INVESTIGATION OF THE ROLE OF INSULIN-LIKE GROWTH FACTOR-II RECEPTOR IN 3T3-L1 PREADIPOCYTE DIFFERENTIATION

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Thesis
entitled

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INVESTIGATION OF THE ROLE OF INSULIN-LIKE GROWTH FACTOR-II RECEPTOR IN 3T3-L1 PREADIPOCYTE DIFFERENTIATION

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ABSTRACT

Insulin-like growth factors (IGF-I and IGF-II) play important roles in cell growth, differentiation and development. The biological effect of IGF-II is mediated by signaling through the IGF-I receptor (IGF-IR) and the insulin receptor (IR). IGF-IIR plays a role in controlling the availability of IGF-II via internalization and degradation of excess IGF-II. Furthermore, IGF-IIR acts as a receptor for the mannose-6-phosphate containing proteins. While IGF-I and IGF-IR have been shown to be crucial for adipocyte differentiation, the role of IGF-IIR in adipocyte differentiation has never been investigated. Here we investigate the expression pattern of IGF-IIR during differentiation of 3T3-L1 preadipocytes to mature adipocytes. The levels of IGF-IIR mRNA were highly abundant in preadipocytes but were gradually decreased during differentiation toward mature adipocytes. Suppression of IGF-IIR expression by 75% in 3T3-L1 preadipocytes did not appear to impair their differentiation to mature adipocytes as judged by Oil red O staining. Furthermore, gene expression analysis of adipogenic marker genes PPARγ-1, PPARγ-2, PC, aP2, GLUT4 and CEBPα confirmed that their expression was not significantly different between the IGF-IIR knockdown and control cells during differentiation. Our results suggest that IGF-IIR is not crucial for adipocyte differentiation.

KEY WORDS: INSULIN-LIKE GROWTH FACTOR-II RECEPTOR (IGF-IIR)/ 3T3-L1 PREADIPOCYTE CELL LINE/ ADIPOCYTE DIFFERENTIATION

72 pages

การตรวจสอบหาบทบาทการทำงาน ของหน่วยรับสัญญาณของอินซูลินไลส์โกรด์แฟคเตอร์ 2 ในระหว่างการเปลี่ยนแปลงรูปร่างของเซลล์ไขมันเริ่มต้น 3T3-L1
INVESTIGATION OF THE ROLE OF INSULIN-LIKE GROWTH FACTOR-II RECEPTOR IN 3T3-L1 PREADIPOCYTE DIFFERENTIATION

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บทคัดย่อ

อินซูลินไลท์โกรด์แฟคเตอร์ 1 และ 2 มีบทบาทหน้าสำคัญในการเจริญเติบโต การเปลี่ยนแปลงรูปร่าง และการพัฒนาของเซลล์ ผลกระทบทางชีวภาพของอิน ซูลินไลท์โกรด์แฟคเตอร์ 2 ทำหน้าที่เป็นตัวกลางในการกระตุ้นส่งสัญญาณผ่านทางหน่วยรับสัญญาณของอินซูลินไลท์โกรด์แฟคเตอร์ 1 และ หน่วยรับสัญญาณของอิซูลินเท่านั้น ในขณะที่การมีปฏิสัมพันธ์ระหว่างอินซูลินไลท์โกรด์แฟคเตอร์ 2 และหน่วยรับสัญญาณของอินซูลินไลท์แฟคเตอร์ 2 มีบทบาทสำคัญในการควบคุมปริมาณของอินซูลินไลท์โกรด์แฟคเตอร์ 2 โดยวิธีการทำให้เสียสภาพเมื่อมีปริมาณของอินซูลินไลท์โกรด์แฟคเตอร์ 2 มากเกินไปภายในเซลล์ นอกจากนี้ยังพบว่าหน่วยรับสัญญาณของอินซูลินไลท์แฟคเตอร์ 2 ยังมีบทบาทในการเป็นหน่วยรับสัญญาณของโปรตีนที่ประกอบด้วยโมเลกุลแมนโนสซิกฟอสเฟตอีกด้วย จากงานวิจัยก่อนหน้านี้พบว่า อินซูลินไลท์โกรด์แฟคเตอร์ 1 และหน่วยรับสัญญาณอิซูลินไลท์โกรด์แฟคเตอร์ 1 แสดงให้เห็นว่ามีความสำคัญอย่างยิ่งในกระบวนการเปลี่ยนแปลงรูปร่างของเซลล์ไขมัน ขณะที่หน่วยรับสัญญาณอิซูลินไลท์โกรด์แฟคเตอร์ 2 ยังไม่มีการค้นพบบทบาทที่เกี่ยวข้องกับกระบวนการเปลี่ยนแปลงรูปร่างของเซลล์ไขมัน ดังนั้นทางผู้วิจัยจึงทำการศึกษาและตรวจสอบรูปแบบการแสดงออกของหน่วยรับสัญญาณอินซูลินไลท์โกรด์แฟคเตอร์ 2 ระหว่างการเปลี่ยนแปลงรูปร่างของเซลล์ไขมัน จากผลการทดลองพบว่า ระดับการแสดงออกของ mRNA ของหน่วยรับสัญญาณอินซูลินไลท์โกรด์แฟคเตอร์ 2 มีการแสดงออกที่สูงมากในช่วงของเซลล์ไขมันเริ่มต้น แต่จะค่อยๆลดการแสดงออกในระหว่างที่เซลล์ไขมันเริ่มต้นมีการเปลี่ยนแปลงเป็นเซลล์ไขมันที่โตเต็มที่ และจากการทดลองลดการแสดงออกของอินซูลินไลท์โกรด์แฟคเตอร์ 2 ที่ร้อยละ 90 ของการแสดงออกปกติ พบว่า ไม่ปรากฏว่ามีผลต่อการเปลี่ยนแปลงรูปร่างของเซลล์ไขมันเมื่อใช้วิธีการย้อมสีของไขมันที่เกิดขึ้นภายในเซลล์ไขมันด้วยสีย้อมออยเรดโอ และจากการวิเคราะห์การแสดงออกของยีนที่มีความจำเพาะต่อเซลล์ไขมัน ได้แก่ PPARγ-1, PPARγ-2, PC, aP2, GLUT4 และ CEBPα เป็นการยืนยันว่า การแสดงออกยีนที่จำเพาะต่อเซลล์ไขมันทั้งหมดไม่มีการแสดงออกที่แตกต่างกับอย่างมีนัยสำคัญ เมื่อเปลี่ยนเทียบการแสดงออกระหว่างเซลล์ไขมันที่ถูกระงับการแสดงออกของยีนหน่วยรับสัญญาณอินซูลินไลท์โกรด์แฟคเตอ2์ และ เซลล์ไขมันควบคุมในระหว่างการเหนี่ยวนำให้มีการเปลี่ยนแปลงรูปร่างของเซลล์ไขมัน ดังนั้นจากผลการทดลองทั้งหมดแสดงให้เห็นว่าหน่วยรับสัญญาณาอินซูลินไลท์โกรด์แฟคเตอ2์ไม่สำคัญต่อการเปลี่ยนแปลรูปร่างของเซลล์ไขมัน

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LIST OF ABBREVIATIONS

Abbreviations used throughout this thesis are listed below.

AIM I	Adipogenic-induced medium I
AIM II	Adipogenic-induced medium II
aP2	Fatty acid binding protein
C	Degree celcius
cDNA	Complementary DNA
C/EBPs	CCAAT/enhancer binding protein member family
DEX	Dexamethasone
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
dNTPs	Deoxynucleotide 5' triphosphate
EDTA	Ethylenediamine tetraacetic acid
E.coil	Escherichia coli
FBS	Fetal bovine serum
GLUT4	Glucose transport isoform 4
IBMX	3-Isobutyl-1-methylxanthine
LB	Luria Bertain (medium)
mL	milliliter
mM	millimolar
MOPS	3-(N-morpholino)-propanesulfonic acid
Min	Minute
NCS	Newborn calf serum
PC	Pyruvate carboxylase
PPARγ	Peroxisome proliferator activated receptor-γ
RNA	Ribonucleic acid

LIST OF ABBREVIATIONS (cont.)

rRNA	ribosomal Ribonucleic acid
shRNA	short hairpin RNA
siRNA	Small interfering RNA
µg	Microgram
µl	Microliter
µM	Micromolar
V	Votage
v/v	Volumn/volumn

CHAPTER I
INTRODUCTION

1.1 Motivation

Insulin-like growth factors (I and II) are pleiotropic mitogenic polypeptide and are involved in many biological processes. Both ligands exerts their biological effect via IGF-I receptor (IGF-IR) and insulin receptor (IR). Previous report has demonstrated that IGF-I is essential for adipocyte differentiation whereas IGF-II plays an important role in mammalian growth by stimulating fetal cell division, differentiation, cell proliferation and also acts as anti-apoptotic properties. However, interaction of IGF-II with its own receptor (IGF-IIR) does not activate any biological process . Conversely, IGF-IIR plays an important role in the internalization of IGF-II ligand and transportation of mannose 6 phosphate containing proteins from Gogi to endosome, including the degradation of many proteins. The role of IGF-IIR in adipocyte differentiation has never been studied.

Furthermore, Prof. Dr. Boonsong Ongphiphadhanakul's laboratory at the Endocrinology unit at the Ramathibodi Hospital has studies whole genome analysis of patients who developed type 2 diabetes in Thai population and identified the association between single nucleotide polymorphism (SNP) of the *IGFIIR* gene in the morbid obese who developed type 2 diabetes while this SNP is not associated with lean type 2 diabetic patients. This raises the hypothesis that the *IGF-IIR* gene may be associated with adiposity in type 2 diabetic patients. Studying the function of IGF-IIR during adipocyte differentiation may shed some light how IGF-IIR is involved in adipogenesis. In this thesis, I aim to study the loss of function of IGF-IIR expression in adipocyte using murine 3T3-L1 adipocyte as a model. I propose that loss of IGF-IIR in 3T3-L1 cells would retard the differentiation. In this study, the specific siRNA of IGF-IIR was performed to suppress the IGF-IIR in 3T3-L1 preadipocyte cell lines by transiently transfection technique. In vitro differentiaton assay and gene expression profile analysis by real time PCR were performed in theIGF-IIR knocked down cells versus the control cells.

1.2 Objective

1.2.1 To study the expression patterns of insulin-like growth factor-I and II and their receptors (IGF-IR and IGF-IIR) during 3T3-L1 preadipocyte differentiation.

1.2.2 To study the loss of function of insulin-like growth factor-II receptor (IGF-IIR) in duing adipocyte differentiation.

CHAPTER II
LITERATURE REVIEW

2.1 Adipose tissue

2.1.1 Role of adipose tissue as an energy storage

Adipose tissue is the largest storage organ for energy in the form of triglycerides albeit the liver and muscle can also store fat. Dietary fat is transported with the chylomicrons and very-low-density lipoprotein (VLDL) to both liver and adipose tissue. The storage fat in adipose tissues is counter balanced by lipogenesis and lipolysis (83,53). The *de novo* fatty acid synthesis constitutes a major pathway of lipogenesis, this pathway involves the conversion of glucose to triglycerides during fed conditions, especially during excess carbohydrate intake. Lipogenesis occurs both in liver and adipose tissue. While liver is a short-term storage organ, adipose tissue is a long-term storage site (83). Recent studies have found that the accumulation of fat in liver is increased after consuming high carbohydrate diet (1,26,46,62). The newly synthesized fat from liver is transported to adipose tissue via VLDL. Although *de novo* fatty acid synthesis also occurs in adipose tissue due to the presence of *de novo* fatty acid synthesis enzymes including fatty acid synthase (FAS) and acetyl-coenzyme A carboxylase 1 (ACC1), the rate of synthesis is much less than the liver (64,84,3). Lipogenesis in liver and adipose tissue is stimulated by insulin but inhibited by glucagon. In contrast, lipolysis is the pathway in which the triglycerides stored in the lipid droplets are hydrolyzed by lipase to free fatty acids and glycerol. This biochemical process is important for the supply of free fatty acids for β-oxidation in the skeletal muscle during prolonged fasting. Lipolysis is also regulated by several hormones including glucagon and catecholamines which are elevated during starvation (Fig 2.1) (53). The proper balance between lipogenesis and lipolysis constitutes a key mechanism to maintain energy homeostasis. Dysregulation of these two pathways can lead to the development of obesity and type 2 diabetes.

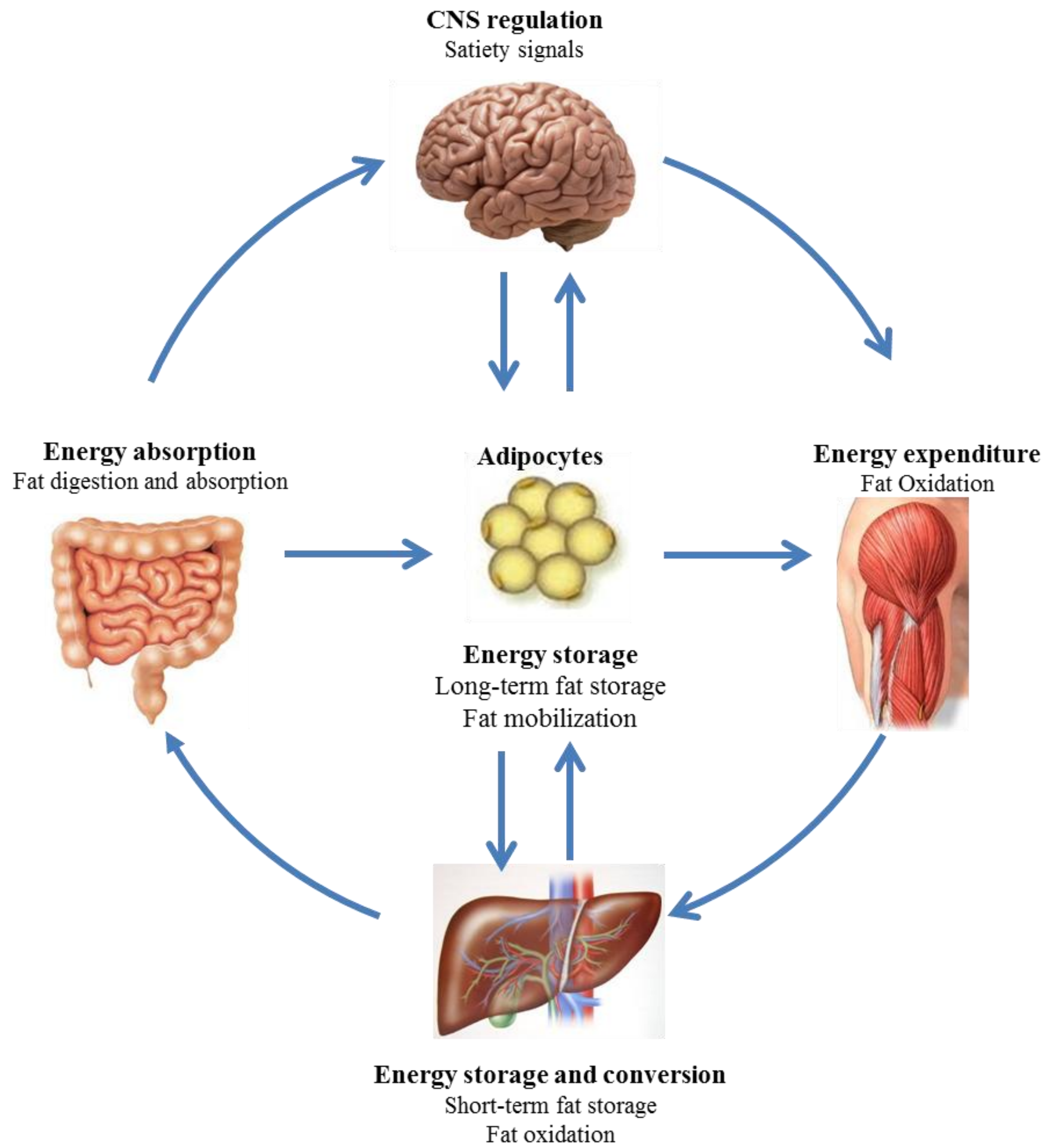


Figure 2.1 The main function of adipose tissue for maintaining the energy for other organ (83)

