a 1:3 ratio. Individual clones were isolated using a standard cloning ring technique.

Doubling time

Doubling time was determined as described by Singhatanadgitet al. (Singhatanadgit et al., 2009). Briefly, hADSCs (1.25 X 10^4 cells) were seeded into six-well plates, cultured in growth mediumfor 96 h, trypsinized, and counted using a hemocytometer(Bright-LineTMHemacytometer; Sigma). The populationdoubling time was calculated according to the following equation:

$$\text{Doubling time} = \frac{\text{Time in culture (h) x ln (2)}}{\text{ln (the number of harvested cells/thenumber of seeded}}$$

Colony forming unit assay

To test the colony forming unit–fibroblast (CFU-F) capability of the isolated cells, single-cell suspensions (500cells) were seeded into 35-mm-diameter culture dishes and maintained in growth medium for 14 days. Cells were then fixed in 10% buffered formalin for 10 min, washed twice with PBS and stained with methylene blue. Aggregates of approximately ≥ 50 cells were counted under a microscope and scored ascolonies.

Differentiation assay

For adipogenic differentiation, 2.5×10^4 cells were plated on48-well-plates and maintained in adipogenic medium [growth medium containing insulin (0.1 mg/ml), dexamethasone (1µM), 3-isobutyl-1-methylxanthine (1 mM), and indomethacin (0.2 mM)] for 14 days. The cells in control condition were maintained in normal growth medium.Medium was changed every 72 h. The cells were then fixed in 10% buffered formalin, rinsed twice with PBS, and stained with 0.2 % Oil Red O (OR) in propanol for 5 min. The cells were examined under a microscope to identify the presence of lipid droplets. Subsequently, the samples were extracted with 100% propanol, and the absorbance was measured at 490 nm. In other similarly cultured samples, RNA was isolated and evaluated for adipogenic marker gene expression using RT-PCR methods as described below.

To examine osteoblast differentiation, the cells were seeded at a density of 2.5

$x10^4$cells/well on 48-well-plates and maintained in an osteogenic medium (growth medium supplemented with ascorbic acid (50 μg/mL), dexamethasone (100nM), and sodium phosphate (2mM) or beta-glyceraphosphate (10mM)). The cells were cultured for 14 days with the medium changed every 48 h. The cells in control condition were maintained in normal growth medium.Mineralization was evaluated using Alizarin Red S (AR) staining. Calcium deposition was quantified by destaining with 10% cetylpyridinium chloride monohydrate (Sigma, USA) in 10 mM sodium phosphate at room temperature for 15 min and determinedspectrophotometically at 570 nm. Osteoblast marker gene expression was investigated using the RT-PCR methods described below.

To determine the role of Notch signaling on cell differentiation, hADSCs were treated with aγ-secretase inhibitor (DAPT; Sigma) at 25 μM to inhibit the cleavage of the Notch intracellular domain, thus, inhibiting the Notch signaling cascade. In some experiments, hADSCs were plated on an indirect affinity immobilization of Notchligand, Jagged-1 (R&D Systems, USA) according to the protocol previously reported(Beckstead et al., 2009).

Transfection and retroviral transduction (Osathanon et al., 2013a)

The retroviral plasmid for expression of dominant negative mastermind-like transcriptional factor (DN-MAML; MSCV-Mam(12-74)-EGFP), a kind gift from Dr. Warren Pear (University of Pennsylvania, PA, USA) and Associate Professor Dr. TanapatPalaga (Chulalongkorn University, Thailand) or the control vector (MSCV-IRES-GFP, Addgene plasmid 20672) was co-transfected with packaging construct pCL-Ampho (Imagenex) into HEK293 cells using the FuGene® HD transfection reagent (Roche, USA) according to the

manufacturer's instructions. Culture supernatants containing retroviruses were harvested twice at 48 and 72 hours after transfection and were used to transduceADSCs.

Immobilization of Jagged-1 (Osathanon et al., 2013a)

Surface-bound Notch ligand, Jagged-1, was fabricated by an indirect affinity immobilization method previously reported by our group(Osathanon et al., 2013a; Osathanon et al., 2013b). Briefly, recombinant protein G (50 μg/mL: Zymed, USA) was incubated with the tissue culture plate surface for 16 hours and subsequently, incubated with BSA (10 mg/mL; Sigma, USA) for 2 hours. The surface was further incubated with recombinant human Jagged-1 (10 μM: R&D Systems, USA) for 2 hours. The surface was washed three times with sterile PBS between each step. An equal amount of human IgG-Fc fragment (Jackson Immunoresearch Laboratory, USA) was employed as a control. Prior to seeding the cells, the surfaces were washed once with culture media.

Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted with Trizol reagent (Roche Diagnostics, USA) according to the manufacturer's instructions. RNA samples (1 μg) were converted to cDNA by avian myeloblastosis virus (AMV) reverse transcriptase (Promega, USA)for 1.5 hours at 42 $^{\circ}$Cand, subsequently, the reaction was stopped by incubating in 99°C for 2 minutes. A semi-quantitative polymerase-chain reaction (PCR) was performed using Tag polymerase (Tag DNA Polymerase, Invitrogen, Brazil)with PCR volume of 25 μL. The reaction mixtures contained 25 pM of primers and 1 μL of cDNA. The PCR working conditions were set at a denaturation for 1 minute at 94°C, primer annealing for 1

minuteat 60°C, and chain elongation for 1.45 minutes at 72°C on a DNA thermocycler (MJ Mini personal thermocycler, Biorad, USA).The oligonucleotide sequences of the primers are shown in Table 1(Chuenjitkuntaworn et al., 2010; Conley et al., 2000; Osathanon et al., 2011; Patel et al., 2005; Phi et al., 2010; Schilling et al., 2007; Valbuena et al., 2006; Wongkhantee et al., 2008; Xu et al., 2004). Theamplified DNA was then electrophoresed on a 1.8% agarose gel and visualized with ethidiumbromide (EtBr; Bio-Rad, USA) fluorostaining.The band density was evaluated using ImageJ software. The number of gene expression was normalized to GAPDH. The normalized expression value of the control had set as1 in each gene.The relative expression value was further compared to the control.

Statistical analyses

Theexperiments were performed in triplicate. Data are expressed as mean±standard deviation. Statistical analyses were performed using independent t-tests for two group comparisons and a one-way analysis of variance (ANOVA), followed byDunett test for multiple group comparisons. Differences at $p<0.05$ were considered to be statistically significant.

Chapter 4

Results

Morphology, doubling time and stem cell marker expression.

Homogeneous single cell derived clones wereisolated and cultured using a cloning ring technique. Eighty-five hADSCs clones were isolated.Of these, fourteen clones were able to proliferate through passage 4 for use in the characterization study.Cellular morphology was different among these clones (Fig. 6A). In general, the cells had a spindle, fibroblast-like morphology. However, clone 1 and 9 exhibited a stellate and epithelial-like morphology, respectively.Individual clones showed variationsin doubling time and colony forming unit ability, ranging from approximately 50 h–250 h and from approximately 20–180 colonies, respectively (Fig. 6B and C). The average doubling time and number of colony forming units were97.81±53.85 h and 61.97±48.00 colonies, respectively. The mRNA expression of embryonic and mesenchymal stem cell markers (Oct4, Nanog, Rex 1, CD73, and CD105) was found in all clones (Fig. 7).While the CD73 and CD105 mRNA expression levels were relatively similar among these clones,different mRNA expression levels ofthe embryonic stem cell markers Oct4, Nanog, and Rex1 were noted.Among all the clones, clones 7 and 8 had the highest mRNA expression of the embryonic stem cell markers we assayed.

