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The role of Aortic Carboxypeptidase-Like Protein (ACLP) in 3T3-L1 Preadipocyte and Adipocyte Function

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The Role of Aortic Carboxypeptidase-Like Protein (ACLP) in 3T3-L1 Preadipocyte and Adipocyte Function.

Arjeta Gusinjac

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies, in partial fulfillment of the requirements for the M.Sc. degree in Biochemistry

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ABSTRACT

Aortic carboxypeptidase-like protein (ACLP) is a 175kDa secreted protein that is downregulated with adipogenesis. I engineered 3T3-L1 preadipocytes that overexpressed ACLP protein and sustained the overexpression with differentiation. I assessed the role of sustained ACLP overexpression on 3T3-L1 adipogenesis, with a focus on possible ACLP interaction with collagen-I during differentiation.

ACLP overexpression did not affect 3T3-L1 adipogenesis under standard cell culture conditions. ACLP overexpression did not affect subconfluent preadipocyte proliferation, apoptosis in serum-supplemented preadipocytes, or serum-deprived preadipocyte cell death. Sustained ACLP overexpression did not affect collagen I/III protein expression, or the activity of matrix metalloproteinase (MMP)-2 and MMP-9 extracellular degrading enzymes.

However, sustained ACLP overexpression on collagen-I coated dishes inhibited the expression of three key differentiation markers, peroxisome proliferator-activated receptor γ, CCAAT enhancer-binding protein α and fatty acid synthase and decreased insulin-stimulated Akt/protein kinase B phosphorylation. This suggests that a collagen-I-rich environment is necessary for ACLP to alter preadipocyte function.

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LIST OF ABBREVIATIONS

ACLP – Aortic carboxy-peptidase like protein
AEBP1 - Adipocyte enhancer binding protein 1
ALBP – Adipocyte lipid binding protein
Akt/PKB – Protein kinase B
BAT – Brown adipose tissue
BSA – Bovine serum albumin
cAMP – Cyclic AMP
C/EBP – CCAAT enhancer-binding protein
CREB - cAMP response element binding
CS – Calf serum
DMEM – Dulbecco's modified Eagle's medium
DMSO – Dimethyl sulfoxide
DDR – Discoidin domain receptor
E. Coli – Escherichia Coli
ECM – Extracellular matrix
EDHB - ethyl-3,4-dihydroxybenzoate
ERK1/2 – Extracellular signal-regulated kinase 1/2
FAS – Fatty acid synthase
FBS – Fetal bovine serum
FFA – Free fatty acid
Gas – Growth arrest specific
GLUT-4 – Glucose transporter 4
Grb2 – Growth factor receptor-bound protein 2
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX – 3-Isobutyl-1-methylxanthine
IOD – Integrated optical density
IPF - Idiopathic Pulmonary Fibrosis
IRS – Insulin receptor substrate
KLF4 - Kruppel-like factor 4
KRH – Krebs-Ringer-Hepes
FOXO – Forkhead box containing O
LAIR-1 - Leukocyte-associated immunoglobulin-like receptor 1
LB - Luria-Bertani
MAPK – Mitogen-activated protein kinase
MMP - Matrix metallopeptidase
p300/EP300 - E1A binding protein p300
PBS – Phosphate-buffered saline
PKA – Protein kinase A
PKC – Protein kinase C
PDK1 - Phosphatidylinositol-dependent protein kinase 1
PI – Phosphoinositide
PPAR – Peroxisome proliferator-activated receptor
PS – Penicillin/Streptomycin

PTB – Phosphotyrosine binding
PTEN – Phosphatase and tensin homolog
pTyr – Phosphotyrosine
SDS-PAGE - Sodium dodecyl sulfate Polyacrylamide gel electrophoresis
SH2 – Src homology 2
SHC - Src homology 2 domain containing
SOS – Son of sevenless
SREBP-1 – Sterol regulatory element binding protein 1
SVF – Stromal vascular fraction
TG - Triacylglcyerol
TGFβ – Transforming growth factor β
TNFα – Tumour necrosis factor α
UCP-1 - Uncoupling protein-1
VSMC – Vascular smooth muscle cells
WAT – White adipose tissue

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INTRODUCTION

Obesity and the adipose tissue

Obesity has reached epidemic proportions and is the leading cause for conditions such as type 2 diabetes, cardiovascular disease, and even certain types of cancer (1, 2). The main problem associated with obesity is insulin resistance, where the normal function of insulin stimulated glucose uptake into cells is impaired. This forces β cells to make more insulin, and when β cells can no longer compensate, there is a state of relative insulin deficiency, and blood glucose increases beyond normal (3, 4). This is often accompanied by an increase in fatty acids and triglycerides in the blood, as insulin is less capable of preventing lipolysis, which leads to more lipid deposition in the liver (termed fatty liver) (5).

Obesity is defined as excess body fat, which develops when energy intake exceeds energy expenditure. The largest storage of usable energy, adipose tissue, also functions as an endocrine organ that releases a number of cytokines, hormones and proteins that affect other tissues and organs in the rest of the body. Leptin for example is an adipose tissue secreted hormone that regulates body weight by acting on the receptors on the hypothalamus, which possibly send out satiety signals to decrease food intake and increase energy expenditure. During obesity leptin levels increase to an exceedingly high level, causing leptin resistance and hence problems with appetite suppression and further obesity (6, 7). It is the double function of adipose tissue as a storage tissue and an endocrine organ that deems it important to study.

Adipose tissue can be separated in two fractions, the adipocyte fraction and the stromal vascular fraction (SVF) containing preadipocytes, blood cells and endothelial cells

(8). Though the number adipocytes usually stays constant in adulthood, obesity can lead to an increase in the total number of adipocytes called hyperplasia, as well as fat accumulation in existing adipocytes called hypertrophy (9). Fat accumulation in adipose tissue occurs when fibroblastic preadipocytes convert to rounded shape and accumulate lipid droplets to form mature adipocytes. The lipid droplets, mainly containing triacylglycerol (TG), are coated with a protein called perilipin that participates in the regulation of lipolysis (9-11).

Fat storage in human adipose tissue occurs mainly in white adipose tissue (WAT) and to a lesser extent in brown adipose tissue (BAT). WAT contains unilocular lipid droplets, with few mitochondria, that can be broken down for energy that the whole body can use when in need (12). WAT is distributed throughout the body, with visceral depots in the omentum, intestines and perirenal areas and subcutaneous depots primarily the in buttocks, thighs and abdomen (13). Brown adipose tissue, on the other hand, contains multilocular lipid droplets, with numerous mitochondria, and is used for thermogenesis. The uncoupling protein-1 (UCP-1), in BAT mitochondria, uncouples respiration from ATP synthesis, leading to heat production. BAT is found in fetuses and newborns in axilary, cervical, perirenal and periadrenal regions (14). Previously, it was thought that BAT is rare and irrelevant in adults (14) but recent articles showed that the presence of BAT is much more common in adults, more so in women than men (15) and that the presence of BAT is cold-activated in healthy men (16).

3T3-L1 adipocyte differentiation

The most common in vitro model of adipose tissue development used is the immortalized murine 3T3-L1 preadipose cell line. They were isolated from disaggregated

17-19 day Swiss mouse embryo cells (17). 3T3-L1 preadipocytes reflect the in vivo differentiation process closely including the expression of the genes required for fatty acid and TG synthesis and the ability to carry out lipolysis, lipogenesis, and insulin-dependent glucose transport (18).

3T3-L1 preadipocytes are generally grown in medium supplemented with 10% calf serum (CS). When they reach confluence they undergo growth arrest and withdraw from the cell cycle. Though cell confluence leads to growth arrest, cell to cell contact is not required for growth arrest to occur (19). 3T3-L1 preadipocytes can spontaneously differentiate after several weeks post-confluence, but their differentiation, also termed adipogenesis, can be accelerated by using differentiation inducers. These inducers include either IGF-1 or insulin for the first four days, and dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) for the first two days of a typical eight day differentiation (20-22) (Fig.1).