2.1.2. *In vitro* models of preadipocyte differentiation

In vitro differentiation of adipocyte is an extensive technique used to study program gene expression (76,38). Under appropriate conditions (hormonal induction), preadipocyte can be differentiated to mature adipcoytes. This process involves both biochemical and morphological changes including the expression of lipogenic enzymes and the accumulation of intracellular lipid droplets (76). The preadipocytes are originated from the pluripotent fibroblast stem cells which are mesodermal origin (15). These pluripoten stem cells can be converted into various tissue types including preadipocytes, cartilage, bone and muscle. Studying of preadipocyte differentiation *in vivo* is tedious because fat tissue is associated with other connective tissues and white blood cells, making it is technically difficult to isolate preadipocytes from others (34). Furthermore primary culture of preadipocytes has limited life span and the number of preadipocytes from individual is limited, making isolation of preadipocytes is not suitable for study the differentiation. Preadipocyte cell lines have recently been used because they are homogeneous (65). Several preadipocyte cell lines have recently been used and are shown in Table 2.1 3T3-L1 and 3T3-F442A have been extensively used as models to study adipogenesis. They were established from Swiss 3T3 cells, deriving from mouse embryos (37). The confluence of each preadipocyte model can be induced to differentiate by appropriate treatments, leading to the morphological and biochemical characteristics of cells changed. Changing in the morphology of each cell type depends on the specificity and acquirement of cell types needed, following by inducing agents used in the Table 2.1 below.

Table 2.1 The most frequently used preadipocyte cell lines in vitro. (38)

Cell Lines	**Origin/stage of development**	**Inducing Agents Used for differentiation**
Es cells	Mouse blastocyst	Retinoic acid
CH3 10T1/2	Mouse embryo	Demethylating agent 5'-azacytidine
TA1	Derived from 5-azacytidine treated 10 T1/2	10% FBS, insulin and DEX
3T3-L1	17 to 19 disaggregated mouse embryo	10%FBS, DEX and MIX, insulin (high concentration)
3T3-F442A	17 to 19 disaggregated mouse embryo	10% FBS, insulin
Ob17	Epididymal fat pads of adult ob/ob mouse	8% FBS, insulin and T3
Primary cultures	**Source/Age**	**Inducing Agents Used for differentiation**
Rat	Subcutaneous epididymal, retroperitoneal/newborn (48 h), 4 wk old or adult	Insulin(low concentration in 10%FBS, high concentration in serum free, accelerated)
Mouse	Subcutaneous/8-12 day old	Serum free; insulin, HDL, DEX
Rabbit	Perirenal/ 4 wk old	Serum free; insulin, DEX
Pig	Perirenal, subcutaneous/fetal, newborn (1-7 day old)	Serum free; insulin with or without glucocorticoids
Human	Subcutaneous (abdominal)/variable age	Serum free; insulin (high concentration) and glucocorticoids

2.1.3 3T3-L1 Preadipocyte differentiation

Adipocyte differentiation starts from a pluripotent stem cell precursor which later becomes a mesenchymal precursor; however a full molecular event underling the conversion of pluripotent stem cell to mesenchymal precursor is not completely understood (38,65). Confluent 3T3-L1 preadipocyte can be differentiated to mature adipocytes by appropriate adipogenic cocktail including insulin, dexamethasone, IBMX (an agent that increases intracellular levels of cAMP (65). Insulin stimulates adipogenesis by promoting *de novo* fatty acid synthesis and anti-

lipolytic. Similar with the biochemical action of insulin, dexamethasone (DEX), a synthetic glucocorticoid promotes *de novo* fatty acid synthesis thus increasing the lipid storage. Lastly, 3-isobutyl-1-methylxanthine (IBMX) is phosphodiesterase inhibitor (65) which enhances adipocyte differentiation by increasing the intracellular cAMP. Elevating levels of cAMP was reported to stimulate the expression of adipogenic transcription factors such as C/EBP-β and PPAR-γ during an early stage of adipocyte differentiation (38,76,72). Moreover, cAMP-activated transcription factor, CREB is a crucial transcription factor required for adipocyte differentiation. The combination of the above factors induces the expression of C/EBP-β and PPAR-γ during adipocyte differentiation (76,72). This leads to expression of a large array of lipogenic enzymes, resulting in morphological change of fibroblasts to become mature adipocytes.

After 24 hours of induction by adipogenic agents, 3T3-L1 preadipocyte cells pass through post-confluent and growth arrest. Day 2 after differentiation, the cells already undergo the post- confluent mitosis and go on an unusual growth arrest called, G_D (8,82). At this time point, several pro-adipogenic transcription factor genes including peroxisome proliferator activated receptor-γ, PPARγ and CCAAT/enhancer binding protein member, C/EBPα are turned on. At day 3 of differentiation, the late makers including lipogenic enzymes are expressed, resulting in of the formation of small intracellular lipid droplets. At days 5-7, the immature adipocytes undergo terminal differentiation to mature adipocytes as judged by the accumulation of larger lipid droplets inside the cells (65). The molecular cascade leading to adipocyte differentiation is shown in Figure 2.2

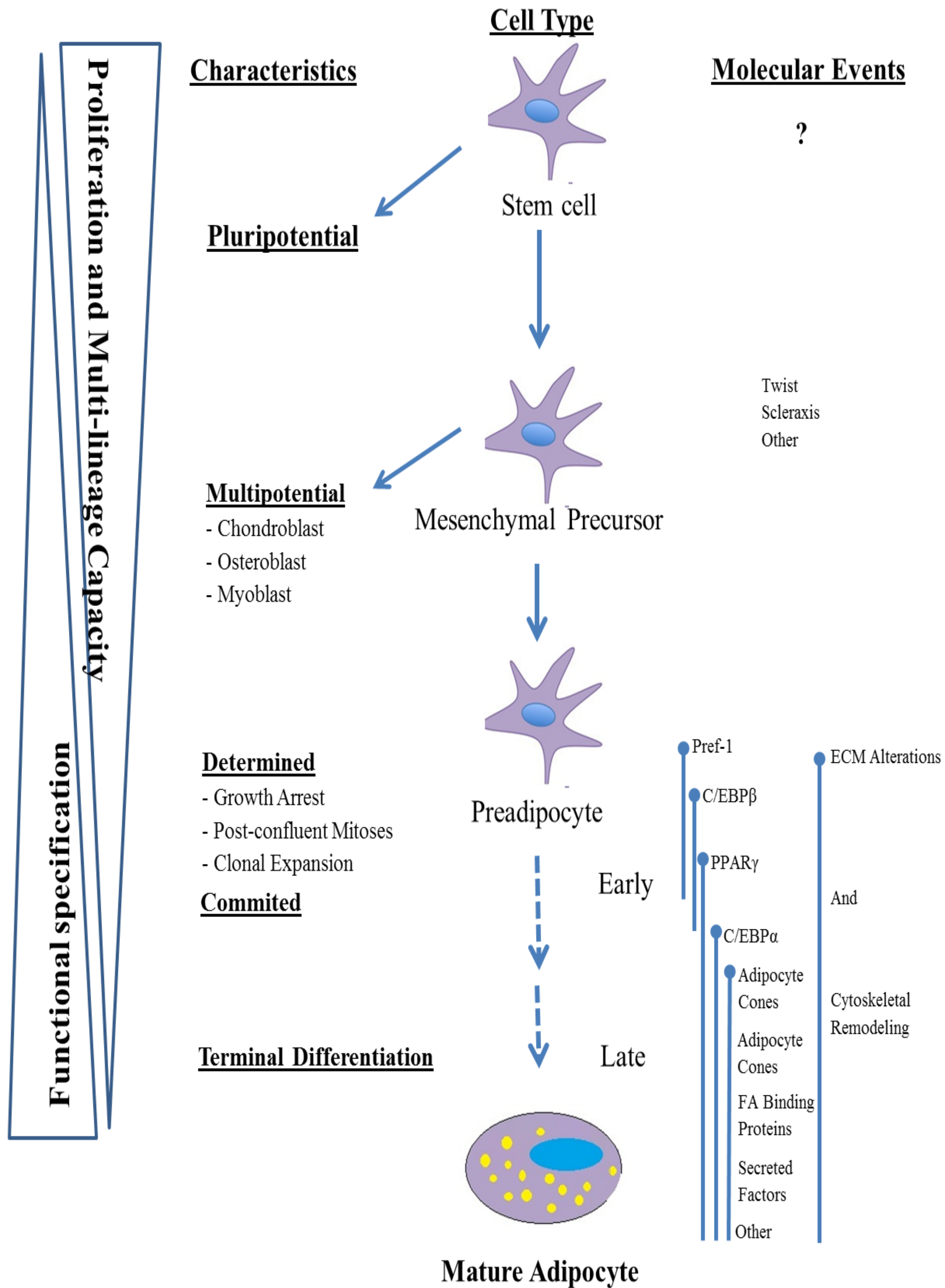


Figure 2.2 Stages of adipocyte differentiation (38)

2.1.4 Program gene expression during 3T3-L1 differentiation

Figure 2.3 shows the sequential expression of genes involved in adipocyte differentiation. In the earliest step, the expression of the lipoprotein lipase (LPL) is induced by cell-cell interaction at homogenously confluence 3T3-L1 preadipocyte cells (16,21). This is considered as an early sign of adipocyte differentiation. The lipoprotein lipase (LPL), released by mature adipocytes plays an important role in regulating fat storage (17,36). Because expression of LPL is not acquired for the induction of adipogenic agents suggesting that LPL expression may be involved in growth-arrest stage rather than starting an early differentiation step (18,91). Approximately 1 hour after exposing the confluence 3T3-L1 to adipogenic agents, the expression of c-fos, c-jun, junB and c-myc, CCAAT/enhancer binding protein (C/EBP) β and δ are turned on in the early stage of differentiation

C/EBPs are major transcription factors required for adipogenesis. C/EBPs are members of the basic-leucine zipper transcription factor family. They can form homodimers or heterodimers with other family members (56). During 3T3-L1 preadipocyte differentiation, it has been shown that C/EBPβ and C/EBPδ are immediately expressed during 3T3-L1 preadipocyte while C/EBPα is induced later during differentiation. After removal of adipogenic cocktail, the expression of C/EBP δ is disappeared within 48 hours whereas C/EBP β is gradually declined until day 8 of differentiation (76). The activity of both C/EBPβ and C/EBPδ are the mediator for inducing the expression of peroxisome proliferator-activated receptor γ (PPARγ) (13,98). which is induced during 2 days after differentiation. Previous study has shown that ectopic expression of both C/EBPβ and C/EBPδ can accelerate differentiation of 3T3-L1 cells (99). Although genetic ablations of C/EBP β or C/EBP δ genes in embryonic fibroblasts slightly affect adipocyte differentiation, ablations of both genes severely affect the development of adipose tissue *in vivo* (88).

C/EBPα functions as an inducer of several other adipocyte-specific genes (76). Ectopic expression of C/EBPα in the 3T3-L1 cells stimulates the conversion of 3T3-L1 preadipocytes to mature adipocytes whereas suppression of C/EBPα expression retards the differentiation of in 3T3-L1 adipocytes. Moreover, deletion of C/EBPα gene severely reduces the formation of white and brown adipose tissues in mice (32,58,96).

Peroxisome proliferator-activated receptor γ (PPARγ) plays a major role as a regulator of adipogenesis. Overexpression of PPARγ in non-adipogenic mouse fibroblasts promotes them to differentiate to mature adipocytes (90). PPARγ consists of 2 isoforms, PPARγ1 and PPARγ2 which are generated by alternative promoter usage of the same gene. PPARγ1 contains additional 30 amino acids at the N terminus PPARγ1 is expressed in several tissues while PPARγ2 is only expressed in adipose tissue. Previous study has shown that PPARγ2 can be functionally substituted by PPARγ1 (30). Furthermore, the PPARγ2 ablated mouse shows a reduced fat tissue (100), suggesting that PPARγ2 may play a selective role in regulating insulin sensitivity (30). It appears that both PPARγ and C/EBPα can govern the overall adipose tissue development (76). Moreover, PPARγ and C/EBPα can individually induce the transcription of several adipocyte genes whose products are involved in the establishment and maintenance of the adipocyte phenotype (38).