Effect of passage number on colony forming unit, doubling time and stem cell marker expression.

Four clones (C2, C3,C8 and C55) were selected to continue passage until passage 10. The colony forming unit, doubling time and stem cell marker expression were examined to compare between passage 4 and 10. The results illustrated that there is no significant different of doubling time and colony forming unit between passage 4 and 10. (Table 2). In addition, the levels of mRNA expression were comparable in both passage, except C3 which the decrease of *OCT4* and *CD105* mRNA levels was noted in passage 10 compared to passage 10 (Fig. 8).

Osteogenic and adipogenic differentiation.

All fourteen hADSCsclones were able to differentiate to osteogenicand adipogenic lineages (Figs. 9, 10). All clones exhibited an increase in mineral deposition when cultured in osteogenic medium for 14 days (Fig. 9A and B). We classified clones with quantified AR staining of less than 1 as low osteogenic clones (clones 1,2,4, and 7) and those with staining values of 2 and above as high osteogenic clones (clones 4, 5, 6, 8, 9, 36, 37, 55, 63, and 70). Using clones 1 and 5 as representativelow and high osteogenic clones, respectively, an increase in the mRNA expression of the osteogenic marker genes ALP and OPN was noted in both clones after culturing in osteogenic conditions (Fig. 9C). Concerning adipogenic differentiation, all clones were able to differentiate to the adipogenic lineage. An increase in intracellular lipid accumulation was observed in cells exposed to adipogenic induction medium for 14 day (Fig. 10A and B). We classified clones with quantified OR staining of less than 0.2 as low adipogenic

clones (clones 1–9) and those with staining values of 0.3 and above as high adipogenic (clones 36, 37, 55, 63, and 70). Although the differentiation capacitydiffered among these clones,a markedincrease in themRNA expression of the adipogenic marker genes LPL and PPARɣ2 was noted in both representative high (clones 55 and 63) and low (clones 4 and 5) adipogenic clones.Notably, clones 36, 37, 55, 63, and 70 were both high osteogenic and adipogenic clones, clones 1,2,3,and 7 were low osteogenic and adipogenic clones, and clones 4,5,6, 8, and 9 were high osteogenic but low adipogenic clones. Together, these data imply that each clone had different basal properties with some intrinsic factorscontrolling their differentiation capacity.

Intrinsic mRNA expression of Notch signaling components.

As it has been reported that Notch signaling is involved in the differentiation of stem cells, the intrinsic mRNA expression of Notch pathway participants was evaluated. Figure 11 shows the mRNA expression levels in both high and low osteogenic and adipogenic clones. Interestingly, high adipogenic clonesexhibited lower mRNA expression of Notch receptors and ligands (Notch 2, 3, 4, Jagged1 and Delta1) compared to low adipogenic clones. In contrast, there was no apparent difference in the mRNA expression of Notch receptors and ligands between low and high osteogenic clones.We next evaluated the expression of these Notch signaling molecules upon adipogenic differentiation. We found decreased expression ofNotch receptor mRNA in both high, clone 55, and low, clone 4,adipogenic clones (Fig. 12).

Intrinsic mRNA expression of Integrin, extracellular matrix protein and osteoblast regulation factors.

In Fig. 13, higher mRNA expression of *integrin alpha5*, *SPARC*, *TWIST1*, *TWIST2*, and *TGF-beta1* was noted in low osteogenic clones compared to those of high osteogenic clones. While low adipogenic clones seems to have less of these genes compared to high adipogenic clones.

Notch signaling inhibits adipogenic differentiation.

To further evaluate the involvement of Notch signaling in the adipogenic differentiation of human adipose derived mesenchymal stem cells, clones 4 and 55 were again used as representatives of low and high adipogenic clones, respectively. A γ-secretase inhibitor (DAPT) was employed to inhibit the cleavage of the intracellular domain of the Notch receptor, resulting in the inhibition of Notch signaling. Treatment with DAPTenhanced the adipogenic differentiation of both low and high adipogenic clones (Fig. 14). Correspondingly, theseclones seeded on Jagged1 bound surfaces exhibited lower intracellular lipid accumulation, indicating that their adipogenic differentiation was attenuated by Notch signaling (Fig. 15).

Chapter 5

Discussion

In the present study, we found Notch signaling inhibited the adipogenic differentiation of single-cell-derived mesenchymal stem cell clones isolated from human adipose tissue. We found thathighadipogenic clones exhibited lower intrinsic mRNA expression of Notch receptors and ligands compared to lowadipogenic clones. Inhibiting Notch signaling resulted in an enhancement of adipogenic differentiation, while the exposure of cells to a Notch ligand resulted in an attenuation of the adipogenic differentiation in both high and low adipogenic clones.

The multipotential differentiation capacity of stem cells is a main valuable characteristic for regenerative medicine. Using standard isolation method, heterogeneous population of stem cell was usually obtained.However, upon exposing heterogenous population of stem cells in differentiation medium, only subpopulation of cells was undergone differentiation into desired lineage. For example, it has been indicated that mesenchymal stem cells differentiating into cardiomyocytes was less than thirty percents (Wei et al., 2011). In addition, selected subpopulation of mesenchymal stem cells, which had high cardiomyogenic differentiation capacity, had low adipogenic, osteogenic and chondrogenic differentiation potency (Wei et al., 2011). In adipose tissue derived mesenchymal stem cells, it has been demonstrated that selected cell subpopulation isolated from adipose tissues had different differentiation potency (Paredes et al., 2011; Rada et al., 2011). In this respect, clones that had low basal

expression of osteogenicgenes exhibited high differentiation capacity towardadipogenic lineages (Paredes et al., 2011), implying that basal gene expression may pre-determines cell fate decision. In the present study, we observed a relationship between the intrinsic expression of Notch signaling components and the adipogenic differentiation potential of human adipose derived mesenchymalsingle-stem-cell clones. Clones with a low intrinsic mRNA expression of Notch ligands and receptors had a higher adipogenic differentiation potential. Together, these data emphasize the influence of intrinsic factor of stem cells on their differentiation potency. Thus, selecting stem cell subpopulation based on their specificintrinsic gene expression may facilitate differentiation procedure in regenerative therapeutic approach.

From a heterogeneous adipose tissue population, a subpopulation of the stromalvascular fraction has been identified expressing Notch1, Delta1, Delta4, Jagged1, Numb and Hes1(Andersen et al., 2008; Bray, 2006). Notch signaling influenced the growth as well as the osteogenic and adipogenic differentiation of a heterogeneous population of adipose stem cells(Huang et al., 2010; Jing et al., 2010).In this regard, it has been shown that Notch signaling induced cell proliferation and reduced osteogenic differentiation in murine adipose stem cells (Jing et al., 2010). Moreover, it has been reported thathuman mesenchymal stem cell clones, which highly expressed Notch ligand, had a high mineralization potential (Fujita et al., 2008). In contrast, we foundno obvious difference in the baseline mRNA expression of Notch receptors and ligands between low and high osteogenic clones. This discrepancy may be due to the tissue source of the mesenchymal stem cells obtained.