In the first four days of 3T3-L1 differentiation, the clonal expansion stage occurs, where two more rounds of mitosis take place, as well as a profound reorganization of the cytoskeleton and extracellular matrix (ECM) (19). Regulation of the cell cycle before clonal expansion and during it seems to be different. The expression of the retinoblastoma proteins, pRB, p107 and p130, is known to regulate cell cycle arrest. In contrast to preconfluent proliferation, during the clonal expansion stage of 3T3-L1 adipogenesis there is an increase in p107 and a decrease in p130 protein level (23). Additionally, growth arrest specific (gas) 6 genes seems to be preferentially expressed during clonal expansion versus preconfluent proliferation (24). The expression of phosphatase inhibitor HA2 is increased during clonal expansion, which blocks differentiation during the clonal expansion stage but not during the terminal differentiation stage (25). Following clonal expansion, from days 4 to 8 of 3T3-L1

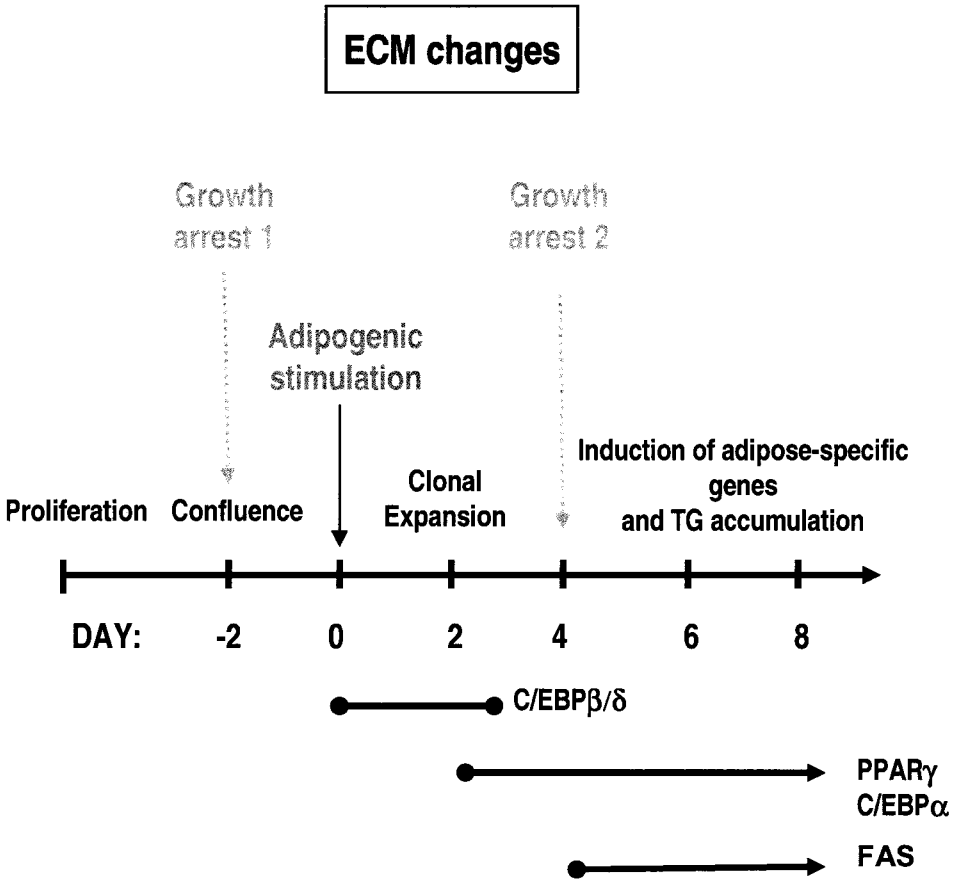

ECM changes
Growth arrest 1
Growth arrest 2
Adipogenic stimulation
Induction of adipose-specific genes and TG accumulation
Proliferation
Confluence
Clonal Expansion
DAY:
-2
0
2
4
6
8
C/EBPβ/δ
PPARγ
C/EBPα
FAS

Figure 1: The 8 day differentiation program of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes are grown in CS media until confluence. 2 days post-confluence (day 0) differentiation is induced using either IGF-1 or insulin, and dexamethasone and IBMX. In the duration of a typical 8 day differentiation, clonal expansion occurs in the first four days followed by TG accumulation in days 4-8 of differentiation. Differentiation is marked by the expression of different adipogenic genes and changes in the extracellular matrix.

differentiation, adipocytes become more sensitive to insulin, allowing more uptake of glucose and de novo lipogenesis that leads to accumulation of lipid droplets (18).

Differentiation of 3T3-L1 preadipocytes is coordinated by a number of transcription factors, the most crucial being the peroxisome proliferator-activated receptor γ (PPARγ) nuclear receptor and the family of CCAAT enhancer binding proteins (C/EBP) transcription factors. PPARγ has been shown to be both necessary and sufficient for adipocyte differentiation (26, 27). Two isoforms of PPARγ (PPARγ1 and PPARγ2) are present in adipose tissue, generated by alternative splicing and promoter usage of the same gene. In a study where both isoforms of PPARγ were inhibited, the overexpression of PPARγ2, but not PPARγ1, was able to induce 3T3-L1 adipogenesis (28). In another study the addition of either PPARγ1 or PPARγ2 to PPARγ null fibroblasts was able to stimulate adipogenesis, but the addition of PPARγ2 lead to a much more potent adipogenesis (29). This suggests that both PPARγ isoforms have a role in adipogenesis, but PPARγ1 is more important.

The family of C/EBP transcription factors consists of five members: C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ and CHOP (30). Three members, C/EBPα, C/EBPβ and C/EBPδ, are needed for efficient adipose tissue differentiation. C/EBPβ and C/EBPδ expression is high during the early stage of 3T3-L1 differentiation (day 0-2) and is reduced in the later stages of differentiation, while the expression of C/EBPα is very low in this early stage of differentiation and is increased with differentiation. The early expression of C/EBPβ and C/EBPδ has been shown to induce the expression of C/EBPα and PPARγ (31, 32). C/EBPα is incapable of inducing adipogenesis in the absence of PPARγ (33). Forced PPARγ expression can overcome C/EBP deficiency by promoting a normal level of lipid filled

adipocytes, but the formed adipocytes have low sensitivity to insulin (34). Hence, a combination of C/EBPα and PPARγ expression is needed for normal adipogenesis.

Insulin signaling

Insulin signaling occurs in both preadipocytes and adipocytes. Insulin binds to the insulin receptor (IR), composed of two heterodimeric subunits, each containing an extracellular α-subunits and a transmembrane β-subunits (35), leading to the transphosphorylation of tyrosine residues on the IR (36). This promotes the intracellular tyrosine kinase domain of IR to phosphorylate tyrosine residues in insulin receptor substrate 1(IRS1) protein. The IRS family also contains IRS2, IRS3 and IRS4 but IRS1 is the most studied and most highly expressed in preadipocytes. IRS1 phosphorylation leads to downstream signaling by binding to SRC homology 2 (SH2) domains in effector proteins. One such protein is phosphoinositide 3-kinase (PI3-kinase), which produces phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate. These products recruit phosphatidylinositol-dependent protein kinase 1 (PDK1) and protein kinase B (Akt/PKB) to the plasma membrane, where Akt/PKB is phosphorylated by PDK1 (37).

Akt/PKB can in turn phosphorylate a number of proteins, including glycogen synthase kinase 3β, leading to activation of glycogen synthase and thus glycogen synthesis (38). Phosphorylation of Akt/PKB also leads to the translocation of glucose transporter-4 (GLUT-4) vesicles from the cytosol to the plasma membrane, where GLUT-4 allows insulin-stimulated glucose uptake into the cell (39). The phosphorylation of Akt/PKB can also activate sterol regulatory element binding protein (SREBP)-1, which in turn activates fatty acid synthase (FAS) that synthesis free fatty acids (FFA) (40). FFA can be esterified with

glycerol to form triacylglcyerol (TG), termed lipogenesis (41). The opposite reaction, TG break down into FFA and glycerol is termed lipolysis.

IRS1 can also interact with SOS/GRB2 (Son of sevenless/Growth factor receptor-bound protein 2) complex, which leads to sequential phosphorylation of Ras, RAF, MEK and ERK1/2 (Extracellular signal-regulated kinase 1/2; also known as p42/44 MAPK). IR can also phosphorylate Src homology 2 domain containing (SHC) proteins that will then interact with GRB2 and activate ERK1/2 pathway independently of IRS1 (43, 44). The ERK1/2 pathway affects cell proliferation, mainly by increasing cyclin D1 mRNA and stabilizing c-Myc protein (45, 46).

Extracellular matrix (ECM) and adipogenesis

The differentiation of 3T3-L1 preadipocytes requires extensive changes in the ECM, allowing fibroblastic preadipocytes to assume a more rounded shape to optimize lipid accumulation (47). A number of studies have looked at the role of ECM on differentiation. It has been shown that the expression of some ECM proteins is decreased with preadipocyte differentiation including: collagen I and III and fibronectin. However, the expression of other ECM proteins is increased with differentiation including: collagen IV, entactin and laminin (48-50). The growth of 3T3-F442A preadipocytes on fibronectin matrices showed inhibited lipogenic gene expression and TG accumulation (51). This suggests that preventing the differentiation-associated decrease in fibronectin inhibits differentiation from occurring. Similarly, a potent inhibitor of differentiation, transforming growth factor-β (TGFβ), has been shown to increase the expression of fibrillar collagen and fibronectin, suggesting a possible link between adipogenesis and altered ECM (52)

A large family of proteins present in the ECM is that of collagen. Collagens consist of three α chains, which can be identical or different depending on the collagen type. The human genome codes for at least 43 different α chains, that construct 28 types of collagen (53). The α chains of fibrillar collagens, I, II, III, VI, and XI, are synthesized in the endoplasmic reticulum (ER). At this point they are called procollagen α chains and contain a C and N-propeptide. C-propeptidases initiate the interaction between the three α chains, and as the α chains line up the interaction occurs at the N-propeptide forming a triple helix. The secretion of collagen to the extracellular matrix leads to the removal of the C and N-propeptides by the corresponding procollagen proteinases. The final step in the assembly of mature collagen involves the crosslinking of different α chains to form collagen fibrils (54).