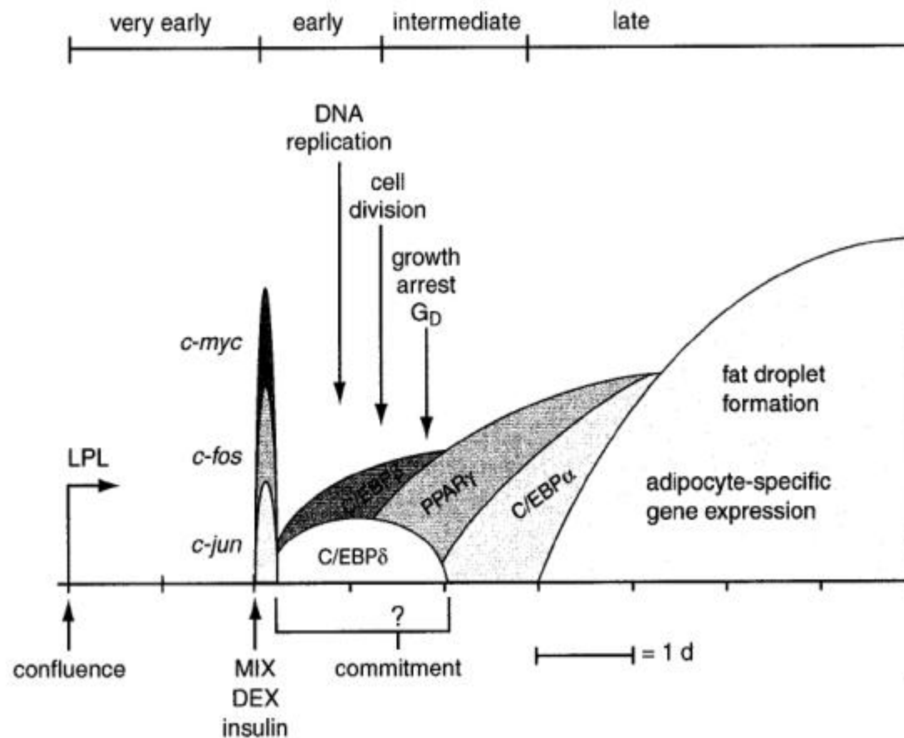


Figure 2.3 Transcription factors regulation during 3T3-L1 preadipocyte differentiation (66)

2.2 Insulin like growth factors (IGFs) and their receptors

Insulin like growth factors (IGF-I and IGF-II) are involved in several biological processes in adipocyte, hepatocytes and myocytes (29). Both growth factors share the similar structures to pro-insulin. IGFs also act in autocrine and paracrine fashion to control cell proliferation, cell death, differentiation and chemotaxis (41). Both IGF-I and IGF-II can bind to their own receptors, IGF-IR, IGF-IIR or insulin receptors as shown in Fig 2.4

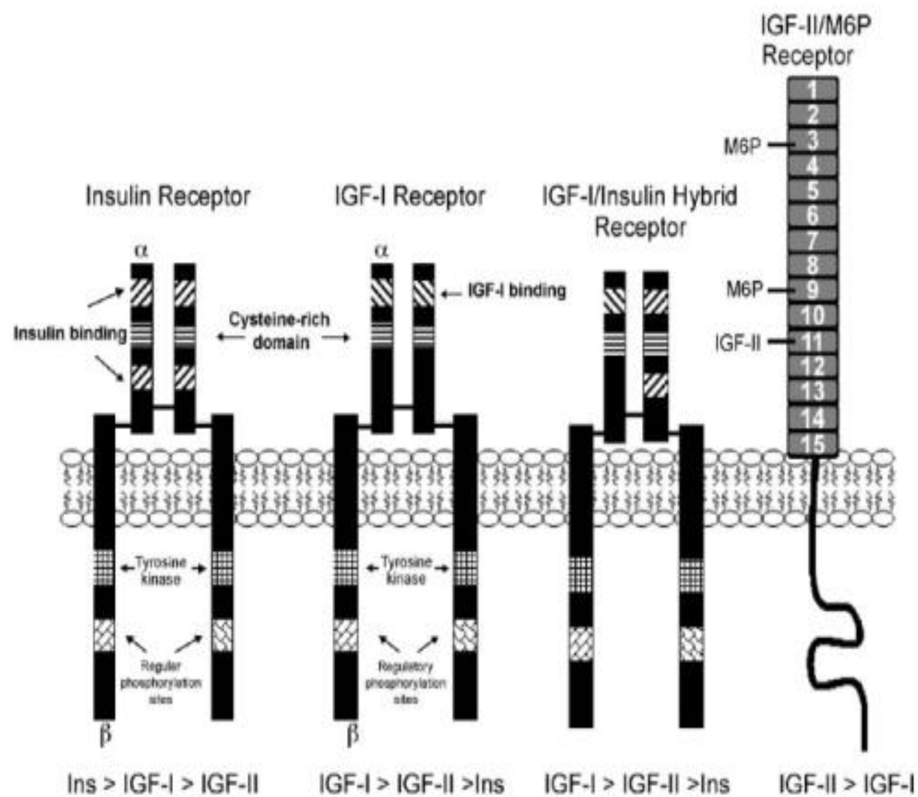


Figure 2.4 Structures of IGF-I, IGF-II, insulin and IGF/insulin hybrid receptors

IGF-I receptor (IGF-IR) is a member of the tyrosine kinase receptor family and structurally homologous to the insulin receptor. IGF-IR consists of two heterodimers of the α-subunit and the β-subunits. The α-subunit is located extracellulary, forming a ligand binding domain while the β-subunit is anchored in the plasma membrane. The two heterodimer subunits are connected together by the disulfide bonds. Binding of ligand to the receptor results in the conformational change, causing autophosphorylation of the tyrosine residues located in the β-subunit. This results in the signal transduction cascade, leading to alteration of cell growth and metabolic response. There appears to be a cross talk between IGF-I, IGF-II and insulin to their receptors. Previous experiments have shown that insulin, IGF-I and IGF-II can bind to IGF-IR (29,41), however IGF-I ligand binds to its own receptor with higher affinity than the IGF-II and insulin do. IGF-I is mainly produced by liver and many tissues, particularly in adipose tissues (89). Exposure of 3T3-L1 preadipocytes to IGF-I promotes growth and differentiation to mature adipocytes (79,24,86). Moreover, IGF-I also stimulates glucose oxidation in 3T3-F442A (80). Binding of IGF-II to IGF-IR also stimulates cell growth and proliferation, especially during embryonic development (74). In addition, binding of IGF-I or IGF-II to insulin receptor (IR) also produces the similar biological effect as binding via IGF-IR. The IR possesses two isoforms; IR-A and IR-B, which are the products of differential splicing of exon 11 (64,28). IR-B is bound by only insulin and displays the metabolic effects of insulin downstream signaling whereas IR-A is expressed in fetal and tumor tissues and promotes proliferative rather than metabolic effects (23). Recently, the several researches have reported that IGF-II binds to IR-A with higher affinity than it does to IR-B and also stimulates the mitogenic effect in fibroblasts (31,78,81).

In contrast, the structure of IGF-IIR is remarkably different from both IGF-IR and IR. Furthermore IGF-IIR lacks an intrinsic tyrosine kinase activity. The IGF-IIR shows a high affinity for IGF-II more than the IGF-I but does not bind to insulin (41). IGF-IIR is a type I transmembrane glycoprotein and composed of 3 main structural domains, the N-terminal extracellular region, the single membrane spanning region and the small cytoplasmic tail. IGF-IIR functions as a homodimer with molecular weight of 400 kDa. The N-terminal extracellular region consists of 15

repeated segments of approximately 147 amino acids. Each repeating segment displays a remarkably similar structure to the mannose-6-phosphate (M6P) receptor known as the cationic-dependent mannose-6-phosphate receptor. However, only the third and the ninth repeating units contain M6P binding site, which is essential for carbohydrate recognition. The C-terminus of the ninth repeating unit can bind to M6P at pH 6.4-6.5 and is specific for phosphomonoester and M6P containing ligands. The N-terminus of the third repeating unit can bind to M6P at pH 6.9-7.0 and essential for M6P-OCH_3 phosphodiester and manose-6-sulfate containing ligands (19,61). Unlike repeating units third and ninth repeating units, the eleventh repeating unit contains the IGF-II binding site -2. Previous study has also shown that IGF-II binding site of eleventh repeating unit is nearby to thirteen repeating unit, containing the fibronectin type II like insert which is efficient to enhancement of IGF-II binding in eleventh repeating unit (11,92).

Additionally, the extracellular region in the repeating segments also contains the cysteine residues which mediate the formation of disulfide bonds required for proper receptor folding (19,43,97). The second region spans through the plasma membrane while the third region is a small cytoplasmic tail, containing different phosphorylation sites by various kinases including protein kinase C (PKC), cAMP-dependent protein kinase and casein kinase I and II (19,50,59). Additionally, IGF-IIR also found in cytoplasm where the receptor can be recycled to the cell surface. This can be induced by many agents such as IGF-I, IGF-II, insulin, other growth factors and chemical compounds (41). Glucose is a major agent that causes the redistribution of IGF-IIR from cytoplasm to cell surface by increasing the binding of IGF-II to IGF-IIR. This redistribution of IGF-IIR is also associated with the increase in the binding and the uptake of exogenous lysosomal enzymes (41). However, the mechanism of IGF-IIR redistribution from cytoplasm to cell surface is unknown.

As IGF-IIR contains two distinct binding sites for IGF-II and mannose-6-phosphate containing proteins. The major role of IGF-IIR can be divided into two distinct functions. Firstly, IGF-IIR functions as the mediator for endocytosis of M6P-containing ligands for activation or degradation in the lysosome by targeting the newly

synthesized lysosomal enzymes to the *trans*-face of the Gogi complex, TGN to export to specific organelles or outside the cell (44,51) via the formation of clathrin-coated vesicles in a process mediated by the interaction between clathrin associated adaptor protein AP2 and the single tyrosine-based internalization motif YSKV, located on the cytoplasmic tail of IGF-IIR. Both proteins are involved in generating the clathrin-coated vesicles progression (54,20,70)

The second function of IGF-IIR is involved in the degradation of IGF-II. This function is a general mechanism used to regulate the levels of circulating IGF-II which targeted to lysosome. IGF-II plays an important role in mammalian growth by promoting fetal growth and development (12,93). IGF-II is also involved in the tumor development. Secretion of IGF-II from tumor and tumor-derived cell lines indicates that IGF-II might be control cell proliferation (69). Interestingly, the biological activity of IGF-II is mediated by IGF-IR and IR whereas IGF-II is degraded to lysosomal enzyme by IGF-IIR (12). There is evidence showing that loss of function of IGF-IIR in mice causes fetal overgrowth whereas the ectopic expression of this receptor results in growth retardation both *in vivo* and *in vitro* (42). Even though, the internalization of IGF-II by IGF-IIR was clearly observed but the biological effect of IGF-II though IGF-IIR including how IGF-IIR is involved in adipocyte differentiation remains unclear. The biological function of IGFs and their receptors are shown in Fig 2.5

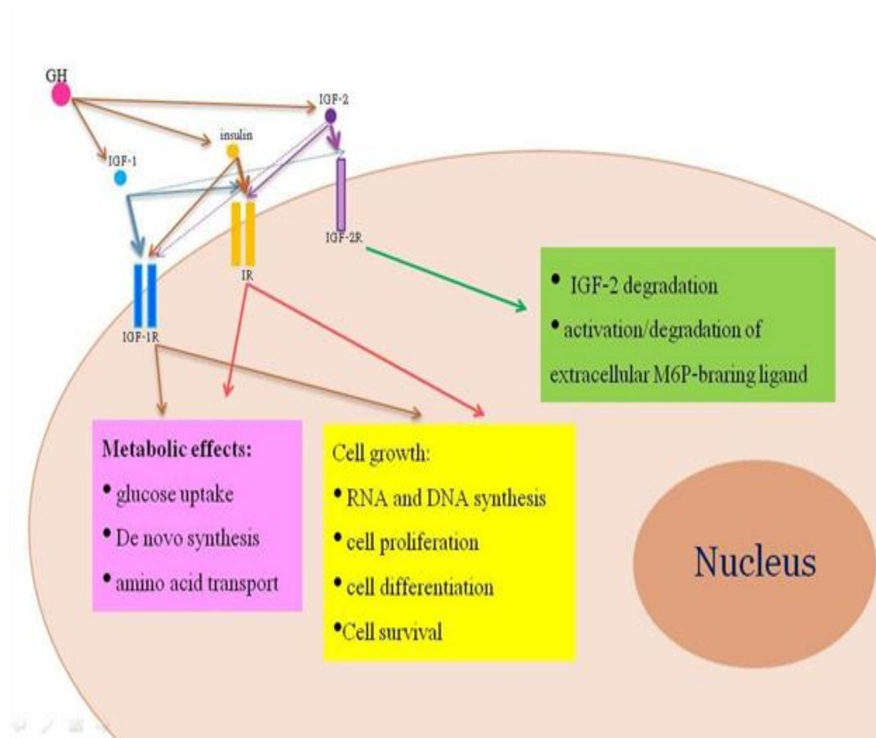


Figure 2.5 Overview of the interaction of insulin and IGFs with their receptors and the biological effects in the cell

2.3 The biological effects of insulin and insulin-like growth factors (IGF-I, IGF-II)

The binding between insulin and specific plasma membrane receptors (IR and IGF-I receptors) produces two biological effects; the metabolic effect which is defined as the changes associated with metabolic processes/pathways such as glucose uptake, glycogen synthesis, promoting *de novo* fatty acid synthesis and inhibiting lipolysis From the above metabolic functions of insulin, it has been reported that it plays important role in induction of adipocyte differentiation by induction the phenotypic change and accumulation of lipid droplets in mature adipocyte at terminal stage of differentiation. While mitogenic effect is defined as the changes associated with cell growth, proliferation and differentiation. The latter one is important for fetal growth, organogenesis, tissue repair and regeneration. (Table 2.2) (41).

Table 2.2 Some metabolic and growth-promoting effects of insulin (47)

Metabolic effects
Stimulation of glucose transport and metabolism
Stimulation of glycogen synthesis
Stimulation of lipogenesis
Inhibition of lypolysis
Stimulation of ion flux
Growth-promoting effects
Stimulation of DNA snsthesis
Stimulation of cell growth and differentiation
Mixed metabolic and growth effect
Stimulation of amino acid influx
Stimulation of protein systhesis
Inhibition of protein degradation
Stimulation of RNA synthesis

Similar with insulin, the biological effects of IGF-I is activated by passed though IGF-IR and IR. This activation is achieved via the activation of Ras cascade (6). Activation of IGF-IR by IGF-I leads to phosphorylations of the two distinct intracellular signaling molecules, the insulin receptor substrate (IRS 1-4) and src homology 2/collagen alpha proteins (SHC). Both proteins interact with several cytoplasmic proteins, containing an src homology (SH2) domain. This in turn results in stimulation of the PI3 kinase/Akt and MAP kinase pathways (27,14), resulting in preventing apoptosis, activation of cell proliferation, cell differentiation and glucose transportation (27). Furthermore, IGF-I is also essential for adipocyte differentiation of two adipocyte cell lines. This mechanism passed though activation of IRS-1/PI3K/Akt signaling pathway and then stimulates the expression of adipogenic marker genes, including peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer

binding protein (C/EBP). The loss of function of this signaling impairs the adipose tissue information. In addition, IGF-I can overcome the inhibition of differentiation mediated by Pref-1 in preadipocyte (45,76). Pref-1 is up-regulated in preadipocyte and down-regulated in adipocytes (76). Therefore, IGF-I is a key regulator for adipocyte differentiation.