Regarding, adipogenic differentiation, inhibition of the Notch signaling pathway in murine adipose derived mesenchymal stem cells promotedtranscription of PPAR-gamma, a marker of adipogenic differentiation, and suppressed transcription of DLK-1/Pref-1, a marker of undifferentiated pre-adipocytes(Huang et al., 2010). In support of our study, these results indicate that the pharmacological blocking of Notch signaling induced adipogenesis in a heterogeneous population of adipose stem cells.In addition, the supplement of γ-secretase inhibitior, a Notch signaling inhibitor, enhances adipogenesis in human mesenchymal stem cells, while no effect on osteogeneis was noted (Vujovic et al., 2007). Overexpression of Notch intracellular domain or Notch ligand, Jagged-1, potently inhibited intracellular lipid accumulation and decreased PPAR-gamma, fatty acid bonding protein-4 and adiponectin expression (Ugarte et al., 2009). In the present study, adipogenic differentiation in both low and high adipogenic clones was enhanced after treating the cells with a γ-secretase inhibitor, but was attenuated when the cells were exposed to an exogeneous Notch ligand, demonstratingthat Notch signaling controls the adipogenic differentiation of adipose derived single-cell clones.

Currently, we used γ-secretase inhibitor for the inhibition experiments. Several limitation of this approach was noted i.e. non-specific inhibition effects, and some toxicity at high dose. To further avoid these limitations as well as to specific inhibition of Notch signaling in genetic levels, dominant negative mastermind liked protein (DN-MAML) was employed in the study using the retroviral transduction. DN-MAML retroviral transduction inhibits mastermind activity, thus resulting in the inhibition of Notch target gene expression. In the present study, we also performed the stable inhibition of Notch

signaling in the heterogeneous population using DN-MAML retroviral transfection to inhibit intracellular Notch signaling. However, the examination of Notch target gene expression upon transfection illustrated that forced expression of DN-MAML cannot effectively inhibit Notch signaling in these cells (Fig 16). This may due to the difficulty of retroviral transfection in human primary cells. The genetic manipulation to further investigate the influence of Notch signaling in adipose-derived mesenchymal stem cell is indeed necessitated. The future study will have to address this point.

The differential expression of Notch receptor impacts cell behaviors. In this regard, cell fate determination in olfactory epithelium was regulated by the alteration of Notch receptor as shown in olfactory epithelium (Carson et al., 2006). Moreover, it has been shown that Notch1, Notch2 and Notch3 involved in inflammatory kidney diseases but the role of these receptors was not redundancy(Djudjaj et al., 2012).Notch 1 selective inhibition resulted in the reduction of cancer cell proliferation and angiogenesis while inhibition of Notch1 and Notch2 resulted in severe toxicity (Wu et al., 2010). In the present study, we demonstrated that Notch1 expression was relatively higher than Notch2, Notch3, and Notch4. This differential intrinsic expression pattern of Notch receptors in both low and high adipogenic clone may influence cell behaviors. In this respect, it has been reported that Notch1 played important role in adipogenic differentiation. The decrease of Notch1 expression was noted upon adipogenic induction of pre-adipocyte cell line (3T3-L1) and the inhibition of Notch1 expression using Notch1 antisense attenuated adipogenesis (Nueda et al., 2007). Correspond to the present study, we showed that Notch1 expression was relatively similar between high and low

adipogenic clone and decreased upon adipogenic differentiation, confirming the functional role of Notch1 in adipogenesis. Thus, these observations may imply that Notch1 expression is required for initiation adipogenic differentiation and, subsequently, the downregulation of Notch1 is necessitated during maturation of adipogenesis. Further investigation is indeed essential to provide and confirm the role of Notch1 in adipogenic differentiation.

In the present study, we focused only the canonical pathway of Notch signaling. However, non-canonical pathway also plays roles in cell behaviors. The results from this study can limited explain partial roles of Notch signaling in single-cell-derived adipose mesenchymal stem cells. The studies on non-canonical pathway should be further examined.

Chapter 6

Conclusion

In summary, Notch signaling regulated adipogenesis in adipose tissue-derived mesenchymal stem cells. In addition, the intrinsic expression of Notch receptor is related to adipogenic differentiation potency, but not osteogenic differentiation. Notch signaling activation resulted in the attenuation of adipogenesis while the inhibition of Notch signaling resulted in the enhancement of adipogenic differentiation. This knowledge could be applied to identify subpopulations of adipose derived stem cells that are susceptible to adipogenic differentiation, using expression of Notch signaling components as markers. These cells may be of use in adipose tissue engineering to fulfil soft-tissue augmentation demands.

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Table 1 Primer sequences

Gene	Primer sequences(forward)	Primer sequences(reward)
OCT4	5' AGACCCAGCAGCCTCAAAATC 3'	5' GCAACCTGGAGAATTTGTTCCT 3'
NANOG	5' GGAAGAGTAGAGGCTGGGGT 3'	5' TCTCTCCTCTTCCTTCTCCA 3'
REX1	5' AGAATTCGCTTGAGTATTCTGA 3'	5' GGCTTTCAGGTTATTTGACTGA 3'
CD73	5' ACACTTGGCCAGTAAAATAGGG 3'	5' ATTGCAAAGTGGTTCAAAGTCA 3'
CD105	5' CATCACCTTTGGTGCCTTCC 3'	5' CTATGCCATGCTGCTGGTGGA 3'
NESTIN	5' CTGCGGGCTACTGAAAAG TT 3'	5' AGGCTGAGGGACATCTTGAG 3'
SOX9	5' GAACGCACATCAAGACGGAG 3'	5' TCTCGTTGATTTCGGTGCTC 3'
SOX2	5' ACCAGCTCGCAGACCTAC AT 3'	5' ATGTGTGAGAGGGGCAGTGT 3'
NMD	5' CACTGATAACTCGCCGTCCT 3'	5' CTCTTCAGCTTGGCTGCTCT 3'
LPL	5' GAGATTTCTCTGTATGGCACC 3'	5' CTGCAAATGAGACACTTTCTC 3'
PPARγ2	5' GCTGTTATGGGTGAAACTCTG 3'	5' ATAAGGTGGAGATGCAGGCTC 3'
ALP	5' CGAGATACAAGCACTCCCACTTC 3'	5' CTGTTCAGCTCGTACTGCATGTC 3'
OPN	5' AGTACCCTGATGCTACAGACG 3'	5' CAACCAGCATATCTTCATGGCTC 3'
NOTCH1	5'-GCCGCCTTTGTGCTTCTGTTC-3'	5'-CCGGTGGTCTGTCTGGTCGTC-3'
NOTCH2	5' CCAGAATGGAGGTTCCTGTA 3'	5' GTACCCAGGCCATCAACACA 3'
NOTCH3	5' TCTTGCTGCTGGTCATTCTC 3'	5' TGCCTCATCCTCTTCAGTTG 3'
NOTCH4	5' AGCCGATAAAGATGCCCA 3'	5' ACCACAGTCAAGTTGAGG 3'
JAGGED-1	5' AGTCACTGGCACGGTTGTAG 3'	5' TCGCTGTATCTGTCCACCTG 3'
DLL1	5' AGACGGAGACCATGAACAAC 3'	5' TCCTCGGATATGACGTACAC 3'