Fibrillar collagen I and III mRNA expression is high in preadipocytes and is reduced with differentiation, while the expression of collagen IV, V and VI is low in preadipocytes and increases with differentiation of 3T3-L1 preadipocytes into adipocytes (55). It was shown that inhibiting active collagen VII synthesis before confluence and during initial stages of differentiation, as well preventing the accumulation of collagen I-IV on the cell surface, by using ethyl-3,4-dihydroxybenzoate (EDHB) inhibits adipogenesis (56, 57). This suggests that collagen has an effect on adipogenesis.

The different collagen proteins are known to interact with a number of membrane receptors including integrins, discoidin domain receptors (DDRs), glycoprotein IV, LAIR-1 (Leukocyte-associated immunoglobulin-like receptor 1), mannose receptor family and leukocyte-associated IG-like receptor-1, to induce intracellular signaling that leads to a number of functions (58). Different collagens interact with specific membrane receptors.

For example, collagen I and III are known to interact with integrin $\alpha_2\beta_1$ and glycoprotein IV on platelets to stimulate intracellular signaling that leads to platelet adhesion (59).

Additionally, fibril forming collagens, including collagen I-III, V, XI, XXIV and XXVII, are known to interact with both forms of DDR (DDR1 and DDR2). DDRs are receptor tyrosine kinases that control cell adhesion and cell motility, and are activated by collagens through the discoidin domains these receptors possess (60-62). This discoidin domain is a motif common in a number of proteins that interacts with not only collagen, but also growth factors, phospholipids and lipids, and galactose or its derivatives (63, 64). In 3T3-L1 preadipocytes, DDR2 overexpression leads to insulin resistance (65), but it is not clear if this depends on a collagen-DDR interaction.

A secreted protein with a discoidin domain similar to DDR is Aortic Carboxypeptidase-Like Protein (ACLP) (66, 67). ACLP was found expressed in a number of cartilaginous tissues, including the adipose tissue (68). The expression of ACLP in 3T3-L1 preadipocytes is downregulated with differentiation, suggesting a role for ACLP in adipogenesis (69, 70). Additionally, the pattern of ACLP expression is similar to other ECM proteins including collagens, decorins and thrombospondin (71, 72). This suggests that ACLP might be an important member of ECM with a role on adipogenesis.

ACLP structure

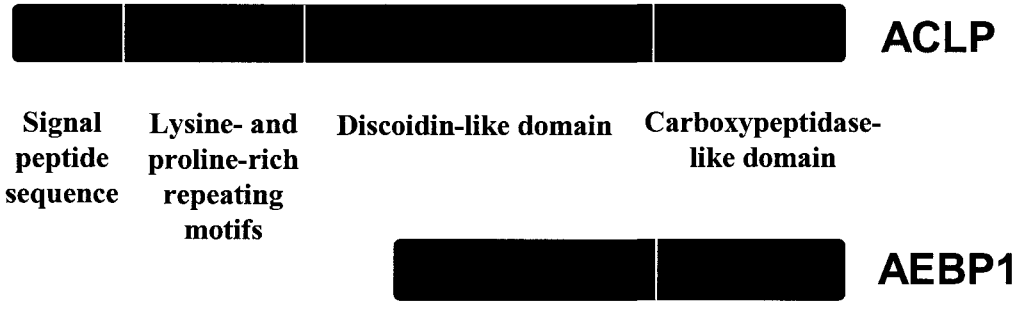
ACLP was originally identified in a screen of potential markers and regulators of smooth muscle cell growth and differentiation. Layne's group screened a human aorta expression library with a labeled E47 fusion protein, which serves as a heterodimerization partner for tissue specific transcription factors that regulate growth and differentiation in

different cell types. One protein isolated from this screen was found to be a truncated clone of ACLP. The full length ACLP cDNA was found to be 3935 base pairs long, coding for 1158 amino acids and a 175kDa ACLP protein (66).

The homologue of ACLP cDNA in mouse was found to be 3633bp long, coding for 1128 amino acids and to be 85% identical and 90% similar to human ACLP cDNA (66). Layne's group cloned the mouse ACLP gene and promoter in vascular smooth muscle cells (VSMCs). It was found that the ACLP gene is composed of 21 exons, where the first exon includes the 5' untranslated region and the initiating methionine while exon 21 contains the termination codon. Additionally, exons 11-14 code for a discoidin-like domain and exons 15-21 code for a carboxypeptidase-like domain (Fig.2). Radiation hybrid mapping had indicated that human ACLP localizes near marker D7S478 on chromosome 7 which is homologous with mouse chromosome 11. Cloning the 2.5kb of the 5'flanking region of the mouse ACLP gene showed that the transcription start site is a CA pair and that the promoter contains multiple GC-rich elements consistent with non-TATA-initiated transcription (67).

To determine which part of the ACLP promoter is crucial for its expression, Layne's group cloned the region from base pairs -2502 to 176 of the mouse ACLP promoter 5'-flanking sequence into a luciferase reporter vector. The largest cloned construct (-2502 bp) exhibited highest luciferase activity. The following 5' deletion constructs -354, -257 and -156bp retained the majority of ACLP promoter activity, while constructs -140 and -122bp decreased reporter activity significantly. Deletion of -100bp did not change activity further, whereas the smallest (-75 and -58bp) retained minimal basal promoter activity. This suggests that the -140 to -122bp ACLP promoter region is mostly responsible for ACLP expression. Further on, using EMSA it was determined that Sp1 and Sp3 bind within -147 and -128bp

Figure 2: The structure of ACLP and AEBP1. ACLP contains a signal peptide sequence that ensures that ACLP is secreted, a lysine- and proline-rich repeating motifs, a discoidin domain which in other proteins is known to bind to collagen, and an inactive carboxypeptidase-like domain. AEBP1 is identical to the N-terminal domain of ACLP, including the complete carboxypeptidase-like domain and part of the discoidin domain.


ACLP
Signal peptide sequence
Lysine- and proline-rich repeating motifs
Discoidin-like domain
Carboxypeptidase-like domain
AEBP1

region of ACLP promoter region causing its transactivation (67). The Sp1 and Sp3 are ubiquitously expressed transcription factors that regulate a number of genes. In cardiac cells they have been shown to form a muscle-specific multiprotein complex by acting with other transcription factors including MyoD and SRF (73, 74). It is not known if additional proteins interact with Sp1 and Sp3 to transactivate the ACLP promoter in VSMC.

In terms of its structure, ACLP was determined to have a signal peptide sequence, an 11 amino acid lysine and proline rich motif repeated four times at the N terminus, a discoidin-like domain and a carboxypeptidase-like domain on its C terminal (66). The ACLP discoidin-like domain is 30% identical to that of slime mold adhesion protein discoidin I, which as previously discussed is a conserved motif known to interact with a number of molecules including collagens (63, 64). The ACLP carboxypeptidase-like domain is 39% identical to that of carboxypeptidase E, but was found to be catalytically inactive (66). Carboxypeptidase E acts as a sorting receptor in the secretory pathway, suggesting that carboxypeptidase domains could have other functions other than catalysis (75). Hence, the carboxypeptidase-like domain of ACLP might function as a binding domain rather for catalysis. The expression of the discoidin domain and the inactive carboxypeptidase-like domain is also found in two related proteins, CPX-1 and CPX-2 (76).

ACLP expression and function outside of the adipose tissue

Layne's group also aimed to determine where the ACLP protein was expressed in the cell. To determine the subcellular localization of ACLP, they attached a c-myc epitope at the C terminus of a mouse ACLP. They saw strong membrane-associated or cytoplasmic staining that shows that ACLP is not in the nucleus (66). Immunofluorescent staining of

smooth muscle cells with an ACLP antiserum generated against its carboxyl terminus showed that ACLP was expressed mainly in perinuclear pattern. Using the same antiserum and fractionated cell extracts, it was determined that ACLP is a secreted protein that associates with the ECM (68).

To characterize the expression of ACLP throughout mouse embryonic development, immunohistochemistry on embryos at different stages of growth was performed. ACLP expression was first detected at 9.5dpc in the developing dorsal aorta and ectoderm (77). An initial screening of adult mice tissues for ACLP mRNA found that it was expressed highly in mouse aorta and present in the colon and the kidney. The ACLP protein however was also highly present in mouse aorta but not in the adventitia, heart, liver, skeletal muscle or kidney suggesting some translational regulation (66). Additionally, immunostaining of mouse embryo sections using ACLP antiserum showed that ACLP was expressed in several tissues, including vascular smooth muscle cells, cartilaginous and bony elements of the developing skeleton, perichondrium of the ribs, airways within the lung and in the dermal layer of the skin. Analysis of ACLP expression in the skin layers showed abundant expression in the dermis layer which is rich in ECM but not in the epidermal layer, and a low level of ACLP expression in the subcutaneous fat (68).

To determine the role of ACLP in mice, ACLP knockout mice were generated and their embryonic development was assessed. Most lethality occurred at birth due to an anterior abdominal wall defect called gastroschisis. ACLP knockout mice were pale and often missing organs such as liver, the intestines, had a bent or looped tail, and the skin contained non-healing wounds. These mice also had a slower rate of healing after a dermal puncture, possibly due to the determined reduced proliferation of isolated dermal fibroblasts,

indicating that ACLP is required for wound repair (68). In another study ACLP expression was shown to be upregulated in VSMC after a mouse carotid injury, in contrast to the suppression of many regulatory genes. VSMCs from mice with carotid injury dedifferentiate and have an increased proliferation, so the increased expression of ACLP suggests a role for ACLP in VSMC proliferation (67).