IGF-II plays important role in embryonic development. The biological effect of IGF-II is mediated by interacting with IGF-I receptor. The interaction between IGF-IIand its receptor (IGF-IIR) is markedly involved in internalization and degradation of IGF-II (67). Previous work has shown that the IGF-II-ablated mice, the fetus show growth retardation (67). Additional, IGF-II also involves in the pancreas development. There is the research reported that IGF-II can regulate the islet growth and differentiation much than IGF-I (67).

2.4 Molecular mechanism of IGF-II via IGF-IIR

Previous studies haves shown that the antibodies blocking IGF-IIR signaling did not suppress the growth-promoting effect of IGF-II. This is supported by the gene silencing experiment in which knocking down of the IGF-IIR increases cell proliferation, fetal over growth and reduction of apoptosis in neonatal rat cadiacmyocytes (41,12). The lack of IGF-IIR signalling in the above experiment would favor binding of IGF-II to IGF-IR thus promoting mitogenic effect (41,12). In contrast suppression of IGF-IR reduces mitogenic activity of IGF-I-mediated signaling, further indicateing the distinct outcomes of IGF-I/IGF-IR and IGF-I/IGF-IIR signaling pathways (33).

Because the cytoplasmic tail of IGF-IIR lacks the kinase activity domain, the intracellular mechanisms mediating by this receptor is still unclear (41). However, several lines of evidence suggest that IGF-II induces proliferation of rat metanephrons and stimulates DNA synthesis in BALB/c 3T3 cells through IGF-IIR (41). These effects can be activated by a G-protein which interacts with IGF-IIR cytoplasmic domain (68). However, the recent study has not confirmed the above results (50). In addition, the interaction of G protein with IGF-IIR can lead to PKC-induced

phosphorylation of intracellular protein, stimulation of MAP kinase pathway and /or decrease in adenylatecyclase activity (101). All of these results suggest that the biological effects of IGF-II may be mediated by activation of G-protein coupled pathway via interaction with IGF-IIR (41).

Table 2.3 The specific ligands bind with IGF-IIR (41)

Non-M6P-containing ligands	Consequences of IGF-IIR/M6P receptor binding
Insulin-like growth factor-II	Endocytosis and Lysosomal degradation, possible signal transduction
Retinoic acid	Growth inhibition and/or apoptosis
Urokinase-type plasminogen	Participation in TGF-β activation at the cell surface; endocytosis and activator receptor lysosomal degradation
Plasminogen	conversion to plasmin and participation of TGF-β activation
M6P-containing ligands	
Lysosomal enzymes	Endocytosis and /or trafficking to lysosomes
Transforming growth factor-β precursor	Cell surface proteolytic activation
Leukemia inhibitory factor	Endocytosis and Lysosomal degradation
Proliferin	Induction of endothelial cell migration and angiogenesis
Thyroglobulin	Endocytosis and lysosomal activation and/or degradation
Renin precursor	Endocytosis and lysosomal activation and/or degradation
Granzyme A	Targeting to lytic granules and possible role in apoptosis
Granzyme B	Internalization and induction of apoptosis
DNAase 1	Possible targeting to lysosomes
CD26	Internalization and T cell activation
Epidermal growth factor	Endocytosis and lysosomal degradation
Herpes simplex viral glycoprotein D	Facilitation of viral entry into cells and transmission between cells
Varicella-zoster viral glycoprotein 1	Facilitation of viral entry into cells

2.5 The signaling transduction of IGF-IIR involved in adipocyte differentiation

Although, IGF-IIR is widely studied in several biological systems, the association of IGF-IIR with adipocyte differentiation is not known. Extracellular IGF-IIR region can be bound by many ligands that are involved in growth, proliferation and differentiation of many types of cell. Interestingly, the binding of some ligands to IGF-IIR may also involve in adipocyte differentiation. The transforming growth factor-β (TGF-β) precursor can be activated into active form upon interacting with cell surface IGF-IIR. The function of TGF-β in active form which is growth inhibitor can regulate the cell differentiation and cell growth of most cell types (41,42). Moreover, TGF-β can also inhibit adipogenesis *in vitro* (73,87). The urokinase-type plasminogen activator receptor (uPAR) which is involved fibrinolysis, cell adhesion and migration, is mediated through TGF-β activation by interacting with IGF-IIR (65,35,55). Previous study has shown that overexpression of IGF-IIR reduces cell growth whereby the receptor acts as a growth inhibitor whereas the loss of function of IGF-IIR can increase IGF signaling by mediated IGF-IR and IR and decreasing the growth inhibitor. TGF-β, uPAR and retinoic acid (RA) which are involved in cell apoptosis in the cell and excess production of lysosomal proteases to increase the cell invasion and metastasis by degradation of basement membrane and extracellular matrix component, generating the tumorgenesis (41,95,22). From the above reports, this suggests that the IGF-IIR is important regulator of growth, proliferation and differentiation of cell through binding of growth factor and growth inhibitor ligands. However, the evidence of correlation of IGF-IIR to mediate to growth factor and growth inhibitor binding mechanism in adipocyte differentiation has been unknown.

Another ligand which is related to IGF-IIR, is a cellular repressor of E1A-stimulated gene (CREG). CREG, a ubiquitously glycoprotein is secreted and plays a role in cell differentiation and suppressing cell proliferation (39). Previous study suggests that the expression of CREG is significantly increased during the conversion of proliferative and synthetic smooth muscle cells (SMCs) to differentiated SMCs *in vitro* whereas CREG knockdown inhibits SMCs maturation (40). The investigation of relationship between CREG and IGF-IIR in NIH3T3 fibroblasts has shown that CREG

can directly bind to IGF-IIR but the exact interaction is still unclear and CREG ligand can inhibit cell proliferation via IGF-IIR-mediated in NIH3T3 cell (39). Moreover, the distribution and trafficking of IGF-IIR is regulated by CREG protein. Additional, the increasing of IGF-II is affected by the loss of CREG whereas the re-adding of CREG protein can inhibit the excess expression of IGF-II stimulated by CREG knockdown in NIH3T3 cell. Therefore, the interaction of CREG with IGF-IIR is involved in the regulation of cell proliferation inhibition which involves regulating translocation of IGF-IIR and altering the IGF-II endocytosis (39). However, the mechanism of binding between CREG and IGF-IIR which regulates the translocation and internalization of IGF-II has been remained unknown (39).

CHAPTER IV
RESULTS

4.1 The expression of insulin- like growth factor-I and -II (IGF-I and IGF-II) and their receptor (IGF-IR and IGF-IIR) during 3T3-L1 differentiation

4.1.1 *In vitro* differentiation of 3T3-L1 preadipocytes to mature adipocytes

In vitro adipocyte differentiation assay is a widely used technique to study the function of genes whose products are required for adipogenesis and lipogenesis. Ideally, suppression of any genes involved in the above process would retard or inhibit the differentiation processes. 3T3-L1 fibroblast is an excellent cell line for the above study. Under appropriate conditions (hormonal induction), 3T3-L1 can accumulate lipids accompanying with increased expression of lipogenic enzymes, resulting in the alteration of the fibrolast-like morphology to mature adipocytes.

As an initial step to investigate expression profiles of IGF-I, IGF-II and their receptors during differentiation as well as loss of function of IGF-IIR, it was necessary to make sure that the differentiation conditions described in literature worked well in this study. 3T3-L1 preadipocytes were induced to differentiate to mature adipocytes by culturing them in the differentiation medium (AIM I) containing insulin, dexamethasone (synthetic glucocorticoid) and an inhibitor of phosphodiesterase, 3-isobutyl-1-methylxanthine (IBMX) for 2 days before the medium was replaced with the medium containing only insulin (AIM II) for the next 8 days.

Figure 4.1 A shows 90-100% confluent 3T3-L1 preadipocytes before inducing with an AIM I containing 1 µM DEX, 0.5 mM IBMX and 10 µg/ml insulin. After they were induced with AIMI, the characteristics of cells were changed from the fibroblast-like shape

to round shape as shown in Figure 4.1B The medium was then changed to AIM II, containing only insulin until they become mature adipocytes. At day 4 of differentiation, 3T3-L1 cells started accumulating lipid droplets inside the cytoplasm as shown in Figure 4.1C After that these cells accumulated more lipid droplets until day 6 at which they were converted to the mature adipocyte concomitant with the accumulated large lipid droplets by day 10 of differentiation as shown in Figure 4.1D and 4.1F

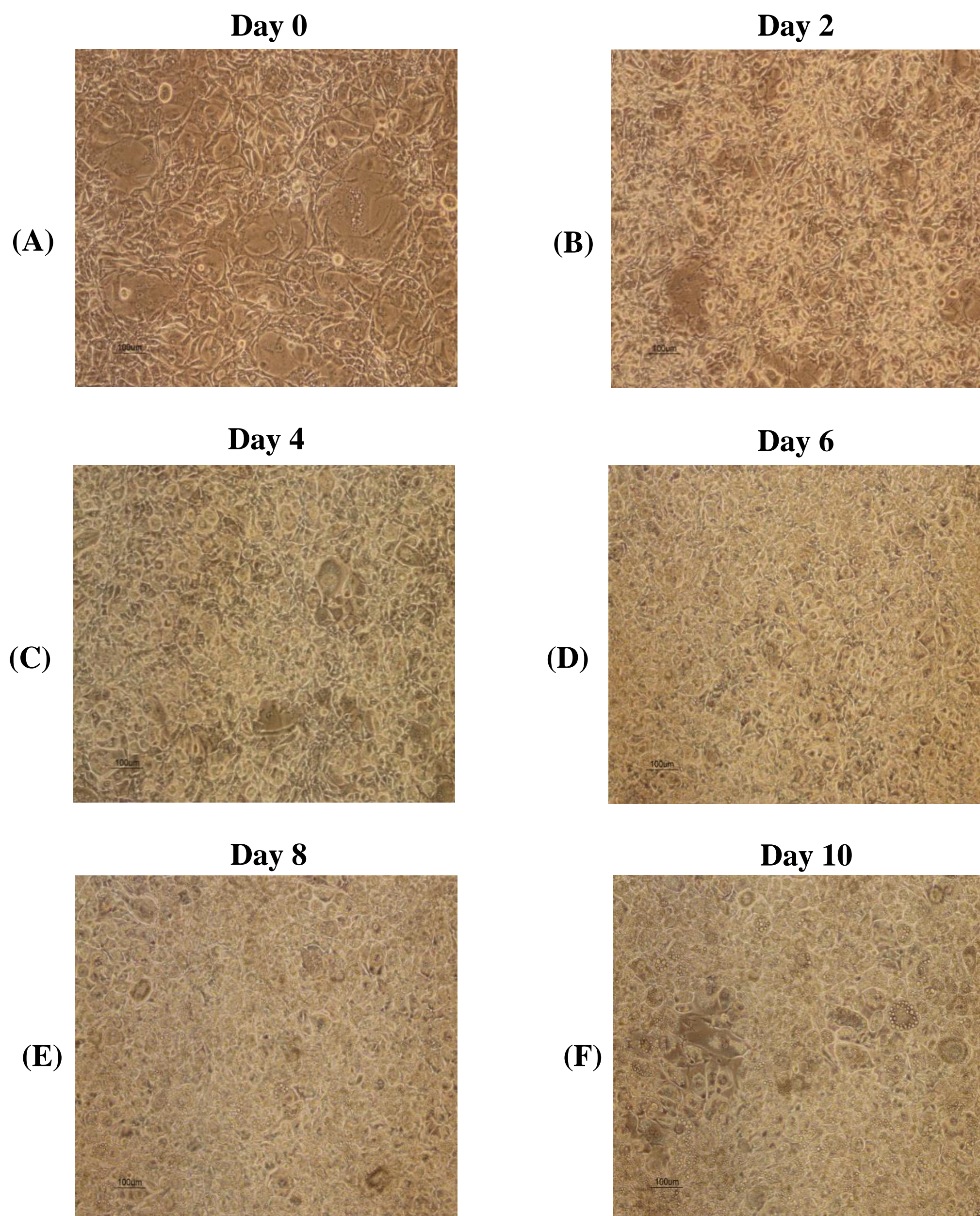


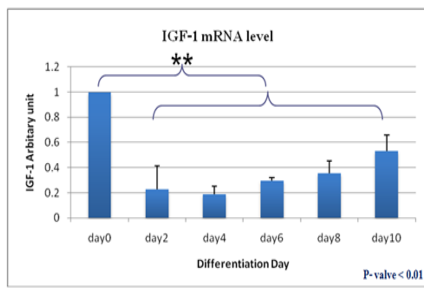
Figure 4.1 The morphological change of 3T3-L1 preadipocytes during differentiation of to mature adipocytes in AIM I and AIM II from day 0 to day10.

4.1.2 Expression of of IGF-I, IGF-II and their receptors (IGF-IR and IGF-IIR) mRNA levels during 3T3-L1 preadipocyte differentiation.

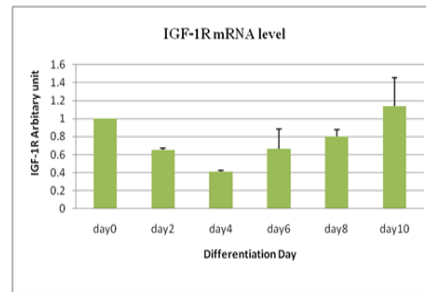
Having shown that 3T3-L1 preadipocytes can be successfully differentiated to mature adipocytes, these cells were collected at various time points and RNA extracted were used to investigate the expression profiles of IGF-I, IGF-II, IGF-IR and IGF-IIR during adipocyte differentiation. This experiment will address whether the expression of the above genes is correlated directly, inversely or constitutively with the progression of adipocyte differentiation.

As shown in Figure 4.2 the expression of IGF-I mRNA was highest in preadipocytes (day 0). However, as soon as 3T3-L1 preadipocytes were induced to differentiate, the IGF-I mRNA level was rapidly decreased by day2,(P-value $\leq$ 0.01). Although the levels of IGF-I mRNA were slightly increased during differentiation (day 2 to day 10), they show no statistical significance, as shown in Figure 4.2A The expression of IGF-IR mRNA was consistent with that of IGF-I mRNA; the levels of IGF-IR mRNA were highest in preadipocytes but gradually decreased once the preadipocytes have started to differentiate to adipocytes, albeit not sharply decreased as the IGF-I at day 2. However, the level of IGF-IR mRNA was increased again thereafter and reached the peak by day 10, as shown in Figure 4.2B These results suggested that the levels of both IGF-I and IGF-IR mRNAs were positively correlated with the progress of 3T3-L1 preadipocyte differentiation. These results were consistent in several previous literature reviews that IGF-I and IGF-IR were essential for proliferation and differentiation in adipocyte cells.