Gene	Primer sequences(forward)	Primer sequences(reward)
HEY1	5' GGAGAGGCGCCGCTGTAGTTA 3'	5' CAAGGGCGTGCGCGTCAAAGT A 3'
HES1	5' AGGCGGACATTCTGGAAATG 3'	5' CGGTACTTCCCCAGCACACTT 3'
Integrin β1	5' AGGAACAGCAGAGAAGCTCA 3'	5' AGCCGTGTAACATTCCTCCA 3'
SPARC	5' GAAAATCCCTGCCAGAACCA 3'	5' AACTGCCAGTGTACAGGGAA 3'
TWIST1	5' TCTTACGAGGAGCTGCAGACGCA 3'	5' ATCTTGGAGTCCAGCTCGTCGCT 3'
TWIST2	5' GCTGCGCAAGATCATCCC 3'	5' GTAGCTGCA GCTGGTCATC 3'
FN	5' GGATCACTTACGGAGAAACAG 3'	5' GGATTGCATGCATTGTGTCCT 3'
TGF-β1	5' GCTAATGGTGGAAACCCACA 3'	5' AGTGAACCCGTTGATGTCCA 3'
GAPDH	5' TGAAGGTCGGAGTCAACGGAT 3'	5' TCACACCCATGACGAACATGG 3'

Table 2 Effect of Passage number on doubling time and colony forming unit

Clone No.	Doubling time (hours)		Colony forming unit (Colony count)	
	P4	P10	P4	P10
C2	178.87±58.72	120.53±59.71	19.66±7.76	18.33±5.50
C3	114.01±13.91	285.15±177.22	34.66±8.38	37.00±7.21
C8	85.14±28.18	75.69±15.40	47.66±17.15	43.66±5.03
C55	70.61±4.70	71.21±13.18	44.66±6.65	26.66±2.51

Morphology — A — 200 Pixel

Stro-1 + cells — B — 50 µm

C — OCT4 NANOG CD44 NESTIN CD73 CD105 GAPDH
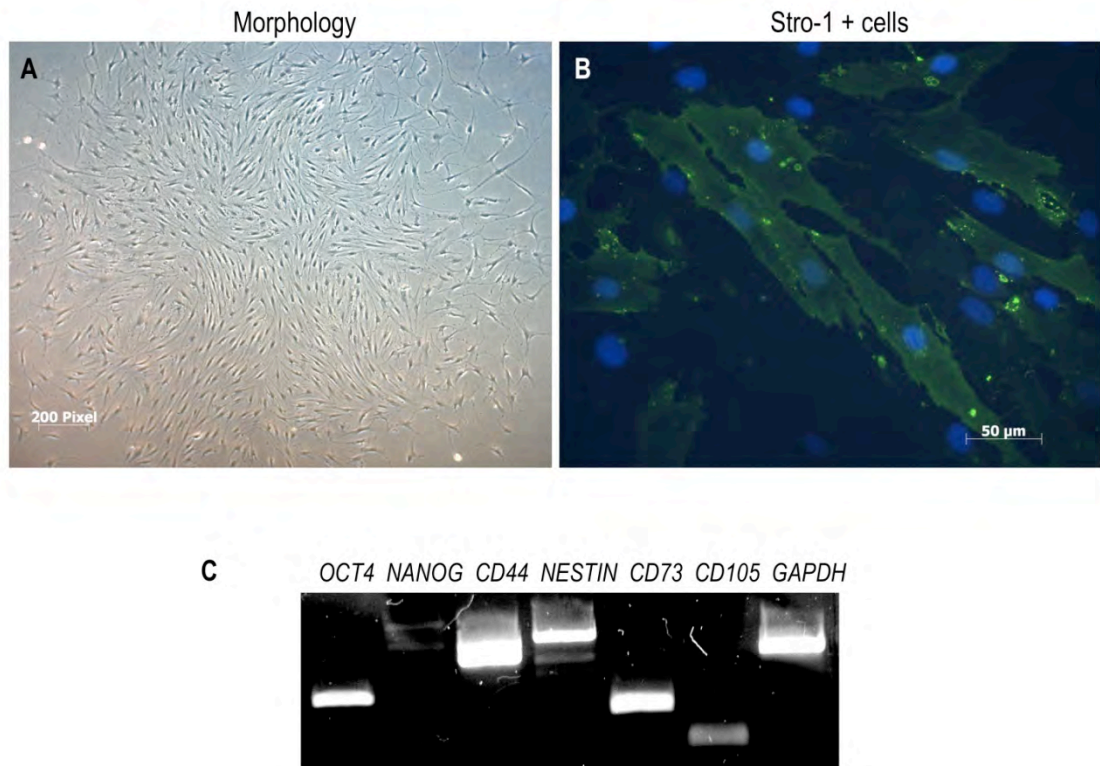
Figure 1 Characteristic of cells isolated from human adipose tissues. (A) Cell morphology observed using phase contrast microscope. (B) STRO-1 positive cell population in isolated cells. (C) The mRNA expression of embryonic and mesenchymal stem cell markers.(Prasitsumrit, 2011)