A recent study by Layne's group implicated ACLP with a disease affecting lung function, termed Idiopathic Pulmonary Fibrosis (IPF). IPF is marked by an increase in fibroblasts, myofibroblasts and ECM. They showed that ACLP expression is significantly increased in the lungs of mice with bleomycin-induced IPF. ACLP deficient mice were protected from bleomycin-induced IPF, including accumulation of myofibroblasts and collagen in the lung. Additionally, ACLP-deficient lung fibroblasts cultured on collagen-I matrix had deficient cell spreading, contraction and proliferation, suggesting a possible ACLP-collagen interaction. This interaction seems to be mediated by the discoidin-like domain of ACLP, as addition of recombinant discoidin-like domain of ACLP to ACLP-deficient lung fibroblasts cultured on collagen-I reversed their effects on cell spreading and contraction (78).

ACLP expression and function in the adipose tissue

ACLP was found expressed at a lower level in the adipose tissue (60). Hence, a number of studies investigated the role of ACLP in *in vitro* models of adipose cells (69, 70, 79). Our laboratory has demonstrated that the ACLP protein is expressed in 3T3-L1 preadipocytes (69, 70). Initial differentiations with medium supplemented with CS showed that ACLP expression is down-regulated early during adipocyte differentiation (day 0-2) and

then rises during late differentiation (day 2-8) (69). However, a more potent differentiation of 3T3-L1 cells with fetal bovine serum (FBS) showed that ACLP down-regulation occurring during days 0-4 and only slightly increasing from day 4-8 of differentiation (70). Northern analysis has shown that ACLP mRNA follows the same trend as the ACLP protein at various stages of the 8 day differentiation process (69).

Another group confirmed the downregulation of ACLP expression with differentiation of 3T3-F442A and 3T3-L1 preadipocytes and found a similar trend with Ob1771 preadipocytes (79). In contrast to 3T3-L1 preadipocytes, ACLP expression during the differentiation of 3T3-F442A cell line decreased more gradually with maximal reduction observed by day 8 (70). Additionally, fractionation of adipose tissue into adipocyte and stromal vascular fraction showed that ACLP mRNA and protein was preferentially expressed in stromal vascular fraction, confirming that ACLP expression is mainly present in preadipocytes (79). The presence of ACLP was also confirmed in the medium of 3T3-L1 preadipocytes and absence in mature adipocytes, confirming that ACLP is secreted (70).

It also seems that ACLP downregulation is a differentiation-associated event, as none of the individual components of the differentiation cocktail could reduce the levels of ACLP. However, ACLP downregulation does not seem to be linked to terminal preadipocyte differentiation. This was shown by looking at the expression of ACLP in differentiating 3T3-C2 preadipocyte, which are capable of entering the early clonal expansion of adipogenesis but they are unable to progress through terminal differentiation. A pattern of ACLP expression in 3T3-C2 similar to that of 3T3-L1 was noted but the extent of ACLP reduction was decreased (69).

The sharp ACLP downregulation during the first 4 days of differentiation suggests that a decrease in ACLP expression may be required for differentiation-induced clonal expansion phase of 3T3-L1 and 3T3-C2 cells during this period. The more prominent clonal expansion phase observed in 3T3-L1 preadipocytes compared to 3T3-C2 preadipocytes may be linked to the steeper decline in ACLP protein expression occurring in differentiating 3T3-L1 cells. Additionally, our group found that ACLP protein expression is unchanged in subconfluent growing cells versus growth-arrested confluent cells, suggesting that the reduction in ACLP expression observed upon adipogenic stimulation is specific to the specialized mitosis associated with clonal expansion and is not a general feature of proliferation (69).

Previous studies from our laboratory have also found that an inhibitor of adipogenesis, TGFβ, increases ACLP expression in 3T3-L1 preadipocytes through a mechanism that requires ERK1/2 activity (70). This is not surprising, as TGFβ is a known modulator of fibrillar matrix proteins production and upregulates other ECM proteins including fibrillar collagen and fibronectin (52). This suggests that ACLP function is similar to collagen and fibronectin, possibly to bind membrane receptors and activate intracellular signaling pathways.

To determine whether the decrease in ACLP expression was absolutely required for adipogenesis, a group overexpressed ACLP on 3T3-F442A cells so that sustained ACLP expression was present during the 8 days of differentiation. They saw inhibition of adipocyte differentiation through reduced Oil Red O staining of lipids, as well as the expression of adipocyte markers aP2 and PPARγ. Additionally, when placed in smooth muscle cell differentiation media these cells showed an elongated spindle-shape morphology and the

expression of smooth muscle specific markers SM22α and SMα-actin, suggesting that ACLP overexpression promotes transdifferentation of preadipocytes into smooth muscle-like cells (79). However, similar studies in our lab showed that overexpression of ACLP in 3T3-L1 and 3T3-F442A preadipocytes does not affect the expression of the transcription factor PPARγ or the accumulation of TG, suggesting no effect on differentiation (69, 70). Hence, the role of ACLP remains elusive.

Adipocyte Enhancer Binding Protein 1 (AEBP1)

ACLP was found to have an identical carboxyl terminus, including part of the discoidin-like domain and the entire carboxypeptidase-like domain, to a previously discovered protein named adipocyte enhancer binding protein 1 (AEBP1) (80) (Fig.2). The ACLP and AEBP1 proteins are encoded by the same 4kb gene (81). Initial sequencing of AEBP1 protein by Ro's group predicted a protein of 719 amino acids and 79kDa (80). However, later sequencing of AEBP1 by Layne's group showed that Ro missed a G residue 11 bases 5' to the start codon, changing the reading frame to that of ACLP (66). Gene bank analysis indicated that the AEBP1 sequence is identical to that of ACLP sequence beginning at ACLP methionine 410, positioned on the discoidin-like domain (80).

Ro's later sequencing of AEBP1 confirmed the missing G predicted by Layne. However, Ro suggests that with the newly determined sequence of AEBP1, ACLP cDNA is missing the 39 bases at the 5' end that are present in the AEBP1 cDNA. These 39 bases in AEBP1 cDNA belong to the 9^{th} intron that is removed in the generation of ACLP cDNA. Thus, the common AEBP1 and ACLP gene undergoes alternative splicing where AEBP1 losses exon 1-9 and retains part of intron 9 while ACLP expresses exon 1-21 but doesn't

retain any part of intron 9. Thus, Ro's group suggests that AEBP1 is encoded by the same gene as ACLP, but is missing the N-terminal portion of ACLP, forming the AEBP1 protein of 748 amino acids and 82kDa (81).

AEPB1 expression in different tissues was also compared with that of ACLP, using RT-PCR. Ro's group found that the expression of ACLP was much higher than that of AEBP1 in almost all tissues examined, including WAT, BAT, liver, kidney, skeletal muscle, brain, small intestine, heart, lung, and spleen, except testis where a similar expression level for both was found. The expression of AEBP1 mRNA was very high in brain and relatively high in WAT, kidney and heart but very low in the other above mentioned organs. The expression of the AEBP1 protein, on the other hand, was the highest in WAT, followed by BAT, liver, lung and spleen, suggesting post-translational regulation (81). AEBP1 mRNA and protein expression, like that of ACLP, is decreased with 3T3-L1 differentiation (80).

AEBP1 was found in a screen of proteins that bind to the promoter of the aP2 gene, which codes for the adipocyte lipid binding protein (ALBP). It was shown that AEBP1 acts as a negative regulator of the aP2 gene by binding to the AE-1 sequence of its promoter. Ro's group proposed that AEPB1 protein contains carboxypeptidase activity which is needed for the repressor activity on aP2 gene (80). Additional roles have been found for AEBP1 including modulation of ERK1/2 activity (82, 83), negative regulation of PTEN (Phosphatase and tensin homolog) (84-86), estrogen signaling pathway (85) and macrophage cholesterol homeostasis and inflammation (87-89).

However, there are a number of controversies concerning AEBP1. Ro's AEBP1/ACLP alternative splicing mechanism was not further analyzed or confirmed by other groups. In fact, many other groups that were able to detect ACLP, were unable to

detect AEBP1 using the antiserum against the common C-terminal portion (66, 69, 79). Additionally, while the Ro suggests that AEBP1 has a catalytically active carboxypeptidase domain (80), other groups were unable detect it (69, 90). Furthermore, a group overexpressed an N-truncated form of ACLP corresponding to AEBP1 sequence and showed that this had no effect on differentiation, conflicting with the repressing role on adipogenesis that Ro has shown (79). This led to a division in groups that believe AEBP1 is an incomplete clone of ACLP (66, 69) and others that believe it is an isoform of ACLP formed by alternative splicing of the same gene (79). Hence, the structure and role of AEBP1 is controversial.