In contrast to IGF-I, the levels of IGF-II mRNA were rather low in preadipocytes and during early to middle stage of differentiation (day 2 to day 6). However, the levels of IGF-II mRNA were sharply increased during terminal differentiation (day 6 to day 8) as shown in Figure 4.2C The expression of IGF-IIR mRNA was high at day 0, but was gradually decreased and reached the lowest level at day 4. However, its expression level was gradually increased again until day 10 of differentiation, as shown in Figures 4.2D and 4.2E These results can be concluded that (i) The levels of IGF-I mRNA and IGF-IR mRNA were similar during 3T3-L1 differentiation. (ii) The levels of IGF-II and IGF-IIR are high in preadipocytes and during terminal differentiation.



(A)



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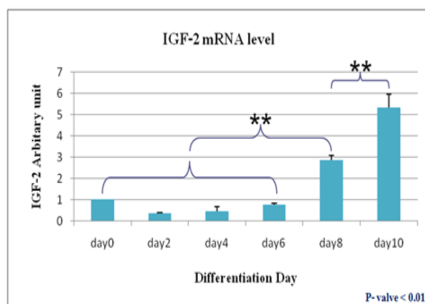
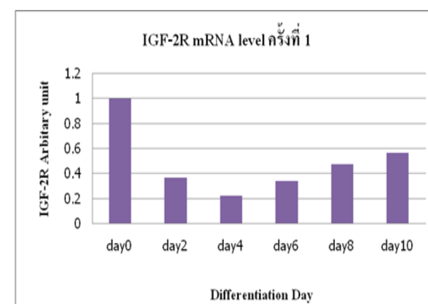
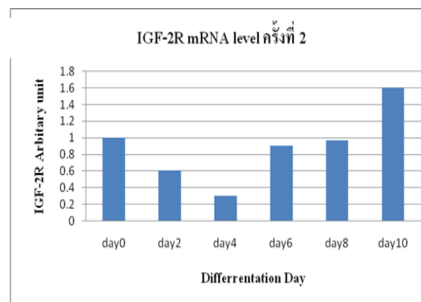


(C)



(D)



(E)

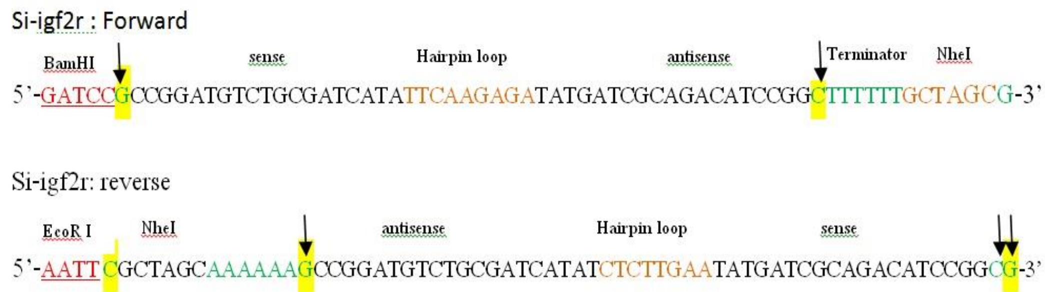
Figure 4.2 The expression levels of IGF-I (A), IGF-IR (B), IGF-II (C) and IGF-IIR (D and E) mRNA during 3T3-L1 differentiation detected by real time PCR **P-value $\leq$ 0.01

4.2 Establishment of the suitable conditions to knockdown IGF-IIR expression in 3T3-L1

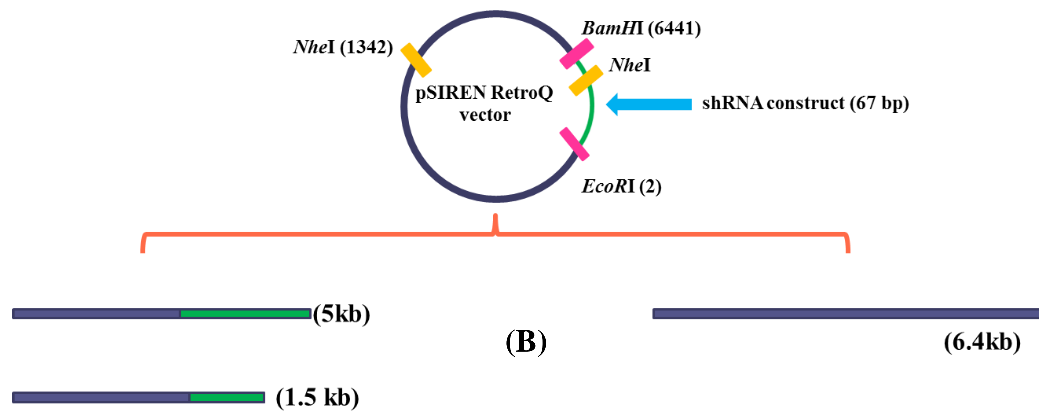
4.2.1. Attempts to knockdown IGF-IIR using shorthairpin RNA

Because the knockdown effect obtained by transient transfections of siRNA generally lasts for few days but differentiation of 3T3-L1 preadipocytes to mature adipocytes takes 8-10 days. This suggests that the transient transfection of IGF-IIR siRNA in this cell line may not be appropriate for this study. Initially, stable 3T3-L2 expressing shRNA for IGF-IIR was planned as constitutive suppression of IGF-IIR expression throughout the differentiation would provide an advantage. To achieve this aim, the siRNA was synthesized in the DNA form and cloned into the retrovirus expression vector as described in Material and Method section. The putative recombinant clones containing IGF-IIR shRNA were screended by restricting their plasmids with *Nhe*I because the *Nhe*I restriction sites were present both in the backbone of pSIREN-retro Q plasmid and inserted- shRNA fragment. After digestion with an *Nhe*I of recombinant plasmids which contained the shRNA and running on agrose gel electrophoresis, I obtained two DNA fragments representing the approximate 1.4 kb and 5 kb, respectively, as shown in Figure 4.3

As shown in Figure 4.3 all seven selected clones when cut with *Nhe*I, released the insert fragments representing the 1.4 kb of shRNA insert cassette. One of these clones (C7) was subjected to nucleotide sequencing. All of selected clone, digesting with *Nhe*I has shown that they contained the gene-specific silencing shRNA sequence. The C7 clone was selected for DNA sequencing using U6_forward_primer as the sequencing primer. The result showed that this clone contained the insert corresponding to the shRNA sequence. Therefore C7 clone was then used to transfect into BOSC cell line which is a packaging cell line. The retroviuses expressing IGF-IIR shRNA were collected after 2 days of transfection. The condition medium containing retroviruses harboring shIGF-IIR was collected and used to infect the 3T3-L1 preadipocytes to generate the 3T3-L1 stable cell line and the stable cell lines were selected in with the medium containing puromycin. Similar experiments in which the scramble control (pSIREN-GFP) and pSIREN-empty plasmid were also transfected to BOSC cells to serve as negative controls. Many attempts to generate 3T3-L1 stable preadipocytes were unsuccessful either there was no retroviruses produced in the condition medium or the infection of retroviruses to 3T3-L1 were not successful. Therefore only the transient transfections were used in the subsequent experiments.



(A)



(B)

Figure 4.3 A schematic draw showing the retrovirus plasmid construct (pSIREN-retro Q) containing shRNA for IGF-IIR (A) Sequence of expression cassette containing sense and antisense of siRNA (in the DNA form), hairpin loop, terminator and restriction enzymes; *Bam*HI*, Eco*RI and *Nhe*I as shown in forward and reverse stands. (B) pSIREN-retro Q, containing inserted DNA (shRNA) after cutting with *Nhe*I, I got two size of DNA fragments, were 1.4 kb, containing inserted DNA and 5 kb, as a linear form of plasmid.

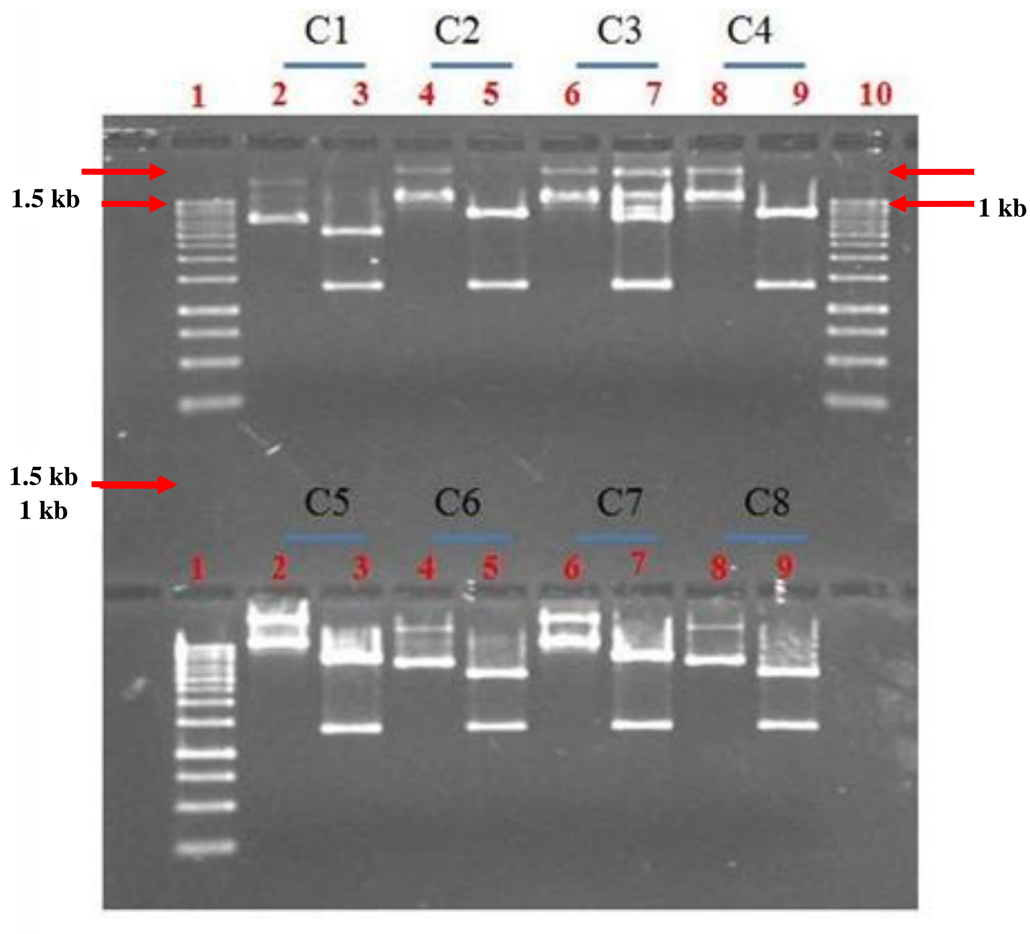


Figure 4.4 Restriction enzyme analysis of the plasmids from selected clones digested with *Nhe*I to verify the presence of shRNA construct. The 1 kb as a DNA marker (Lane 1 and 10), undigested plasmids as a control in each clone (Lanes 2, 4, 6 and 8), linearized plasmids of each clone (lane 3, 5,7) on the top pannel. DNA marker (Lane 1), undigested plasmids in each clone (Lanes 2, 4 and 6) and linearized plasmids in each clone (lane 3, 5, 7) on the bottom pannel.

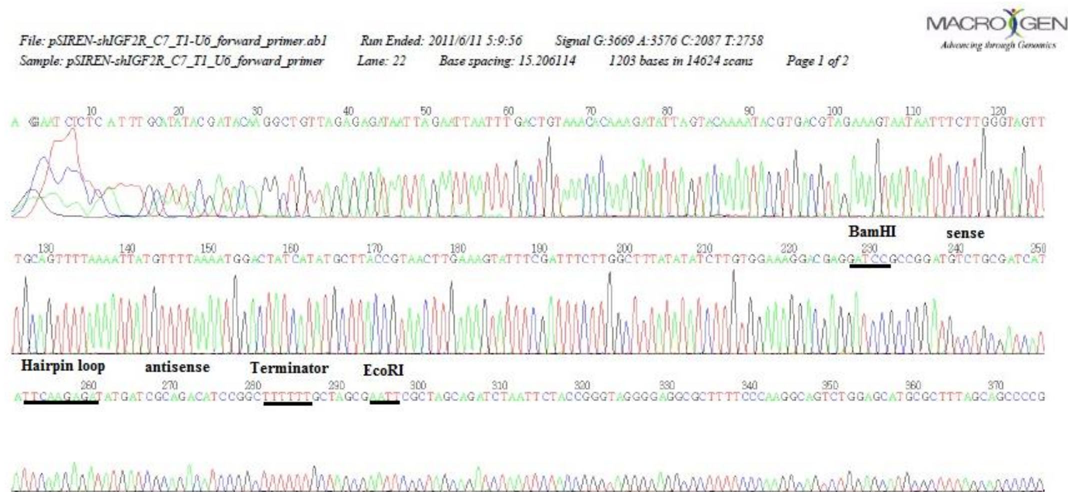


Figure 4.5 The DNA sequencing chromatograms of selected clones: *pSIREN-shIGF2R_C7_T1_U6_forward_primer*

4.2.2. Verification of the knockdown efficiency of commercial siRNA by transient transfection

To verify whether the commercial siRNA was capable of suppressing IGF-IIR expression, two concentrations of IGF-IIR siRNA were transiently transfected to 3T3-L1 preadipocytes. Initially, transfection of IGF-IIR siRNA to the cells plating at the density of $2x10^5$ cells/well did not alter the expression of endogenous IGF-IIR, suggesting that the confluence of the cells had an impact on the transfection (data not shown). However, transfection of siRNA to the cells plating at lower density, i.e. 1 x 10^5 cells/well gave a much better satisfied result. In this experiment, 3T3-L1 preadipocytes plated at the density of 1 x 10^5 cells/well were transfected with 25 nM and 50 nM of IGF-IIR siRNA.. As shown in Figure 4.6 transfections of cells with both concentrations resulted in 75% suppression of IGF-IIR mRNA expression. Due to the economic reason, 25 nM of IGF-IIR siRNA was used in the subsequent experiments.