Control
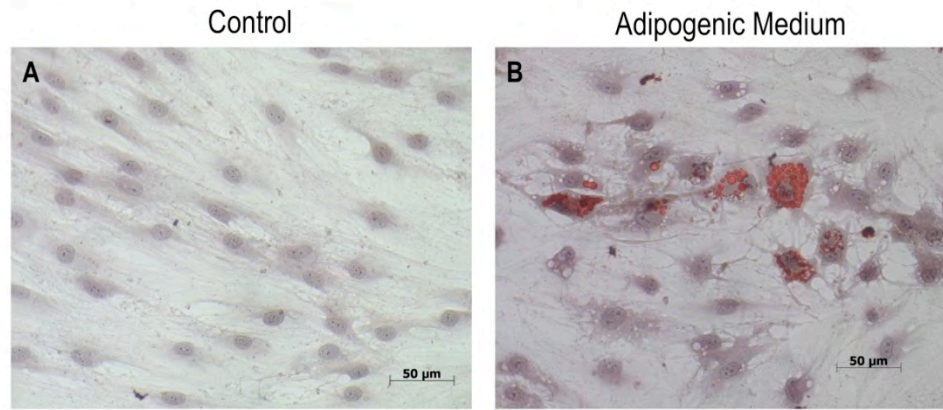
Adipogenic Medium

Figure 2 Adipogenic differentiation. Isolated cells were cultured in adipogenic induction medium for 14 days. (A) Cells in the control growth medium. (B) Cells in adipogenic induction medium illustrating intracellular lipid accumulation.(Prasitsumrit, 2011)
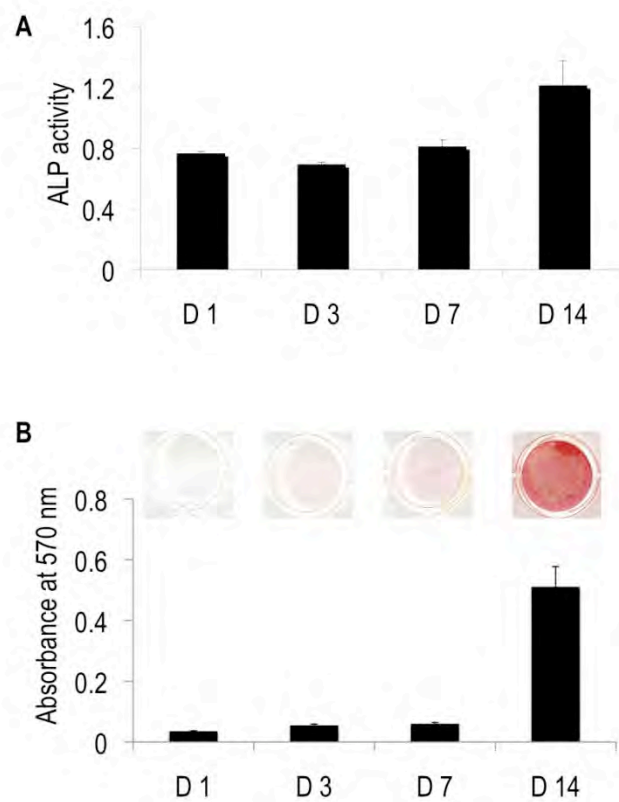
Figure 3 Osteogenic differentiation. Isolated cells were cultured in ostegenic induction medium. (A) The alkaline phosphatase enzymatic activity. (B) The mineral deposition determining by alizarin red S staining.(Prasitsumrit, 2011)
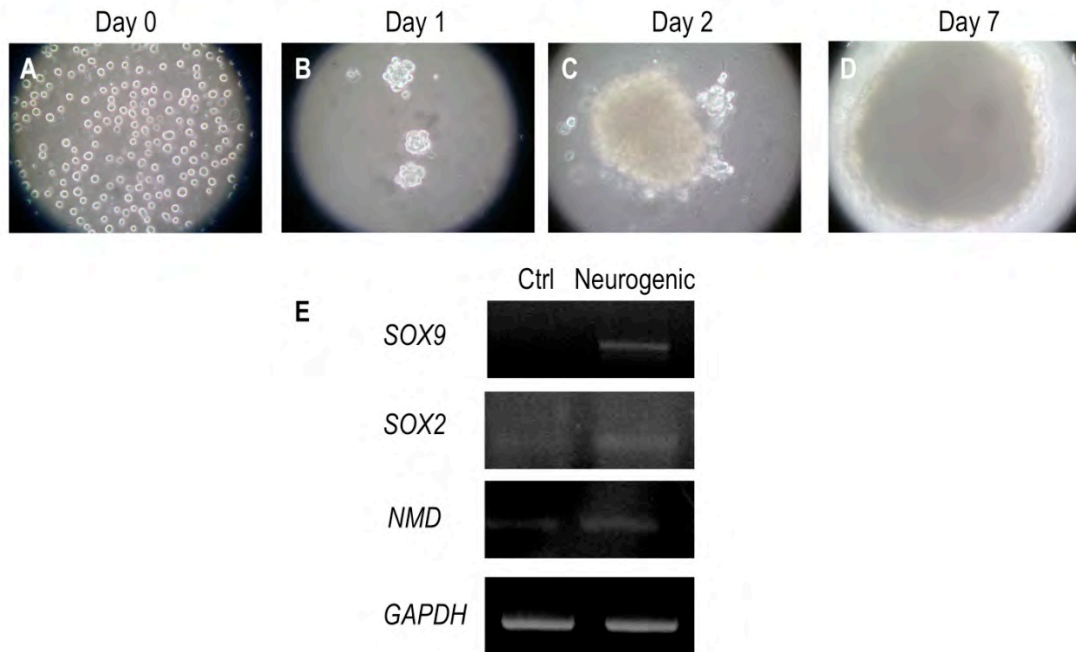
Figure 4 Neurogenic differentiation. Isolated cells were cultured in neurogenic induction medium. The formation of neurospheres was noted at day1 (B), day2 (C), and day7 (D). Floating single cells were observed in day0 (A). The mRNA expression of neurogenic markers was exmanied (E).(Prasitsumrit, 2011)
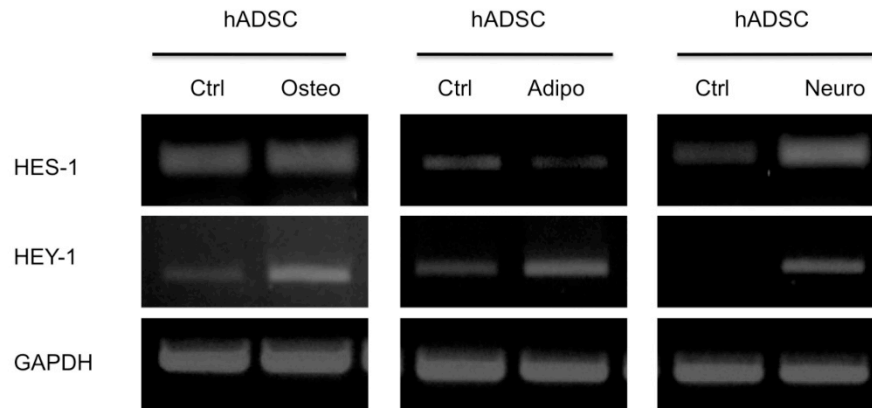
Figure 5 The mRNA expression of Notch target genes upon adipose- derived mesenchymal stem differentiating toward osteogenic, adipogenic and neurogenic lineages.(Prasitsumrit, 2011)
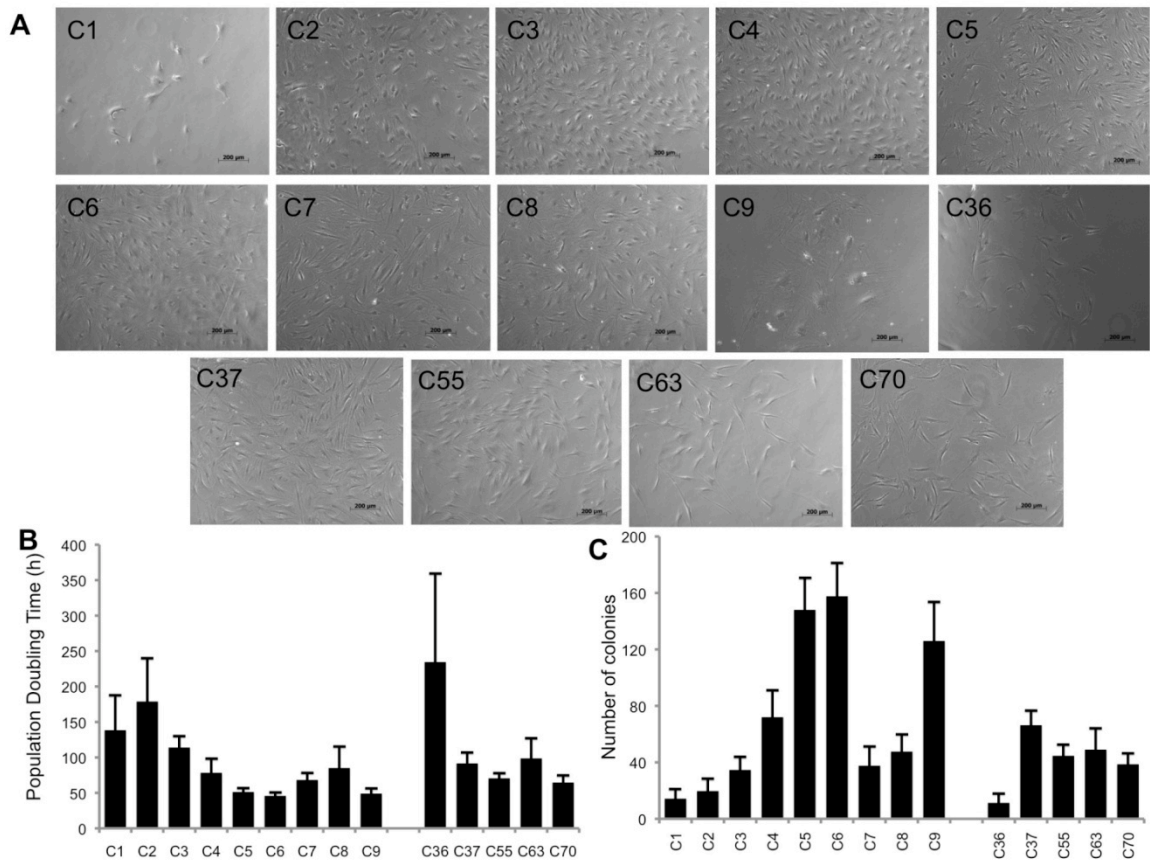