Hypothesis/Reasoning for project

ACLP expression was shown to be vital for the development of mice, as the ACLP knockout mice either died prematurely due to gastroschisis or lived with non-healing skin wounds (68). Though the expression of ACLP is highest in smooth muscle cells, a lower expression was also found in adipose tissue (67). Expression of ACLP was detected in murine 3T3-L1 and 3T3-F442A preadipocytes (69, 70, 79). Similar to the mRNA expression of collagen I and III (55), the expression of ACLP is downregulated with 3T3-L1 adipogenesis (69, 70). Considering that ACLP protein contains the discoidin domain which in other proteins has been shown to interact with collagens (48-50), it is possible that a collagen - ACLP interaction occurs. I will be investigating the possible effect of ACLP on 3T3-L1 adipogenesis, focusing on the potential ACLP interaction with collagen.

Hypothesis

ACLP plays an important role in preadipocyte or adipocyte function

Objective

To determine the role of ACLP in murine 3T3-L1 preadipocytes and adipocytes

Specific aims:

1. Create a 3T3-L1 line that stably overexpresses ACLP in preadipocytes and is sustained with adipogenesis
2. Determine whether sustained ACLP overexpression affects 3T3-L1 preadipocyte and adipocyte functions

MATERIALS AND METHODS

3T3-L1 cell culture and differentiation

The 3T3-L1 preadipocyte cell line was obtained from American Type Culture Collection. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% CS (Gibco), and an antibiotic solution consisting of 100U/ml penicillin and 100mg/ml streptomycin (1% PS) (Gibco) at 37°C and 10% CO_2.

Differentiation was induced 2 days post-confluence (day 0) and continued for 8 days, changing the differentiation medium every 48 hours. The differentiation medium was DMEM supplemented with 10% FBS (Gibco) and 1% PS. Additionally, 0.5mM IBMX (Sigma) and 0.25μM dexamethasone (Steraloids) were added for the first two days of differentiation and 1μM insulin (Sigma) for the first four days of differentiation.

For indicated experiments, 3T3-L1 preadipocytes were seeded on regular plastic dishes or on collagen-I coated dishes (BD Biosciences). Cells were induced to differentiate or kept in control medium for 8 days. A modified, less potent, differentiation medium was used. The modified differentiation medium was DMEM with 1%PS supplemented with 10% CS, and with 0.25mM IBMX and 0.125μM dexamethasone for the first two days of differentiation and 100nM insulin for the first 4 days of differentiation.

ACLP overexpression

The Phoenix-Eco cells (American Type Culture Collection) are a 293T human embryonic kidney cell line engineered to express gag, pol and envelope proteins, needed for retrovirus assembly, using murine leukemia expression vectors. These cells are easy to

transfect, and produce replication-incompetent retroviral particles that can then be used to infect 3T3-L1 preadipocytes. pLXSN and pLXSN-ACLP constructs were a generous donation from Dr. Matthew Layne (54). They were received as a bacterial culture of *E.coli* with geneticin resistance in LB broth with 8% glycerol. Layne generated pLXSN-ACLP by ligating the full length mouse ACLP cDNA (encoding 1128 amino acids) into the EcoRI site of pLXSN.

To carry out the transfection, 293T-derived Phoenix-Eco packaging cells were cultured in DMEM supplemented with 10% FBS, 1%PS and 50 μg/ml nystatin (Calbiochem). In the meantime, 10μg of pLXSN and of pLXSN-ACLP DNA were diluted with 100μl of water and sterilized by precipitation with 1/10 volume of 3M sodium acetate pH 5.2 and 2 volumes of 95% ethanol, and incubated overnight at -20°C. The next day, the 80% confluent Phoenix-Eco cells were changed to fresh medium for 1 hour. The DNA was centrifuged at 3000g, 4°C for 30 minutes and supernatant decanted before allowing the pellet to air dry. Once dry, DNA was resuspended in 438μl of sterile water, and 62μl of 2M $CaCl_2$ was added. The DNA/$CaCl_2$ solution was bubbled into 500μl 2XHBSS (Gibco) for about 15 seconds before applying the mixture, dropwise to the Phoenix-Eco cells. In addition to the pLXSN and pLXSN-ACLP transfection into Phoenix-Eco cells, a sham transfection was also done using the same preparation but without DNA.

Transfected Phoenix-Eco cells were incubated for 24 hours at 37°C, at which time they were washed with medium three times to remove any remaining precipitate before being given fresh medium. The next day, medium was again changed and viral supernatant was collected after 24 hours. Viral supernatant was filtered through 0.45μm syringe filters

and hexadimethrine bromide (polybrene; Sigma) was added to a final concentration of 4μg/ml.

The filtered viral supernatant was added to 50-60% confluent 3T3-L1 preadipocytes, and the infection was allowed to proceed for 24 hours. The cells were then grown in DMEM/10%CS/1%CS medium supplemented with 400μg/ml geneticin (Gibco), until ready to split. Geneticin-selected pLXSN and pLXSN-ACLP cells were grown until 80% confluent before freezing with liquid nitrogen in a solution of DMEM supplemented with 50%CS, 1%PS and 15% DMSO (Sigma). They were then thawed as needed for experiments. Sham cells were also selected with geneticin; a significant reduction in the amount of these cells was seen by the time pLXSN and pLXSN-ACLP cells were 80% confluent.

ACLP detection in the medium

pLXSN and pLXSN-ACLP preadipocytes were grown on 100mm culture dishes until confluence, and the medium was then replaced with DMEM/1%PS supplemented with 1μM insulin. After 48 hours, medium was collected, centrifuged at 500g for 5 minutes, and the supernatant was mixed with equal parts (v:v) of 2X Laemmli (4% SDS, 20% glycerol, 120mM Tris pH 6.8, and 0.004% bromophenol blue) and 5% ß-mercaptoethanol. The resulting solution was boiled for 5 minutes to denature the proteins. After cooling, 70μl of pLXSN and pLXSN-ACLP samples were resolved on a SDS-PAGE gel and immunoblotted with rabbit anti-ACLP antibody (generous donation from Dr. Matthew Layne) as described below.

Cell proliferation

pLXSN and pLXSN-ACLP preadipocytes were plated at a density of $2.5x10^3$ cells/cm^2. After 24, 48, and 72 hours of growing, cells were trypsinized with 0.5ml TrypLE™ Express (stable trypsin replacement enzyme from Gibco) at 37°C until detached from the dish. Once cells were detached, TrypLE™ Express was inactivated by the addition of 0.5ml DMEM/10%CS/1%PS medium. Cells were enumerated in duplicate using a Neubauer hemacytometer and Nikon Eclipse TS-100 microscope. The middle 1mm square represented the number of cells present in 0.1µl of medium.

Assessment of cell death

pLXSN and pLXSN-ACLP preadipocytes were grown to confluence, at which point the medium was changed to either fresh growing medium (DMEM/10%CS/1%PS) or serum-free medium (DMEM/1%PS). After 16 hours, cells were trypsinized with 0.5ml TrypLE™ Express, as described above. The cells were stained with 0.2% Trypan blue stain (Gibco) to assess non-viable cells (<1% of cells). Viable cells were enumerated in duplicate using a Neubauer hemacytometer, as described above.

Assessment of apoptosis using Hoescht staining

pLXSN and pLXSN-ACLP preadipocytes were grown on glass coverslips that were set on standard culture dishes. Confluent cells were washed three times with PBS and fixed with 10% formalin (10% formaldehyde in PBS) for 1 hour. Cells were then washed two more times with PBS, and individual coverslips were placed on a moist filter paper. Each coverslip was stained with 50µL of 1µg/ml Hoescht 33248 (Sigma) solution for 10 minutes.

Coverslips were then washed three times with PBS, and mounted face down on a slide using a drop of Moviol. Slides were dried overnight in the dark. The next day, fluorescence from the Hoescht DNA stain was visualized using an Axiocam digital camera mounted on a Zeiss Axioplan 2 imaging microscope. Photographs were taken of 10 random fields, and the total number of viable and apoptotic cells were counted. Cells were classified as apoptotic based on their smaller and brighter nuclei. Cell counts were done by two independent observers, where one observer knew the treatment conditions but the other did not.

TG assay

pLXSN and pLXSN-ACLP preadipocytes were grown until confluence and then were induced to differentiate or kept in control medium. On day 8, cellular TG was extracted using a solution of 2:3 (v:v) isopropanol:heptane (Fisher) for 30 minutes. Extracted TG was transferred to new tubes and extraction repeated with fresh isopropanol:heptane solution for another 15 minutes. The collected extraction was dried using a SpeedVac to obtain dried lipids in the tubes.

TG accumulation was quantified using the method by Frings (91). Lipids were dissolved in 300µl isopropanol, and TG saponified to glycerol using 150µl saponification reagent (1.78M KOH and 25 % v:v isopropanol in water) for 10 minutes at room temperature. Consequently, glycerol was oxidized to formaldehyde with 300µl of sodium metaperiodate (3.04mM sodium periodate, 1M anhydrous ammonium acetate and 6% glacial acetic acid v:v in water). Formaldehyde was condensed with acetylacetone and ammonia to 3,5-diacetyl-1,4-dihydrolutidine, by addition of 300µl of acetyl acetone reagent (0.4% v:v 2,4 pentadione in isopropanol). The reaction from glycerol to 3,5-diacetyl-1,4-dihydrolutidine was developed for 15 minutes at 65°C. 0.3mg/mL triolein (in isopropanol)

was used as a standard. Total 3,5-diacetyl-1,4-dihydrolutidine was quantified at 410nm using the Ultraspec 3000 UV/Visible spectrophotometer from Pharmacia Biotech.