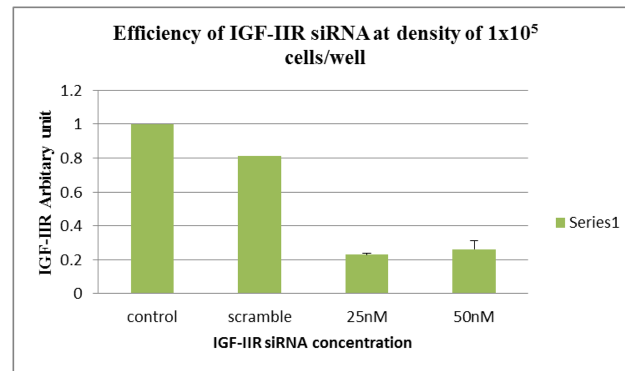


Figure 4.6 Knockdown efficiency of IGF-IIR siRNA at concentrations of 25 nM and 50nM at $1x10^5$ cell/wells detected by real time PCR.

4.2.3 To determine the suitable density of 3T3-L1 preadipocyte for differentiation

Although the result described in the earlier section clearly showed that the appropriate number of cells, i.e. plating cells at the density of 1 x 10^5 cells showed a success of siRNA knockdown, this number of the cells was too low to get a uniform adipocyte differentiation. Therefore in the next experiment, 3T3-L1 preadipocyte cells were plated at the density of $5x10^4$, $1x10^5$, $1.5x10^5$ and $2x10^5$ cells/well. After that these cells were induced to differentiate and the morphology of the cells was observed at days 0, 2, 4 and 6. As shown in Figure 4.7 differentiation to mature adipocytes cannot be observed when 5 x 10^4 cells were used. In contrast, differentiation was obvious when $1x10^5$, $1.5x10^5$ and $2x10^5$ cells were plated in the beginning. Although plating cells at the density of 1 x 10^5 showed uniform differentiation as shown in Figure 4.7 transfecting 3T3-L1 at this density with just the transfection reagent (mock-transfected) did not produce the same result because of a significant number of cell death was observed after the transfection. This lowered the number of cells and hence reduced the differentiation eefficiency. On the other hand, although plating cells at the density of 2 x 10^5 also showed uniform differentiation, transfecting of 3T3-L1 at this density with IGF-IIR siRNA resulted in marginal suppression of IGF-IIR expression (50-60% knockdown) which is likely due to too high confluence of the cells. In contrast, transfecting cells at the density of 1.5 x 10^5 cells produced the most satisfied result, i.e. the transfected cells still retained the capability to differentiate after siRNA transfection (data not shown).

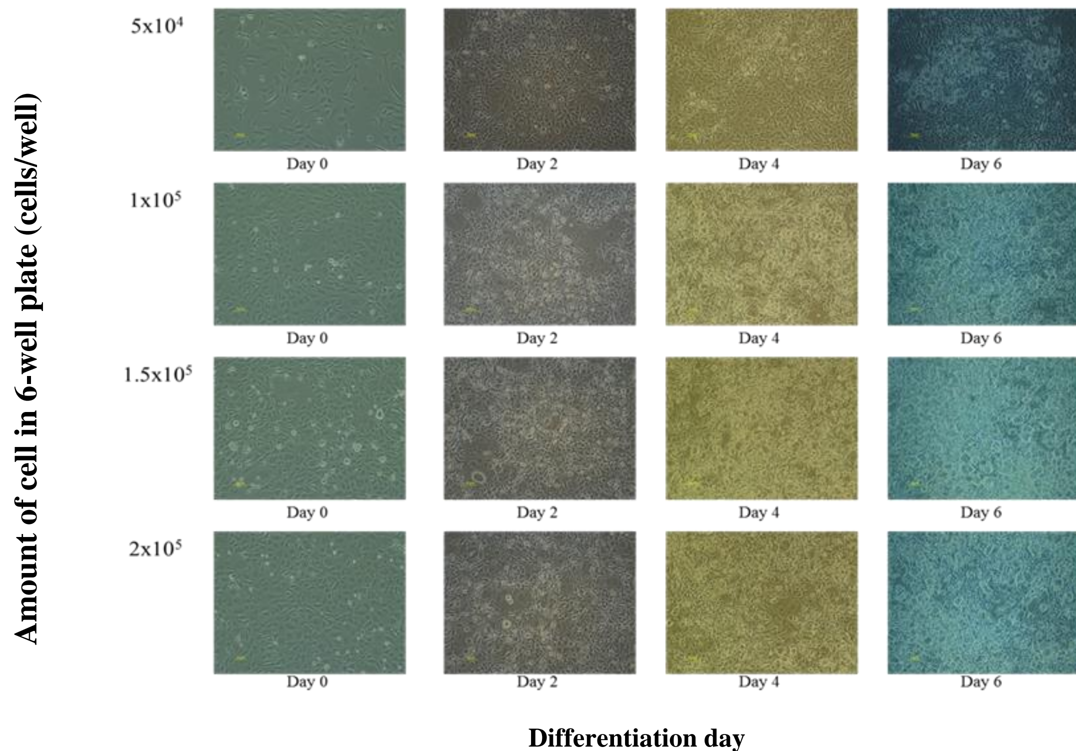


Figure 4.7 Differentiation of 3T3-L1 preadipocytes plating at different cell densities ($5x10^4$, $1x10^5$, $1.5x10^5$ and $2x10^5$ cells/well).

4.3. Effect of knocking down IGF-IIR expression on 3T3-L1 adipocyte differentiation

4.3.1 Effect of suppression of IGF-IIR expression on *in vitro* differentiation

Previous study has shown that IGF-IR is important for adipocyte differentiation (29,41). However, the information about the role of IGF-IIR during adipocyte differentiation remains to be elucidated. I hypothesize that if IGF-IIR was required for adipocyte differentiation, suppression of IGF-IIR would inhibit or retard differentiation accompanied by the decrease expression of adipogenic marker genes. However, if IGF-IIR was not required for adipocyte differentiation, suppression of IGR-IIR would not have any impact on adipocyte differentiation and expression of adipogenic marker genes. As reasoned in the above section, $1.5x10^5$ preadipocytes

were transfected with 25 nM IGF-IIR before they were subject to differentiation. During this period, morphology of the cells was observed and RNA collected for gene expression analysis.

As shown in Figure 4.8 the non-transfected 3T3-L1 preadipocyte as well as cells transfected with scramble control siRNA had started to differentiate to adipocytes at day 4 and fully differentiated to mature adipocytes by day 6. Similarly, 3T3-L1 preadipocytes transfected with IGF-IIR siRNA was also capable of differentiating to adipocytes by day 4 and fully differentiated to mature adipocytes by day 6 albeit they were slightly less number of mature adipocytes compared with the scramble control but very similar to the non-transfected cells. This result suggested that suppression of IGF-IIR in 3T3-L1 preadipocytes marginally affected adipocytes differentiation. Similar experiments were also observed (data not shown)

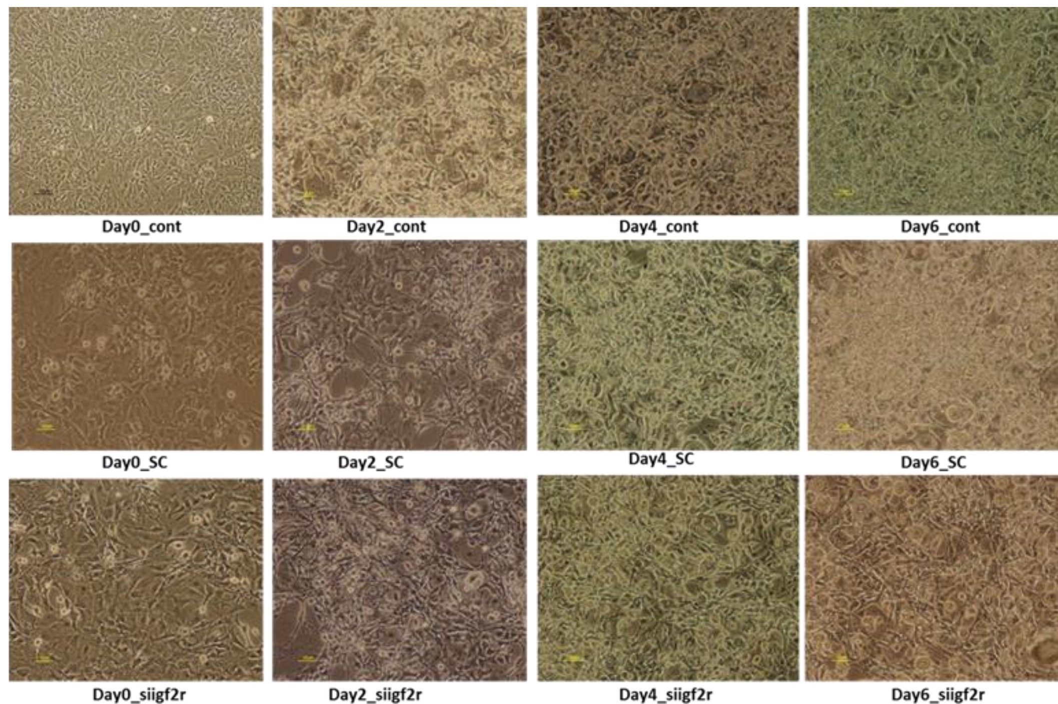


Figure 4.8 Expression of IGF-IIR gene was suppressed by IGF-IIR siRNA during 3T3-L1 preadipocyte differentiation at density of $1.5x10^5$cells/well ; cont (control: non transfected cell), SC (scramble: non-specific siRNA) and siIGF-IIR (IGF-IIR specific siRNA)

4.3.2. Suppression of IGF-IIR gene did not affect to the expression of adipogenic maker genes.

Adipocyte differentiation is governed by a hierarchy expression of adipogenic transcription factors including PPAR-γ1, PPAR-γ2 and C/EBPα (76,65). These three transcription factors are expressed during early differentiation thus considering as the early markers of adipocyte differentiation whereas aP2 (fatty acid binding protein), PC (pyruvate carboxylase) and GLUT4 (glucose transporter isoform 4) are expressed during mid to terminal differentiation thus considering as late markers. To confirm the lack of any effect of suppression of IGF-IIR expression on 3T3-L1 adipocyte differentiation, gene expression profile analysis including those of PPARγ1, PPARγ2, PC, aP2, GLUT4 and C/EBPα were measured by real time PCR. As shown in Figure 4.9A. The expression level of IGF-IIR mRNA was reduced as the result of knockdown, confirming the suppression effect of IGF-IIR siRNA. However the degree of knockdown slightly loss during differentiation, i.e. the level of IGF-IIR

mRNA expression was reduced by 70% at day 2 (4-day post transfection) and reduced approximately by 60% at day 4 and day 6 (6- and 8-day post transfection, respectively).

Early marker genes

In scramble control, the expression pattern of PPARγ1 and PPARγ2 were similar albeit not identical, i.e. PPARγ1 mRNA level was clearly detectable at day 2 and gradually increased until day 6 of differentiation as shown in Figure 4.9B In contrast, the expression of PPARγ2 mRNA was sharply increased to 60-80-fold at day 2 to day 4 and extremely increased to 160-170-fold at day 6, as shown in Figure 4.9C However, in the knockdown cells, the expression of both PPARγ1 and PPARγ2 were also similar to scramble control.

In the scramble control, the expression level of C/EBPα was sharply expressed at day 2 and further increased at day 4 before it declined at day 6 of differentiation. Similar expression profile was observed in the knockdown cells, as shown in Figure 4.9D

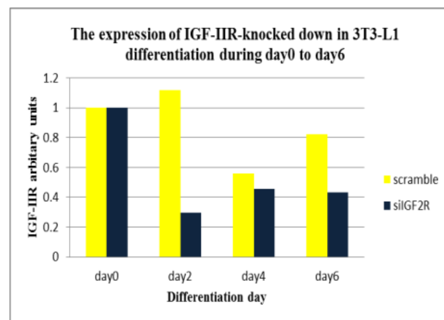
In the scramble control, the expression level of PC was sharply increased at day 2 and further increased at day 4 to day 6 of differentiation. Similar expression profiles were observed in the knockdown cells. In contrast expression level of PC was the lowest in preadipocyte at day 0 of differentiation, as shown in Figure 4.9E

Late marker genes

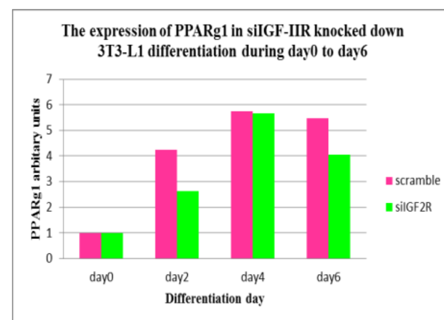
In scramble control cell, the expression level of aP2 was expressed at day 2 and dramatically increased at day 4 to day 6 of differentiation. Similar expression profile was investigated in the knockdown cells. In contrast, expression level of aP2 mRNA was undetectable in preadipocyte (day 0 of differentiation), as shown in Figure 4.9F

In scramble control cell, the expression level of GLUT4 was sharply expressed at day 4 and further increased at day 6 of differentiation Similar expression profile was observed in the knockdown cells whereas the expression of GLUT4 was rarely expressed at day 2 and was not detectable at preadipocyte, as shown in Figure 4.9G

From the results were suggested that suppression of IGF-IIR gene has not altered to expression profile of adipogenic marker genes in initiation and termination stages of 3T3-L1 preadipocyte differentiation.