Figure 6 Adipose tissue derived clone characteristics. Morphology of the fourteen single-cell clones derived from human adipose tissue (A). Population doubling time. (B) Colony forming units C).
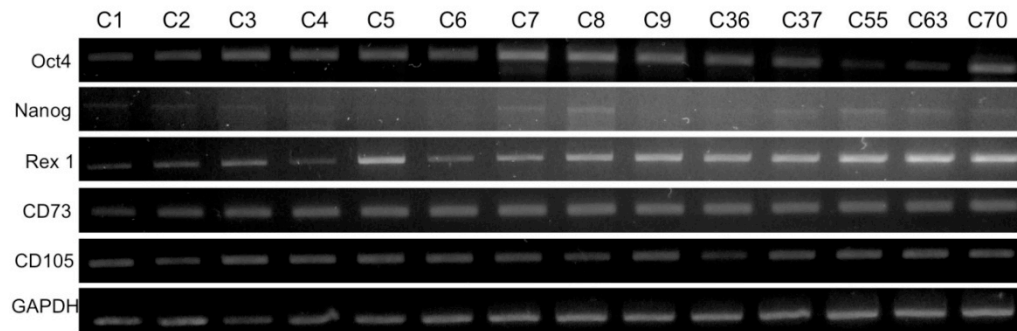
Figure 7 ThemRNA expression of the embryonic stem cell markers (Oct4, Nanog and Rex-1) and the mesenchymal stem cell markers (CD73 and CD105) by human adipose derived mesenchymal single clones.
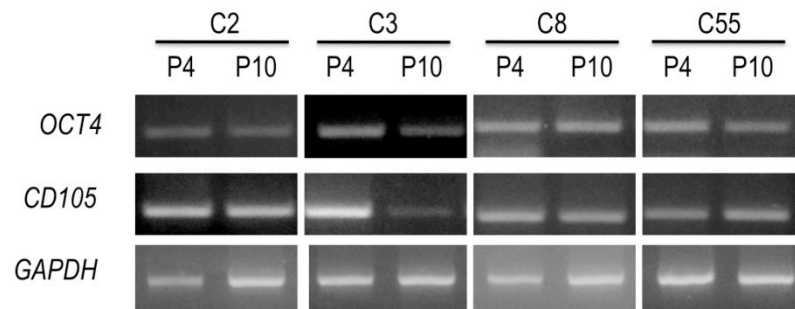
Figure 8 The mRNA expression of embryonic and mesenchymal stem cells expression at passage 4 (P4) and passage 10 (P10)
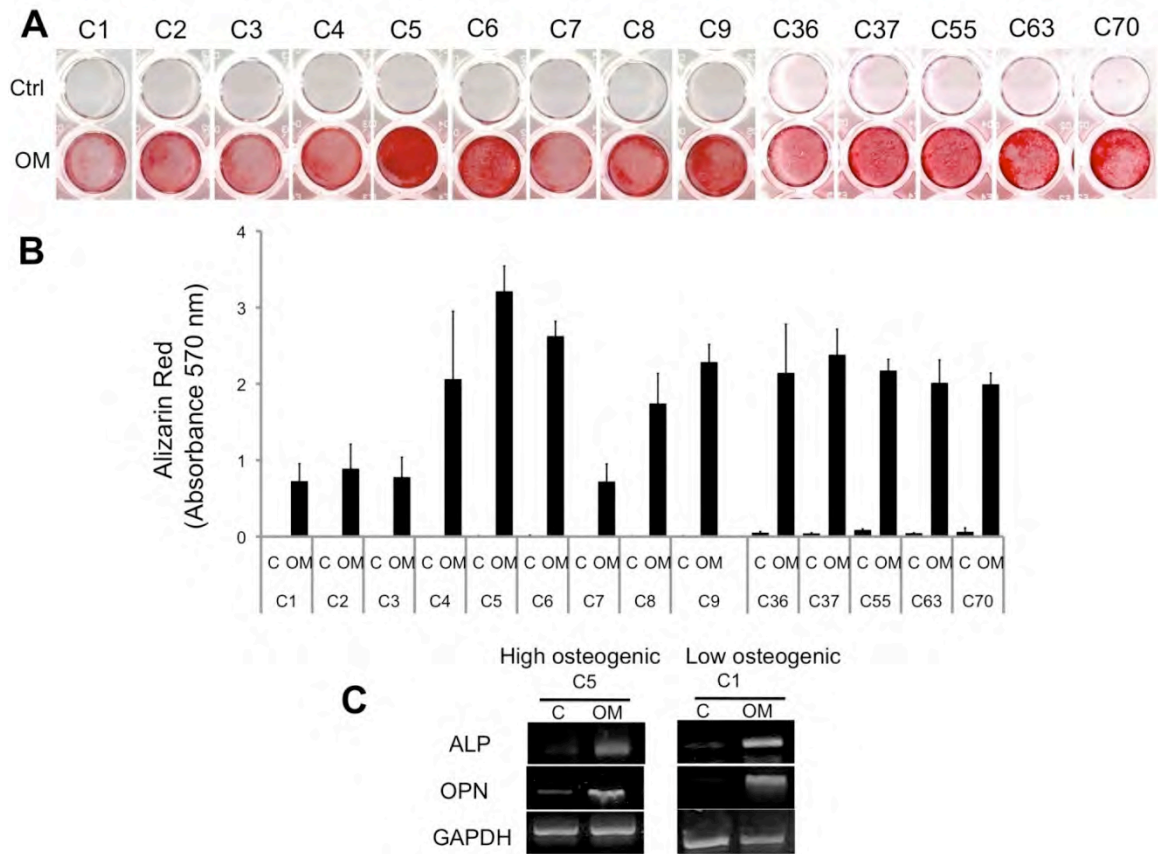
Figure 9 Osteogenic differentiation of human adipose derived mesenchymal single clones. Mineralization evaluated by Alizarin Red S staining after culturing in osteogenic medium (OM) for 14 days (A and B). The osteogenic mRNA marker gene expression of representative low (Clone1) and high (Clone5) osteogenic clones after osteogenic induction (C).
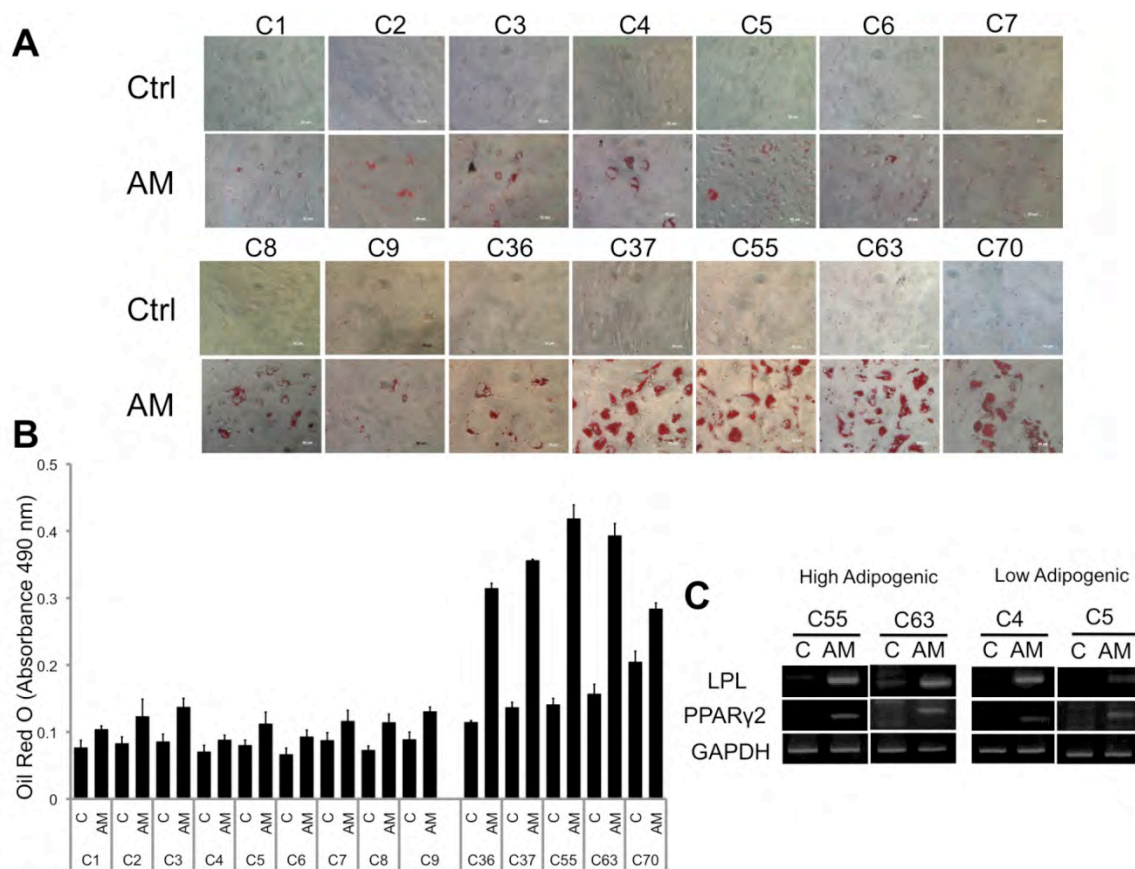
Figure 10 Adipogenic differentiation of human adipose derived mesenchymal single clones. Intracellular lipid accumulation evaluated by Oil Red O staining after culturing in adipogenic