Sample preparation and protein assay

pLXSN and pLXSN-ACLP preadipocytes were grown until confluence and then were induced to differentiate or kept in control medium. At the specified time, cells were washed twice with PBS. Cells were then lysed and proteins solubilized using a solution of 1X Laemmli (2% SDS, 10% glycerol, 60mM Tris pH 6.8, and 0.002% bromophenol blue) and 5% ß-mercaptoethanol. Cells were scraped from the dish, and the DNA was sheared using a 26½ gauge syringe and then the proteins were denatured by boiling samples for 5 minutes before storage at -20°C. Solubilized proteins were quantified using the modified Lowry assay (Bio-Rad Protein assay Kit) with bovine serum albumin (BSA; Fisher) as a standard. Quantification was done using the Ultraspec 3000 UV/Visible spectrophotometer from Pharmacia Biotech.

Immunoblot analysis

Equal amounts of solubilized cellular protein (ranging from 10-50µg depending on the experiment) were resolved on a 7.5% SDS-PAGE gel at 150V. Once completed the proteins from the gel were electrophoretically transferred to a nitrocellulose membrane from Bio-Rad Laboratories at 70V for 1 hour and 45 minutes. Nonspecific antigenic sites on the membrane were blocked with 5% non-fat dried milk in PBS supplemented with 0.1%Tween (Sigma), for one hour at room temperature with gentle shaking. The membrane was then

incubated with the indicated primary antibody overnight at room temperature, with gentle shaking.

The next day, the membrane was washed with PBS/0.1%Tween, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary (Jackson Laboratories) antibody in 5% non-fat dried milk for 1 hour, with gentle shaking. The membrane was again washed with PBS/0.1% Tween. Immobilon Western Chemiluminescent HRP Substrate from Millipore Corporation was then added for 5 minutes. The chemiluminescence was detected by exposing membranes to Bioflex scientific imaging film (Clonex) using the Kodak M35A X-Oman Processor. Specific bands were quantified using the AlphaEaseFC Software from Alpha Innotech. Care was taken to avoid under- or over-exposure of bands of interest. The following antibodies were used for detection: mouse anti- PPARγ (Santa Cruz), rabbit anti-C/EBPα (Santa Cruz), mouse or rabbit anti-FAS (BD Transduction Laboratory), rabbit anti-ACLP, rabbit anti-ERK1/2 (Millipore) and rabbit anti-collagen-I/III (BD Transduction Laboratory). Membranes were probed with more than one antibody, without stripping, if the size of proteins detected was different enough that protein bands did not overlap. When proteins of similar sizes were detected, membrane was stripped of the antibodies for 30 minutes, using a stripping buffer (100mM ß-mercaptoethanol, 2%SDS, and 62.5 mM Tris HCl 6.8) before membrane was placed in the next primary antibody and detection repeated.

Zymography

pLXSN and pLXSN-ACLP preadipocytes were grown until confluence and induced to differentiate or kept in control medium. On day 8, cells were placed in DMEM supplemented with 0.5% BSA and 1% PS for 3 hours. Cell medium was then collected and

spun at 500g for 5 minutes using the Biofuge 15 centrifuge from Heraeus instruments. The supernatant was transferred to new tubes and 40µl of supernatant was mixed with 20µl of 3X non-denaturing bromophenol blue (187.5nM Tris HCl pH 6.8, 30% glycerol, 6% SDS and 4 mg/ml bromophenol blue). Equal volumes of medium were resolved by a gelatin-polyacrylymide gel. The resolving gel contained 370nM Tris HCl pH 9, 8% acrylamide, 0.1% gelatin, 0.1% SDS, 0.08% ammonium persulfate, and 0.1% TEMED, while the stacking gel contained 125nM Tris HCl pH 6.8, 4% acrylamide, 0.1% SDS, 0.05% ammonium persulfate, and 0.17% TEMED.

The resolving gel was incubated 2X for 20 minutes with 40ml 2.5% Triton X-100 (Sigma) with gentle shaking. The gel was then placed in 40ml incubation buffer (2.5mM Tris HCl pH 8.8, 25nM $CaCl_2$, 0.00002% NaN_3 and 250nM $ZnCl_2$) overnight at 37°C, with gentle shaking. The next day, the gel was placed in fixation solution (40ml 45% methanol and 10% acid acetic) for 30 minutes at room temperature, with gentle shaking. The gel was stained with 40mL 0.1% Coommassie blue in fixation solution for 1 hour, room temperature, with gentle shaking. Consequently, the gel was destained with 40mL fixation solution until clear zones appeared (1-2 hours while changing the solution every 20 minutes) at room temperature, with gentle shaking. The gel was then rinsed twice with water, and was then photographed using the Alpha Imager Gel Doc system from Alpha Innotech. Specific bands were quantified using the AlphaEaseFC Software from Alpha Innotech

Lipolysis assay

pLXSN and pLXSN-ACLP preadipocytes were grown until confluence and differentiated. On day 8 of differentiation, lipolysis was quantified using the "Cultured

human adipocyte lipolysis" assay kit from Zen-Bio. Cells were treated with one of four conditions: control (DMSO), 0.1nM isoproterenol, 1nM isoproterenol or 10nM isoproterenol. After 4 hours, medium was collected, centrifuged at 500g for 5 minutes at 4°C and the supernatant was transferred to another tube. Isoproterenol induced the breakdown of TG into glycerol and fatty acids. Glycerol kinase was then added to the medium, which induced the phosphorylation of glycerol by adenosine triphosphate. Produced glycerol-1-phosphate was then oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. Addition of peroxidase catalyzed the coupling of hydrogen peroxide with 4-aminoantipyrine and sodium N-ethyl-N-(3-sulfopropyl)m-anisidine, producing a quinoeimine dye. Spectroscopic readings of the quinoeimine excitation at 540nM was done using the FLUOstar Galaxy spectrophotometer from BMG. Glycerol present in samples was compared to glycerol (Sigma) standards of 100μM, 50μM, 25μM, and 12.5μM.

Glucose uptake assay

pLXSN and pLXSN-ACLP preadipocytes were grown until confluence and differentiated. On day 8 of differentiation, cells were placed in DMEM supplemented with 1% PS for 2 hours. Cells were stimulated with αMEM (Invitrogen) or 10nM insulin (in αMEM) for 30 minutes. 15 minutes after the start of stimulation, duplicate dishes received 1mM $HgCl_2$ to determine GLUT4-indpendent glucose transport. Medium was removed, cells were washed 2X with 37°C HEPES buffer (140mM NaCl, 20mM HEPES pH7.4, 4mM KCl, 2.5mM $MgSO_4$ and 1mM $CaCl_2$) and medium replaced with labeling solution (0.5 nCi/mL ^{3}H-deoxyglucose, 10nM deoxyglucose in HEPES buffer) for 8 minutes. Cells were washed 2X with 0.9% saline (NaCl in water) and lysed with 50mM NaOH for 20 minutes with

gentle shaking. Once cells were lysed and resuspended well, they were placed in scintillation vials with scintillation cocktails and counted using a Beckman LS3801 scintillation counter. The amount of ^{3}H-deoxyglucose in each sample, as well as in the labeling solution was determined and expressed per amount of protein present in the dish, as well as per minute of insulin stimulation. Protein concentration was also determined from the cell lysates, as previously described.

Insulin signaling

pLXSN and pLXSN-ACLP preadipocytes were seeded on regular or collagen-I coated dishes and grown until confluence. In some experiments, where indicated, cells were differentiated for 8 days. At the specified times, cells were placed in DMEM supplemented with 1%CS and 1% PS for 16-18 hours. The next day, cells were stimulated for 5 minutes with vehicle (2mg BSA in 1ml KRH – 125mM NaCl, 4.8mM KCl, 2.6mM $CaCl_2.2H_20$ pH 7.4, 1.2mM $MgSO_4$, and 25 mM Hepes) or 10nM insulin (in BSA/KRH)., Cells were then lysed with 1X Laemmli supplemented with 1 mM sodium orthovanadate, 5 mM EGTA, 5 mM sodium pyrophosphate, 50 mM sodium fluoride and 5% β-mercaptoethanol. Cells were scraped from the dish, DNA sheared using a 26½ gauge syringe and proteins denatured by boiling samples for 5 minutes. Adipocyte lysates were centrifuged for 5 minutes at 3000g and the infranatants were transferred to new tubes. Proteins were quantified and equal amounts were used for immunoblotting, as previously described. Primary antibodies used included mouse phosphotyrosine (pTyr) (Cell Signaling), rabbit pAkt (Cell Signaling), rabbit pERK1/2 (Cell Signaling), rabbit ACLP, rabbit Akt (Cell Signaling), rabbit ERK1/2, and rabbit IRS1 (Epitomics).