(A)



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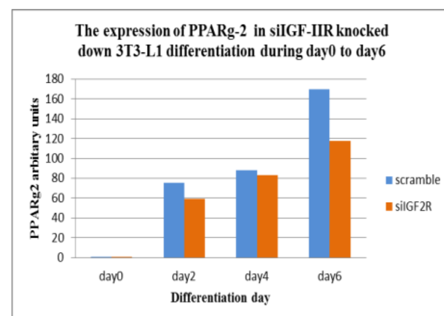
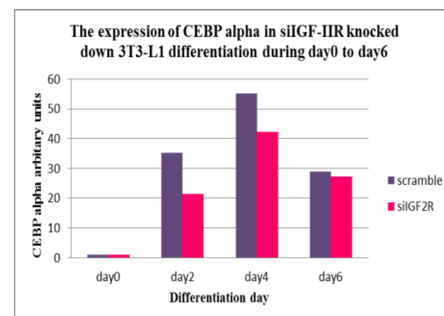
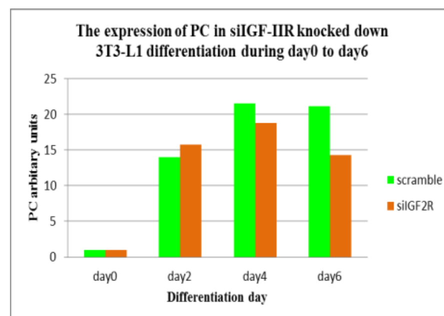


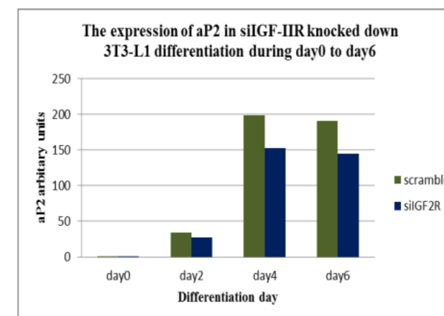
(C)



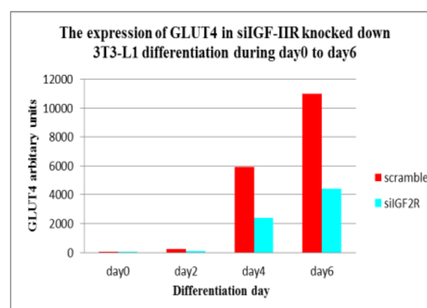
(D)



(E)



(F)



(G)

Figure 4.9 The bar chart shown the expression of mRNA level of adipogenic genes; (A) IGF-IIR, (B) PPARγ1, (C) PPARγ2, (D) C/EBPα, (E) PC,(F) aP2 and (G) GLUT4 in non-specific siRNA transfected 3T3-L1 preadipocyte and IGF-IIR siRNA transfected 3T3-L1 preadipocyte during differentiation at day0 to day6

CHAPTER V
DISCUSSION

Although the use of human preadipocytes is a potential model to study gene function, such use has several limitations including (i) restricted number of preadipocytes can be obtained from individual (2) they can be maintained only as primary culture (iii) the ability of primary adipocytes to differentiate to mature adipocytes appears variable between sources (38,65). As such, alternative cell lines including mouse preadipocyte cell line has become extensively used. Murine 3T3-L1 adipocyte cell line is a widely used model for studying adipogenesis. Under hormonal induction 3T3-L1 preadipocytes can be converted from a fibroblast shape to a round shape concomitant with the lipid accumulation. This is resembled a characteristic of mature adipocytes. During differentiation process, 3T3-L1 preadipocytes pass through the adipogenesis lineage until they become mature adipocytes upon subject to adipogenic hormonal induction including insulin, dexamethasone and IBMX. Insulin signaling via AKT/PKB phosphorylation (60,76) promotes *de novo* fatty acid synthesis, stimulates expression and activity of the lipoprotein lipase (LPL) which hydrolyzes the lipoprotein-derived triglycerides to free fatty acids required for catabolism or storage in adipocytes (53). Dexamethasone (DEX), a synthetic glucocorticoid acts on adipocytes by stimulating lipogenesis (4). Another role of DEX is that it can promote adipogenesis by stimulating the expression of C/EBPδ and PPARγ, key transcription factors required for adipocyte differentiation (98). DEX also suppresses the expression of preadipocyte factor-1 (pref-1) which is an inhibitor of adipocyte differentiation. Lastly, 3-isobutyl-1-methylxanthine (IBMX) is a phosphodiesterase inhibitor. IBMX activates the accumulation of cAMP by inhibiting phosphodiesterase and also activates adenylyl cyclase activity (38). cAMP is a mediator for inducing the expression of PPARγ and C/EBPβ (65). This mechanism exerts via binding of a cAMP-responsive element binding protein (CREB) to the

cAMP-responsive element (CRE) in the promoters of PPARγ and C/EBPβ genes. CREB-ablated mice appear is lean, resulting from the absence of fat tissues (77).

Previous studies have shown that both IGF-I and IGF-II are mitogenic ligands which exhibit structural homology with insulin and they are involved in many biological processes such as cell growth and differentiation, cell death and chemotaxis (29,41). IGF-I is a main regulator for activation biological effects such as stimulating glucose oxidation and adipogenesis via IR and IGF-IR (80,86,13). In the present study, expression levels of IGF-I and IGF-IR mRNAs were high in preadipocytes. Assuming that the expression of both IGF-I and IGF-IR mRNAs reflects the levels of IGF-1 and IGF-II proteins, the present study suggests that both ligands may be required to support rapid proliferation rate prior to mitotic-arrested which is prerequisite for adipocyte differentiation (14,45) . Our finding was also consistent with the previous report which shows that the level of IGF-I is high in preadipocytes and is required for adipocyte differentiation (86). Furthermore earlier studies have also found that the number of IGF-IR is down-regulated while the number of insulin receptor is up-regulated once cells have become adipocytes. This reciprocal change of IGF-IR and insulin receptor makes adipocytes become metabolic responsive rather than mitogenic responsive (45). Similar to IGF-I, IGF-II promotes cell proliferation, cell growth and anti-apoptosis. Especially, IGF-II plays role in human fetal growth, promoting both tissue-specific and developmental regulation. Moreover, IGF-II has also been reported that it is a key factor in progression of many tumors (70). The biological effects of IGF-II are mediated via the interaction with IGF-IR and insulin receptor but never through its receptor. In contrast, IGF-IIR plays a role in degradation and internalization of IGF-II (70, 41, 100). Assuming that the level of IGF-II mRNA also reflects its protein, the present study suggests that this ligand may be essential for terminal stage of adipocyte differentiation although the exact role of IGF-II during terminal stage of adipocyte differentiation still remains unclear. Nevertheless this result is consistent with previous data which reported that the endogenous IGF-II is not important for early commitment phase of adipocyte differentiation and it does not affect to the expression of adipocyte specific transcription factors, PPARγ and C/EBPα which are key transcription regulator for adipocyte differentiation (49). The level of IGF-IIR mRNA expression is similar to that of IGF-II mRNA. It is well

known that IGF-IIR is highly specific receptor for IGF-II. This receptor mops up excess IGF-II but not IGF-I or insulin via receptor internalization (41). Based on the assumption that the expression of IGF-IIR mRNA also reflects the level of protein, it is possible that low levels of IGF-IIR expression during 3T3-L1 differentiation may facilitate the removal of IGF-II from compete binding with insulin and IGF-I to insulin receptor or IGF-IR. As mentioned earlier signaling via IGF-II would promote mitogenic effect rather than metabolic effect. The latter effect is required for adipocytes to accumulate the lipids.

Another biological function of IGF-IIR is known to regulate the transportation of M6P-containing lysosomal enzymes from the Golgi network to lysosome (41). This information brings about the hypothesis that IGF-IIR might be involved in lysosomal enzyme trafficking pathway during adipocyte differentiation. However, the mechanism by which IGF-IIR controlling this process in adipocyte is unknown. Therefore the functional importance of IGF-IIR in adipocyte differentiation was investigated by the knockdown experiment. It was found that suppression of IGF-IIR expression in 3T3-L1 preadipocytes results in only a slight retardation of their differentiation suggesting that IGF-IIR may not be absolutely crucial for adipocyte differentiation. Subsequent gene expression profile analysis of early and later marker genes of adipocyte differentiation including PPARγ, C/EBPα, PC, aP2 and GLUT4 also conducted. The results have shown that the expression of PPARγ (-1 and -2) mRNA levels of the IGF-IIR knocked down 3T3-L1 were similar to the non-specific siRNA transfected 3T3-L1 during differentiation. Furthermore, the expression of C/EBPα mRNA level of IGF-IIR knocked down 3T3-L1 adipocytes was also similar with non-specific siRNA transfected 3T3-L1 adipocytes. Together with the expression of adipocyte-specific marker genes; PC, aP2 and GLUT4 mRNA levels of IGF-IIR knocked down 3T3-L1 were consistent with another although the expression of GLUT4 mRNA level was 2-fold lower in the IGF-IIR knocked down 3T3-L1 adipocytes. These findings suggest that blocking of IGF-IIR expression did not affect and the expression of adipocyte-specific marker genes which is consistent with the little effect of adipocyte differentiation. However it is noted that the lack of any effect of IGF-II knockdown on adipocyte differentiation may be resulted from the loss of siRNA during adipocyte differentiation. This is because the differentiation of

adipocytes to mature adipocytes takes 8-10 days in which the transfected IGF-II siRNA may have degraded or diluted out during this prolonged period. Although the use of short hairpin RNA (shRNA) via stable transfection appears to be a better mean however many attempts to generate 3T3-L1 stable lines expressing IGF-IIR shRNA was not successful. This technical difficulty is in agreement with previous report (48). Future work may require the use of other highly efficient transfection method such as electroporation (48).

Taken together, the present study suggests that IGF-IIR may be involved in other metabolic pathway rather than adipocyte differentiation however. the structure of IGF-IIR is multifunctional transmembrane glycoprotein and has no intrinsic tyrosine kinase activity and is well known as a clearance receptor (41,48). Therefore, the information of IGF-IIR signaling pathway mechanism is still ambiguous. The possible evidence for IGF-IIR in differentiation and maintaining of adipocyte may involve in (i) distribution and localization of GLUT4 vesicle expressed in cell membrane of adipocytes (71). This previous report is consistent with the present result that which showed that suppression of IGF-IIR mRNA lowers the levels of GLUT4 mRNA expression. (ii) Degradation of IGF-II ligand by IGF-IIR may involve the activation of IGF-I and insulin to IGF-IR and IR which are essential for promoting of metabolic and biochemical effects rather than mitogenic effect. Lastly the degradation of IGF-II by IGF-IIR may involve in regulation of induction of leptin which is a main adipokine to maintain adipocyte because it has been reported that IGF-II prevents leptin induction in hemangioma stem cell which differentiate to many types of cell including adipocyte (49).

CHAPTER VI
CONCLUSION

1. 3T3-L1 preadipocyte cell was completely converted into the mature adipocyte which accumulated the lipid droplets by induction of adipogenic agent such as dexamethasone (DEX), isobutylxanthine (IBMX) and particularly insulin.
2. The expression of IGFs and their receptors were dependent on the adipocyte differentiation process. The expression pattern of IGF-I is also similar to its receptor. The expression of IGF-II mRNA level was gradually increased. Additional, the expression pattern of IGF-IIR mRNA was high peak in preadipocyte and gradually decreased in mature adipocyte.
3. Knockdown of IGF-IIR by 90% reduction in 3T3-L1 preadipocyte cell was slight retarded to differentiate from preadipocyte to mature adipocyte when compared with non- specific siRNA transfected and non-transfected 3T3-L1 preadipocyte differentiation.
4. Gene expression analysis of adipogenic marker genes PPARγ-1, PPARγ-2, PC, aP2, GLUT4 and CEBPα supported that their expression was not significantly different between the IGF-IIR knockdown and control cells during differentiation.

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APPENDIX

1. real time PCR raw data of the expression levels of IGF-I, IGF-IR, IGF-II and IGF-IIR mRNA during 3T3-L1 differentiation

1.1. real time PCR raw data of the expression levels of IGF-I mRNA (experiment I and II)

Table 1.1.1 the expression level of IGF-I mRNA in experiment I

Well	Ct (dRn)	igf1 ave	Ct (dRn)	18sRNA ave	normalized	ΔΔCt	fold
day0	29.69	30	15.02	15.12	14.88	0	1
day0	30.31		15.22				
day2	30.51	30.51	13.98	14.15	16.36	1.48	0.358489
day2	30.51		14.32				
day4	31.51	31.09	14.22	14.105	16.985	2.105	0.232451
day4	30.67		13.99				
day6	31.16	31.34	14.72	14.79	16.55	1.67	0.314253
day6	31.52		14.86				
day8	29.97	30.81	14.63	14.695	16.115	1.235	0.424842
day8	31.65		14.76				
day10	30.46	30.69	14.87	15.12	15.57	0.69	0.619854
day10	30.92		15.37				

Table 1.1.2 the expression level of IGF-I mRNA in experiment II

Well	Ct (dRn)	IGF-I ave	18sRNA ave	normalized	ΔΔCT	fold
day0	27.83	27.885	11.935	15.95	0	1
day0	27.94					
day2	31.06	31.34	12.005	19.335	3.385	0.09572
day2	31.62					
day4	31.72	31.485	12.675	18.81	2.86	0.13774
day4	31.25					
day6	29.52	29.86	12.075	17.785	1.835	0.28029
day6	30.2					
day8	30.38	30.22	12.46	17.76	1.81	0.28519
day8	30.06					
day10	29.11	29.34	12.205	17.135	1.185	0.43982
day10	29.57					

1.2 real time PCR raw data of the expression levels of IGF-IR mRNA (experiment I and II)

Table 1.2.1 the expression level of IGF-IR mRNA in experiment I

Well	Ct (dRn)	IGF-IRave	18sRNA ave	normalized	ΔΔCt	fold
day0	27.62	27.625	15.12	12.505	0	1
day0	27.63					
day2	27.09	27.315	14.15	13.165	0.66	0.632878
day2	27.54					
day4	27.85	27.92	14.105	13.815	1.31	0.403321
day4	27.99					
day6	28.28	28.285	14.79	13.495	0.99	0.503478
day6	28.29					
day8	27.51	27.61	14.695	12.915	0.41	0.752623
day8	27.71					
day10	27.59	27.755	15.12	12.635	0.13	0.913831
day10	27.92					

Table 1.2.2 the expression level of IGF-IR mRNA in experiment II

Well	Ct (dRn)	IGF-IR_ave	18s_ave	normalized	ΔΔCT	fold
day0	26.58	26.445	13.99	12.455	0	1
day0	26.31					
day2	26.79	26.98	13.935	13.045	0.59	0.664343
day2	27.17					
day4	27.98	27.895	14.185	13.71	1.255	0.418994
day4	27.81					
day6	26.6	26.615	13.875	12.74	0.285	0.820742
day6	26.63					
day8	26.79	26.74	14.06	12.68	0.225	0.855595
day8	26.69					
day10	26.93	27	14.99	12.01	-0.445	1.361314
day10	27.07					

1.3 real time PCR raw data of the expression levels of IGF-II mRNA (experiment I and II)

Table 1.3.1 the expression level of IGF-II mRNA in experiment I

Well	Ct (dRn)	IGF-II ave	18sRNA ave	normalized	ΔΔCt	fold
day0	28.93	28.92	15.12	13.8	0	1
day0	28.91					
day2	27.64	29.37	14.15	15.22	1.42	0.373712
day2	31.1					
day4	29.62	29.565	14.105	15.46	1.66	0.316439
day4	29.51					
day6	28.74	28.91	14.79	14.12	0.32	0.80107
day6	29.08					
day8	26.82	26.905	14.695	12.21	-1.59	3.010493
day8	26.99					
day10	26.31	26.39	15.12	11.27	-2.53	5.775717
day10	26.47					