medium (AM) for 14 days (A and B). The adipogenic mRNA marker gene expression of representative low (Clone 4 and 5) and high (Clone 55 and 63) adipogenic clones after induction (C).
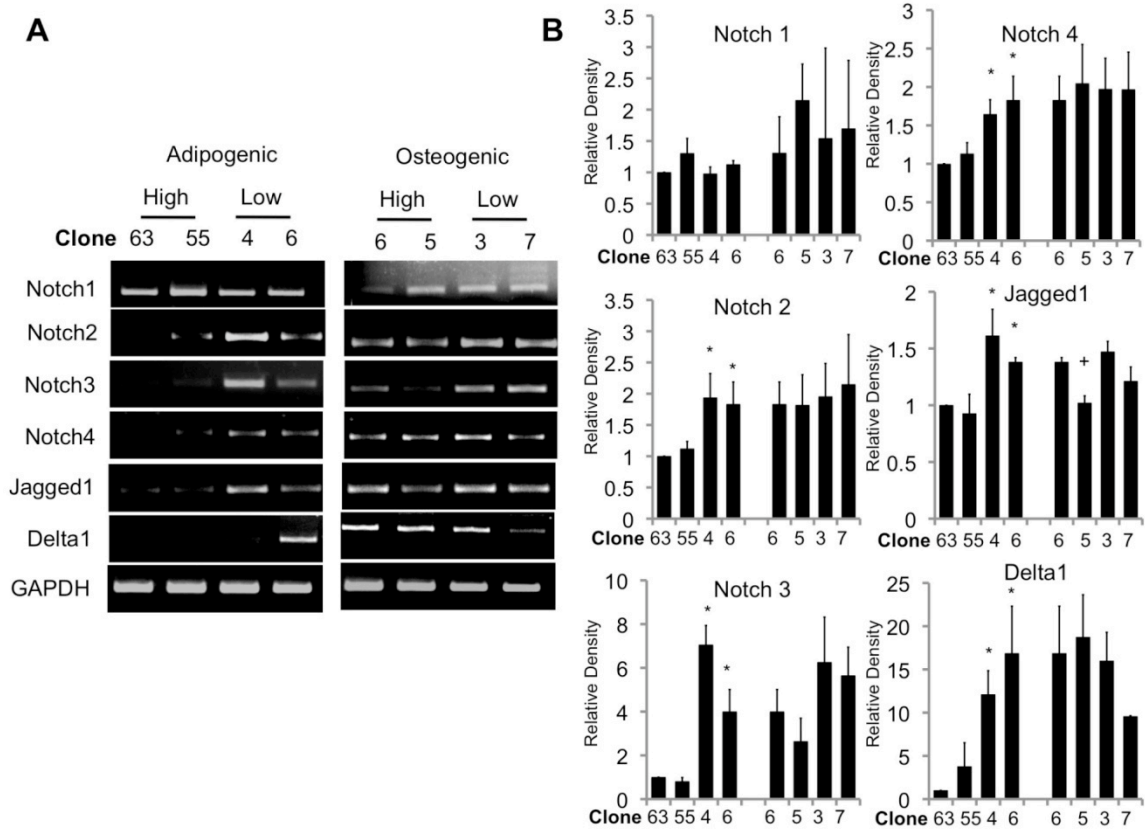
Figure 11 Baseline mRNA expression of Notch receptors (Notch1, 2, 3, and 4) and Notch ligands (Jagged1 and Delta1) in representative low/high adipogenic and osteogenic clones (A). Graphs represent relative density (B). Asterisk (*) indicates a significant difference ($p<0.05$) compared to a representaive high adipogenic clone (Clone 63). Plus sign (+) indicates a significant difference ($p<0.05$) compared to a representative high osteogenic clone (clone 6).
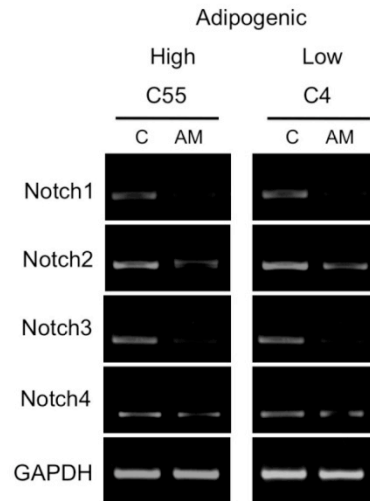
Figure 12 The Notch receptor mRNA expression of high and low adipogenic clones after adipogenic induction for 14 days.
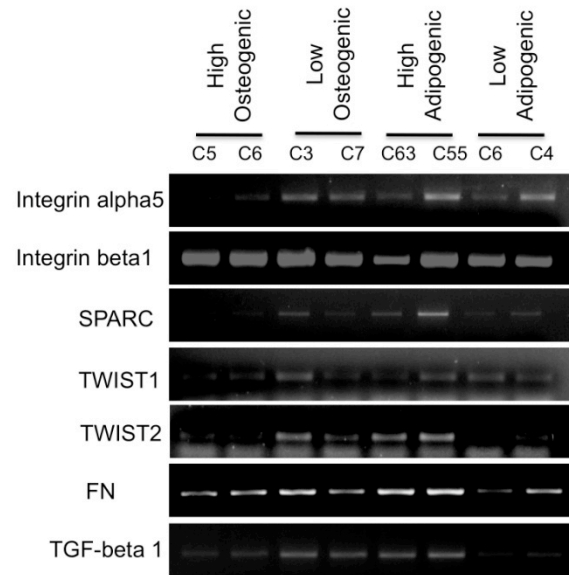
Figure 13 Baseline mRNA expression of membrane receptor (Integrin), extracellular matrix (SPARC and FN) and osteogenic regulation molecule (TWIST and TGF-beta1) in representative low/high adipogenic and osteogenic clones.
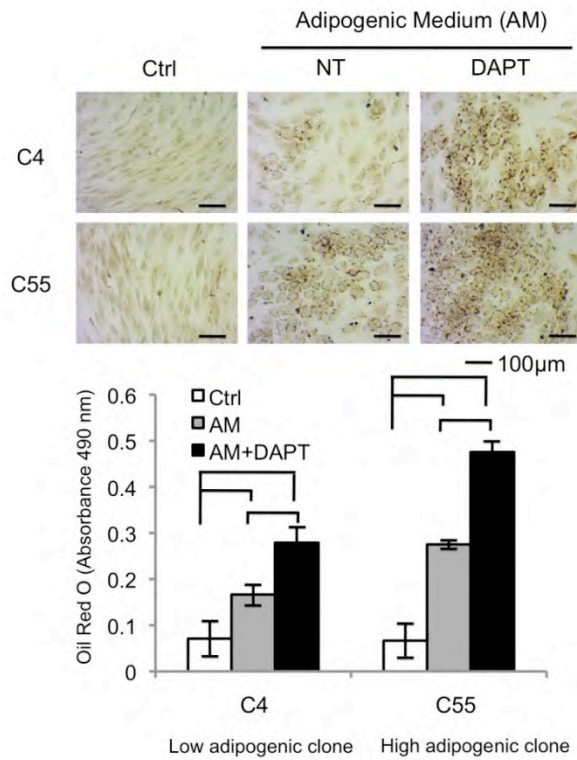