Statistical analysis

Differences between multiple means were analyzed using ANOVA with the Newman-Keuls post-hoc test, and differences between two means were analyzes using a paired t-test (GraphPad InStat software version 3.05). $p<0.05$ was considered significant.

RESULTS

Endogenous ACLP expression during 3T3-L1 adipocyte differentiation

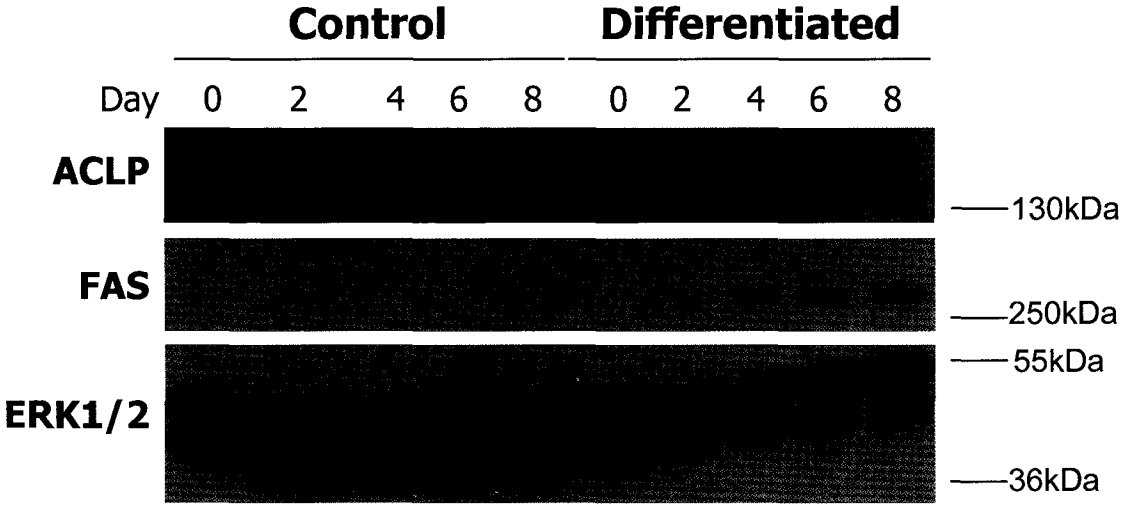
As previously mentioned, ACLP expression is downregulated upon 3T3-L1 adipocyte differentiation (69, 70, 79). Hence, my first experiment was performed to confirm the reduced ACLP expression, by immunoblot analysis, during the 8 day differentiation process of 3T3-L1 preadipocytes into adipocytes. ACLP expression decreased sharply with induction of differentiation, day 0-2 (Fig.3), decreased even further by day 4 of differentiation, slightly increased between days 4-6 and decreased again by day 6-8 of differentiation. The expression of ACLP in 3T3-L1 preadipocytes kept in control medium remained constant during the 8 day period. The expression of FAS on days 4-8 after adipogenic stimulation indicates the successful differentiation of 3T3-L1 preadipocytes into adipocytes. ERK1/2 (expression does not change during differentiation) was used as a loading marker, confirming that the same amount of protein was used for each sample.

Successful ACLP overexpression in 3T3-L1 preadipocytes

Overexpression studies are often used to determine the role of proteins, since a change in protein expression is expected to affect the role of the particular protein in the cell. ACLP overexpression is particularly useful, as it could be hypothesized to prevent the differentiation-induced ACLP downregulation in 3T3-L1 preadipocytes.

ACLP overexpression in 3T3-L1 preadipocytes was achieved by retroviral infection. The empty plasmid/vector (pLXSN) or the plasmid containing the ACLP insert (pLXSN-ACLP) were first transfected into Phoenix-Eco cells for production of retrovirus

Figure 3: Endogenous ACLP expression is downregulated during 3T3-L1 preadipocyte differentiation. Confluent 3T3-L1 preadipocytes (day 0) were either kept in control medium or induced to differentiate as described. On indicated days, cells were lysed, and equal amounts of solubilized protein were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with the indicated antibodies. ERK1/2 is used as a loading control. Representative blots from one experiment are shown.


Control
Differentiated
Day 0 2 4 6 8 0 2 4 6 8
ACLP
FAS
ERK1/2
130kDa
250kDa
55kDa
36kDa

medium, that was then filtered for retrovirus particles and used to infect 3T3-L1 preadipocytes. After selection of infected preadipocytes with geneticin, a 4.9 fold increase in ACLP protein expression was noted in pLXSN-ACLP preadipocytes compared to pLXSN preadipocytes (Figure 4).

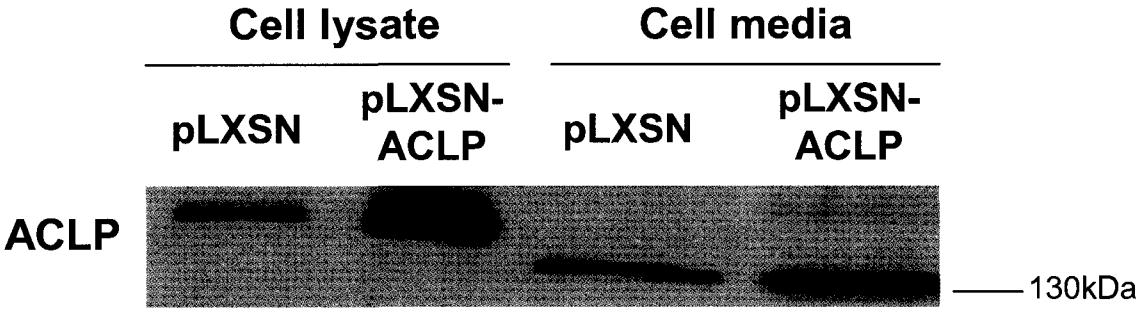
As previously discussed, ACLP is a secreted protein. This was confirmed by detecting the presence of ACLP protein in the medium of 3T3-L1 preadipocytes. As shown in Figure 4, the presence of ACLP protein was 3.2 fold higher in pLXSN-ACLP preadipocyte medium than in pLXSN preadipocytes medium. This confirms that the overexpressed ACLP in pLXSN-ACLP preadipocytes is secreted in the medium.

Assessing the effect of sustained ACLP overexpression on 3T3-L1 adipogenesis

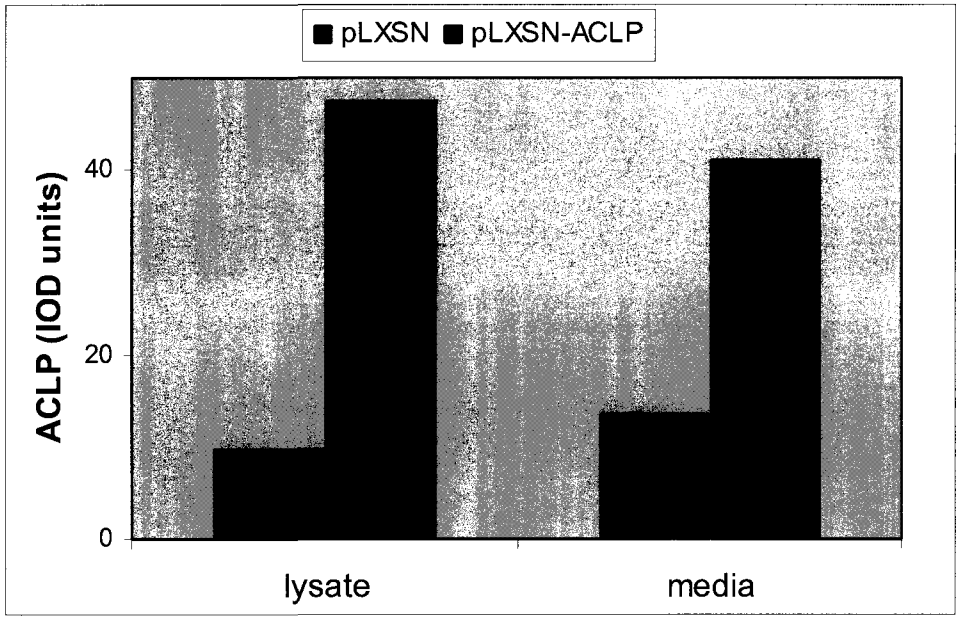
As previously described, the role of ACLP in adipogenesis is controversial. Whereas our group determined that sustained ACLP overexpression has no effect on 3T3-L1 or 3T3-F442A differentiation (69, 70), another group found that sustained ACLP overexpression inhibits 3T3-F442A differentiation (79). Hence, it was important to further evaluate the role of ACLP on adipogenesis. Since ACLP is a secreted protein, I hypothesized that its role could be linked with other extracellular matrix (ECM) proteins. Similar to ACLP, the expression of many ECM proteins is changed with adipogenesis, allowing preadipocytes to mature into adipocytes (38-41). It has been suggested that ACLP interacts with collagen, and that this occurs via the ACLP discoidin-like domain (78). Hence, the effect of sustained ACLP overexpression on 3T3-L1 adipogenesis was assessed, as well, but focus was given to the possible ACLP – collagen interaction.