Table 1.3.2 the expression level of IGF-II mRNA in experiment II

Well	Ct (dRn)	IGF-II ave	ave_18sRNA	normalized	ΔΔCt	fold
day0	27.98	28.05	13.795	14.255	0	1
day0	28.12					
day2	29.58	29.68	13.97	15.71	1.455	0.364755
day2	29.78					
day4	28.92	28.87	13.9	14.97	0.715	0.609205
day4	28.82					
day6	28.59	28.595	13.86	14.735	0.48	0.716978
day6	28.6					
day8	26.87	26.705	13.905	12.8	-1.455	2.741566
day8	26.54					
day10	27.26	27.26	15.3	11.96	-2.295	4.90754
day10	27.26					

1.4 real time PCR raw data of the expression levels of IGF-IIR mRNA (experiment I and II)

Table 1.4.1 the expression level of IGF-IIR mRNA in experiment I

Well	Ct (dRn)	IGF-IIR ave	18sRNA ave	normalized	ΔΔCt	fold
day0	23.75	23.72	15.12	8.6	0	1
day0	23.69					
day2	24.13	24.185	14.15	10.035	1.435	0.369847
day2	24.24					
day4	24.88	24.88	14.105	10.775	2.175	0.221442
day4	24.88					
day6	24.91	24.955	14.79	10.165	1.565	0.337978
day6	25					
day8	24.43	24.365	14.695	9.67	1.07	0.476319
day8	24.3					
day10	24.54	24.54	15.12	9.42	0.82	0.566442
day10						

Table 1.4.2 the expression level of IGF-IIR mRNA in experiment II

Well	Ct (dRn)	IGF-IIR ave	18s_ave	normalized	ΔΔCt	fold
day0	23.73	23.695	13.99	9.705	0	1
day0	23.66					
day2	24.27	24.355	13.935	10.42	0.715	0.609205
day2	24.44					
day4	25.56	25.61	14.185	11.425	1.72	0.303549
day4	25.66					
day6	23.66	23.73	13.875	9.855	0.15	0.90125
day6	23.8					
day8	23.91	23.805	14.06	9.745	0.04	0.972655
day8	23.7					
day10	23.97	24.015	14.99	9.025	-0.68	1.60214
day10	24.06					

2. real time PCR raw data of the mRNA expression levels of IGF-IIR knocked down in 3T3-L1 differentiation during day0 to day6

Table 2 The expression of IGF-IIR knocked down in 3T3-L1 differentiation

Well	IGF2R Ct (dRn)	IGF2R ave	18srRNACt (dRn)	18srRNA ave	normalized	ΔΔCt	fold
cont_D0	22.52	22.48	13.16	13.145	9.335	0	1
cont_D0	22.44		13.13				
cont_D2	22.64	22.635	12.92	12.87	9.765	0.43	0.742261785
cont_D2	22.63		12.82				
cont_D4	23.23	23.23	13.22	13.255	9.975	0.64	0.641712949
cont_D4	23.23		13.29				
cont_D6	23.38	23.435	12.91	12.94	10.495	1.16	0.447512535
cont_D6	23.49		12.97				
sc_D0	22.63	22.53	13.26	13.215	9.315	0	1
sc_D0	22.43		13.17				
sc_D2	22.76	22.765	13.56	13.61	9.155	-0.16	1.117287138
sc_D2	22.77		13.66				
sc_D4	23.27	23.195	13.25	13.035	10.16	0.845	0.556710809
sc_D4	23.12		12.82				
sc_D6	23.57	23.425	13.82	13.83	9.595	0.28	0.823591017
sc_D6	23.28		13.84				
si2R_D0	22.55	22.48	12.57	12.665	9.815	0	1
si2R_D0	22.41		12.76				
si2R_D2	24.5	24.385	12.85	12.81	11.575	1.76	0.295248165
si2R_D2	24.27		12.77				
si2R_D4	24.12	24.015	13.08	13.06	10.955	1.14	0.453759578
si2R_D4	23.91		13.04				
si2R_D6	23.66	23.665	12.65	12.635	11.03	1.215	0.43077308
si2R_D6	23.67		12.62				

3. real time PCR raw data of the mRNA expression levels of early (PPARγ1, PPARγ2 and CEBPα) and late (PC, aP2 and GLUT4) adipogenic marker genes differentiation during day0 to day6

Table 3.1 the expression of PPARγ1 mRNA levels in IGF-IIR knocked down 3T3-L1 differentiation

Well	PPARg1 Ct (dRn)	PPARg1 ave	18sr RNA Ct	18sr RNA ave	normalized	ΔΔCt	fold
cont_D0	23.48	23.405	16.9	16.85	6.555	0	1
cont_D0	23.33		16.8				
cont_D2	21.58	21.625	17.17	17.11	4.515	-2.04	4.112455
cont_D2	21.67		17.05				
cont_D4	21.08	20.86	17.79	17.565	3.295	-3.26	9.57983
cont_D4	20.64		17.34				
cont_D6	20.96	20.86	17.06	16.95	3.91	-2.645	6.254957
cont_D6	20.76		16.84				
sc_D0	23.32	23.195	16.76	16.855	6.34	0	1
sc_D0	23.07		16.95				
sc_D2	21.75	21.77	17.36	17.515	4.255	-2.085	4.242751
sc_D2	21.79		17.67				
sc_D4	20.77	20.565	17.11	16.75	3.815	-2.525	5.755734
sc_D4	20.36		16.39				
sc_D6	20.77	20.61	16.72	16.72	3.89	-2.45	5.464161
sc_D6	20.45		16.72				
si2R_D0	23.27	23.155	16.68	16.54	6.615	0	1
si2R_D0	23.04		16.4				
si2R_D2	21.81	21.815	16.38	16.59	5.225	-1.39	2.620787
si2R_D2	21.82		16.8				
si2R_D4	21.35	21.08	17.21	16.97	4.11	-2.505	5.676493
si2R_D4	20.81		16.73				
si2R_D6	20.77	20.805	15.98	16.21	4.595	-2.02	4.055838
si2R_D6	20.84		16.44				

Table 3.2 the expression of PPARγ2 mRNA levels in IGF-IIR knocked down 3T3-L1 differentiation

Well	PParg2Ct (dRn)	PPARg2 ave	18srRNA Ct	18sr RNA ave	normalized	ΔΔCt	fold
cont_D0	30.38	30.185	16.38	16.3	13.885	0	1
cont_D0	29.99		16.22				
cont_D2	23.5	23.475	15.74	15.685	7.79	-6.095	68.35619
cont_D2	23.45		15.63				
cont_D4	23.63	23.615	16.06	16.125	7.49	-6.395	84.15634
cont_D4	23.6		16.19				
cont_D6	23.02	22.965	15.98	15.985	6.98	-6.905	119.8428
cont_D6	22.91		15.99				
sc_D0	30.02	29.985	16.25	16.245	13.74	0	1
sc_D0	29.95		16.24				
sc_D2	23.99	23.98	16.42	16.475	7.505	-6.235	75.32203
sc_D2	23.97		16.53				
sc_D4	23.31	23.265	15.87	15.98	7.285	-6.455	87.7301
sc_D4	23.22		16.09				
sc_D6	22.76	22.77	16.58	16.435	6.335	-7.405	169.4834
sc_D6	22.78		16.29				
si2R_D0	29.57	29.63	15.8	15.755	13.875	0	1
si2R_D0	29.69		15.71				
si2R_D2	23.66	23.635	15.68	15.65	7.985	-5.89	59.30164
si2R_D2	23.61		15.62				
si2R_D4	23.81	23.71	16.37	16.215	7.495	-6.38	83.28588
si2R_D4	23.61		16.06				
si2R_D6	22.72	22.745	15.74	15.745	7	-6.875	117.3765
si2R_D6	22.77		15.75				

Table 3.3 the expression of CEBPα mRNA levels in IGF-IIR knocked down 3T3-L1 differentiation

Well	CEBP alphaCt (dRn)	CEBP alpha ave	18sr RNACt (dRn)	18srRNA ave	normalized	ΔΔCt	fold
cont_D0	31.5	33.125	15.14	15.18	17.945	0	1
cont_D0	34.75		15.22				
cont_D2	29.02	28.945	15.26	15.085	13.86	-4.085	16.971
cont_D2	28.87		14.91				
cont_D4	26.45	26.89	15.4	15.465	11.425	-6.52	91.77314
cont_D4	27.33		15.53				
cont_D6	27.46	28.705	14.98	14.985	13.72	-4.225	18.70044
cont_D6	29.95		14.99				
sc_D0	31.93	33.475	15.41	15.37	18.105	0	1
sc_D0	35.02		15.33				
sc_D2	28.32	28.295	15.23	15.33	12.965	-5.14	35.26096
sc_D2	28.27		15.43				
sc_D4	27.67	27.84	15.67	15.52	12.32	-5.785	55.13895
sc_D4	28.01		15.37				
sc_D6	27.9	28.265	15	15.01	13.255	-4.85	28.84001
sc_D6	28.63		15.02				
si2R_D0	31.8	32.605	15.35	15.35	17.255	0	1
si2R_D0	33.41		15.35				
si2R_D2	28.47	28.42	15.54	15.59	12.83	-4.425	21.48116
si2R_D2	28.37		15.64				
si2R_D4	27.54	27.255	15.3	15.4	11.855	-5.4	42.22425
si2R_D4	26.97		15.5				
si2R_D6	27.52	27.515	15.1	15.025	12.49	-4.765	27.18992
si2R_D6	27.51		14.95				

Table 3.4 the expression of PC mRNA levels in IGF-IIR knocked down 3T3-L1 differentiation

Well	PC Ct (dRn)	PC ave	18srRNA Ct (dRn)	18srRNA ave	normalized	ΔΔCt	fold
cont_D0	25.18	25.07	16.71	16.86	8.21	0	1
cont_D0	24.96		17.01				
cont_D2	20.96	21.01	16.17	16.15	4.86	-3.35	10.19649
cont_D2	21.06		16.13				
cont_D4	20.78	20.74	16.92	16.87	3.87	-4.34	20.25211
cont_D4	20.7		16.82				
cont_D6	20.74	20.81	16.34	16.345	4.465	-3.745	13.40779
cont_D6	20.88		16.35				
sc_D0	25.51	25.47	16.5	16.675	8.795	0	1
sc_D0	25.43		16.85				
sc_D2	21.79	21.785	16.83	16.795	4.99	-3.805	13.97717
sc_D2	21.78		16.76				
sc_D4	21.1	20.955	16.65	16.59	4.365	-4.43	21.55574
sc_D4	20.81		16.53				
sc_D6	21.4	21.365	16.99	16.97	4.395	-4.4	21.11213
sc_D6	21.33		16.95				
si2R_D0	24.87	24.87	16	15.98	8.89	0	1
si2R_D0	24.87		15.96				
si2R_D2	21.34	21.335	16.37	16.42	4.915	-3.975	15.72513
si2R_D2	21.33		16.47				
si2R_D4	21.24	21.1	16.52	16.44	4.66	-4.23	18.76536
si2R_D4	20.96		16.36				
si2R_D6	20.93	20.95	15.81	15.9	5.05	-3.84	14.3204
si2R_D6	20.97		15.99				

Table 3.5 the expression of aP2 mRNA levels in IGF-IIR knocked down 3T3-L1 differentiation

Well	AP2Ct (dRn)	AP2 ave	18sr RNA Ct	18sr RNA ave	normalized	ΔΔCt	fold
cont_D0	28.41	28.375	16.9	16.85	11.525	0	1
cont_D0	28.34		16.8				
cont_D2	23.41	23.37	17.17	17.11	6.26	-5.265	38.45235
cont_D2	23.33		17.05				
cont_D4	21.89	21.87	17.79	17.565	4.305	-7.22	149.0859
cont_D4	21.85		17.34				
cont_D6	21.15	21.28	17.06	16.95	4.33	-7.195	146.5247
cont_D6	21.41		16.84				
sc_D0	28.58	28.525	16.76	16.855	11.67	0	1
sc_D0	28.47		16.95				
sc_D2	23.95	24.09	17.36	17.515	6.575	-5.095	34.17809
sc_D2	24.23		17.67				
sc_D4	20.74	20.79	17.11	16.75	4.04	-7.63	198.0883
sc_D4	20.84		16.39				
sc_D6	20.82	20.815	16.72	16.72	4.095	-7.575	190.6787
sc_D6	20.81		16.72				
si2R_D0	28.25	28.265	16.68	16.54	11.725	0	1
si2R_D0	28.28		16.4				
si2R_D2	23.44	23.51	16.38	16.59	6.92	-4.805	27.95433
si2R_D2	23.58		16.8				
si2R_D4	21.53	21.44	17.21	16.97	4.47	-7.255	152.747
si2R_D4	21.35		16.73				
si2R_D6	20.72	20.76	15.98	16.21	4.55	-7.175	144.5074
si2R_D6	20.8		16.44				

Table 3.6 the expression of GLUT4 mRNA levels in IGF-IIR knocked down 3T3-L1 differentiation

Well	GLUT4 Ct (dRn)	GLUT4 ave	18sr RNA Ct	18sr RNA ave	normalized	ΔΔCt	fold
cont_D0	39.39	39.295	16.9	16.85	22.445	0	1
cont_D0	39.2		16.8				
cont_D2	30.73	30.87	17.17	17.11	13.76	-8.685	411.5717073
cont_D2	31.01		17.05				
cont_D4	26.62	26.34	17.79	17.565	8.775	-13.67	13034.06975
cont_D4	26.06		17.34				
cont_D6	25.04	25.07	17.06	16.95	8.12	-14.325	20523.65416
cont_D6	25.1		16.84				
sc_D0	No Ct	38.98	16.76	16.855	22.125	0	1
sc_D0	38.98		16.95				
sc_D2	31.85	31.695	17.36	17.515	14.18	-7.945	246.4241774
sc_D2	31.54		17.67				
sc_D4	26.37	26.34	17.11	16.75	9.59	-12.535	5934.867065
sc_D4	26.31		16.39				
sc_D6	25.55	25.42	16.72	16.72	8.7	-13.425	10998.35394
sc_D6	25.29		16.72				
si2R_D0	38.32	38.275	16.68	16.54	21.735	0	1
si2R_D0	38.23		16.4				
si2R_D2	31.84	31.96	16.38	16.59	15.37	-6.365	82.42442429
si2R_D2	32.08		16.8				
si2R_D4	27.57	27.475	17.21	16.97	10.505	-11.23	2401.965976
si2R_D4	27.38		16.73				
si2R_D6	25.96	25.835	15.98	16.21	9.625	-12.11	4420.518857
si2R_D6	25.71		16.44				

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