Figure 14 Effect of Notch signaling on adipogenic differentiation of low and high adipogenic clones. Adipogenic differentiation of representative low (clone 4) and high (clone 55)adipogenic clones in the presence of γ-secretase inhibitor for 7 days. The bars showed statistical significance ($p<0.05$). Ctrl; cells cultured in normal growth medium, NT; cells cultured in adipogenic medium without γ-secretase inhibitor, DAPT; cells cultured in adipogenic medium with γ-secretase inhibitor (25 μM).
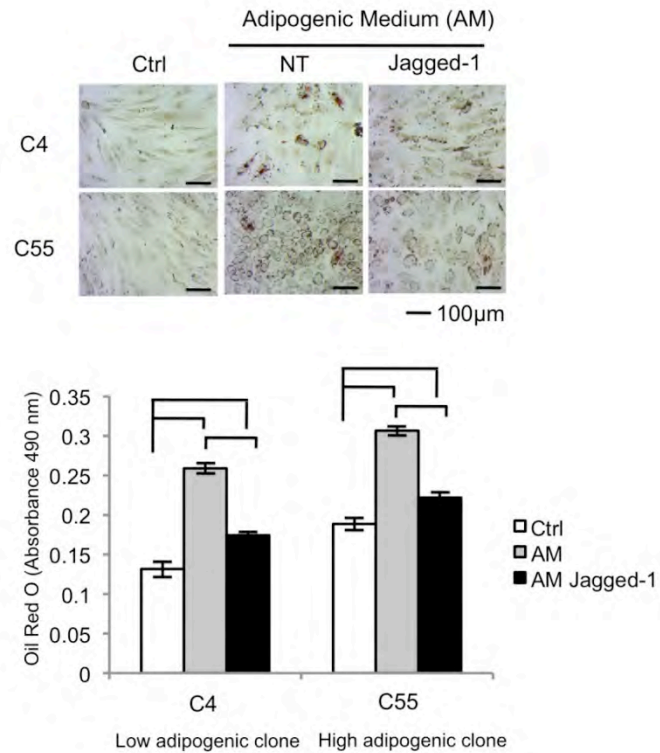
Figure 15 Effect of Notch signaling on adipogenic differentiation of low and high adipogenic clones.Adipogenic differentiation of representative low (clone 4) and high (clone 55) adipogenic clones in the presence of Notch ligands, Jagged-1, for 7 days. The bars showed statistical significance ($p<0.05$). Ctrl; cells cultured in normal growth medium, NT; cells cultured in adipogenic medium on the control human Fc modified surface; Jagged-1; cells cultured in adipogenic medium on the Jagged-1 modified surface (10nM).

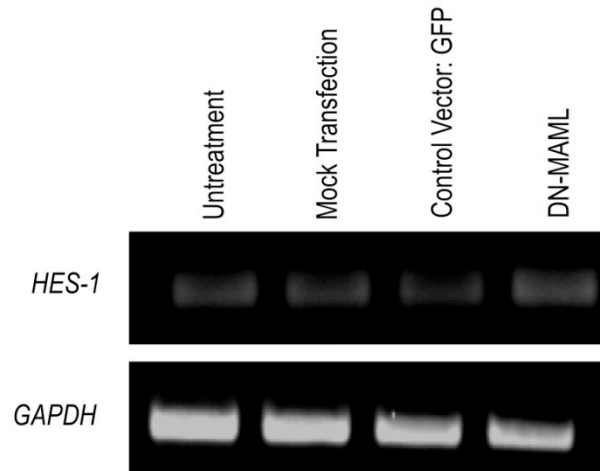**Figure 16** The mRNA expression of Notch target genes in heterogeneous adipose-derived mesenchymal stem cells upon transfection with DN-MAML.

Appendix 1