Figure 4: Increased ACLP expression leads to increased secretion of ACLP in the medium of 3T3-L1 preadipocytes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were grown until confluence. Medium was changed to starvation medium (DMEM/1%PS) supplemented with 1μM insulin. After 48 hours medium was collected, cells were lysed and 5μg of protein from the cell lysates as well as 70μl of protein from the medium were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with the anti-ACLP antibody. A) Representative blot of one experiment is shown. B) Densitometric analysis (IOD = Integrated optical density) of the ACLP band is shown.

A.


Cell lysate
Cell media
pLXSN
pLXSN-ACLP
pLXSN
pLXSN-ACLP
ACLP
130kDa

B.


pLXSN
pLXSN-ACLP
ACLP (IOD units)
40
20
0
lysate
media

Assessing adipogenesis on standard dishes

The effect of sustained ACLP overexpression on 3T3-L1 adipogenesis has already been assessed on standard dishes (69, 70, 79). However, due to the controversy regarding the results, this experiment was repeated to confirm the results obtained in our laboratory. pLXSN and pLXSN-ACLP preadipocytes were either kept in control or differentiation medium for an 8 day period. On day 8, cells were photographed, their TG accumulation was measured and the expression of three key differentiation markers was evaluated.

Morphologically, pLXSN preadipocytes kept in control medium were elongated preadipocytes with no visible lipid droplets, while pLXSN adipocytes were rounded with many cytoplasmic lipid droplets (Fig.5). Similar change in morphology was noted for pLXSN-ACLP preadipocytes differentiation into adipocytes, showing that sustained ACLP overexpression on standard dishes does not affect the morphology of 3T3-L1 preadipocytes or those induced to differentiate into adipocytes.

There was ~3 times more TG in pLXSN adipocytes than in pLXSN preadipocytes (Fig.6). Similar increases in TG levels were noted for pLXSN-ACLP adipocytes compared to preadipocytes, showing that sustained ACLP overexpression on standard dishes does not affect the level of TG in 3T3-L1 preadipocytes or those induced to differentiate into adipocytes.

The expression level of three key differentiation markers, FAS, PPARγ and CEBPα, were quantified by immunoblotting. The expression level of the three key differentiation markers was very low in pLXSN preadipocytes and significantly increased in pLXSN adipocytes (Fig.7). Similar increase in the expression level of the three differentiation markers was noted when pLXSN-ACLP preadipocytes were differentiated

Figure 5: Sustained overexpression of ACLP does not affect the morphology of 3T3-L1 preadipocytes or adipocytes grown on standard dishes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were grown to confluence. Cells were induced to differentiate or kept in control medium for 8 days as described. Photomicrographs (200X) of the various cultures from one experiment, representative of five independent experiments are shown.

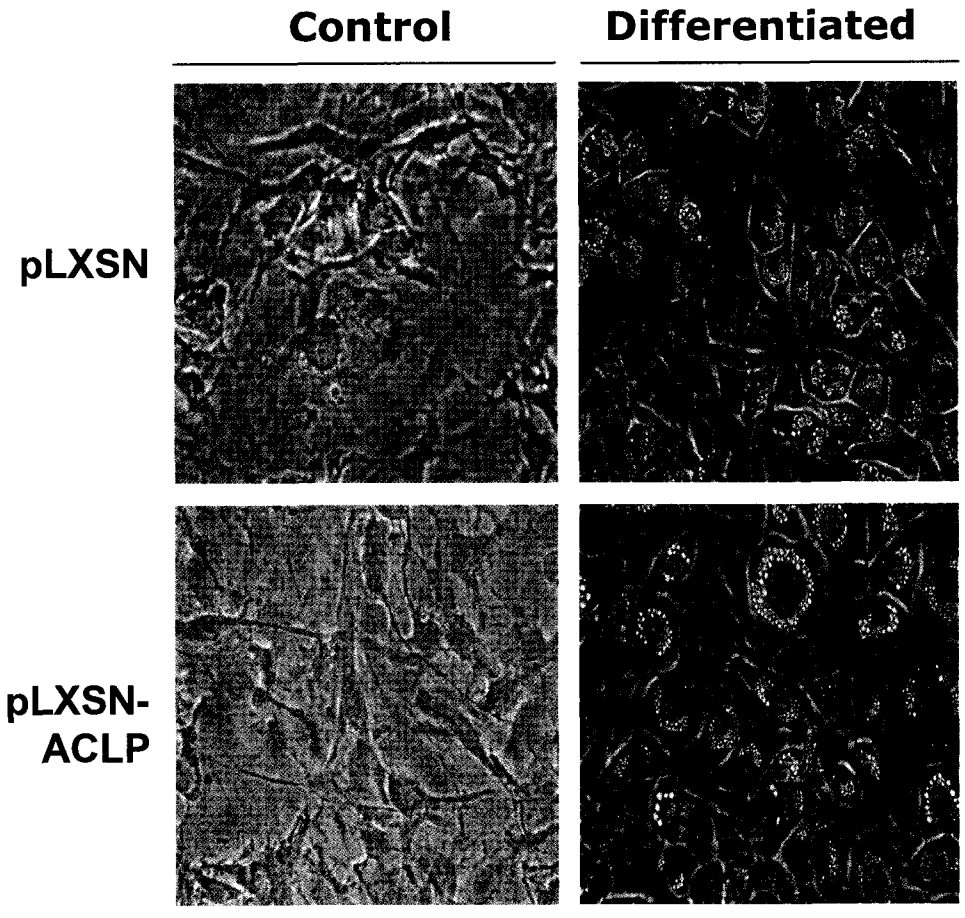

Control
Differentiated
pLXSN
pLXSN-
ACLP

Figure 6: Sustained overexpression of ACLP during 3T3-L1 adipogenesis on standard dishes does not affect TG accumulation. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were grown to confluence. Cells were induced to differentiate or kept in control medium for 8 days as described. TG was extracted, solubilized and quantified as described. Total TG is normalized per total protein present in the dish. Results are the mean ± SD of four independent experiments. Statistical analysis was performed using ANOVA. *** $p<0.001$ vs. respective un-differentiated controls.

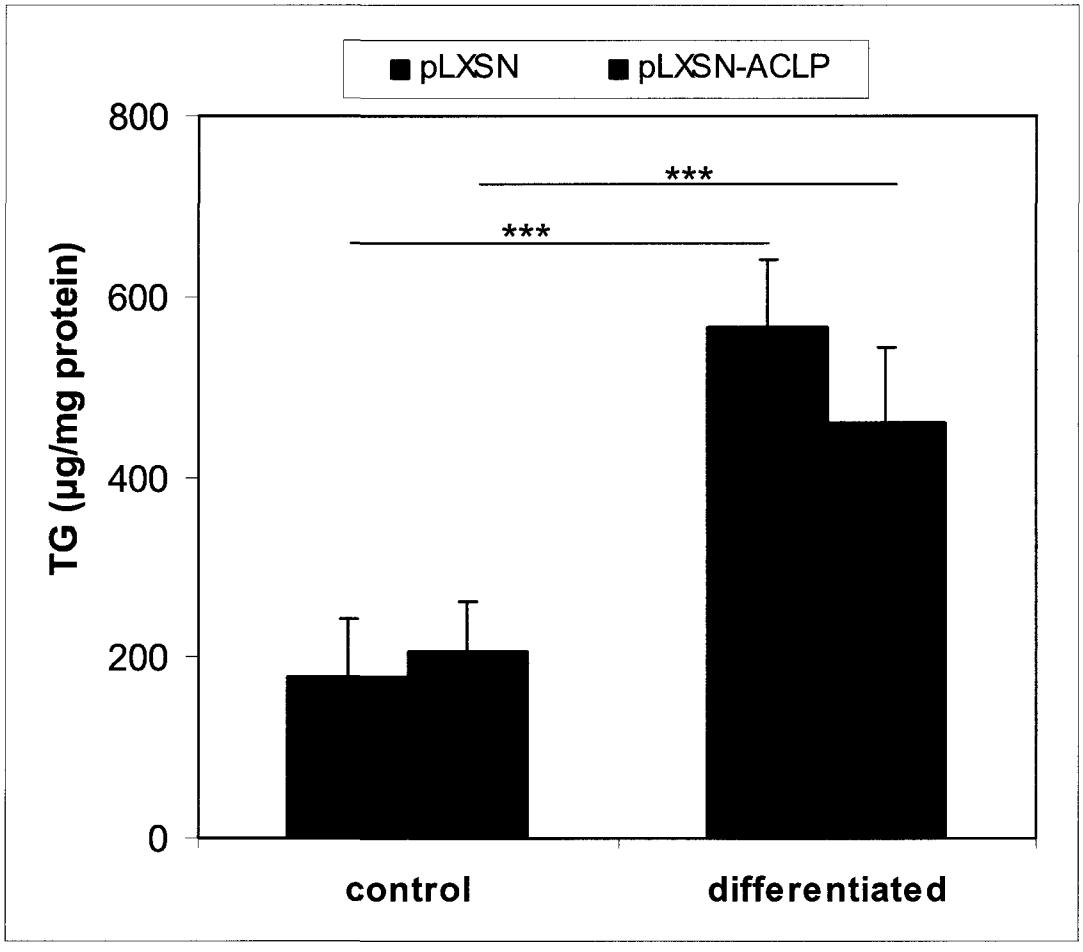
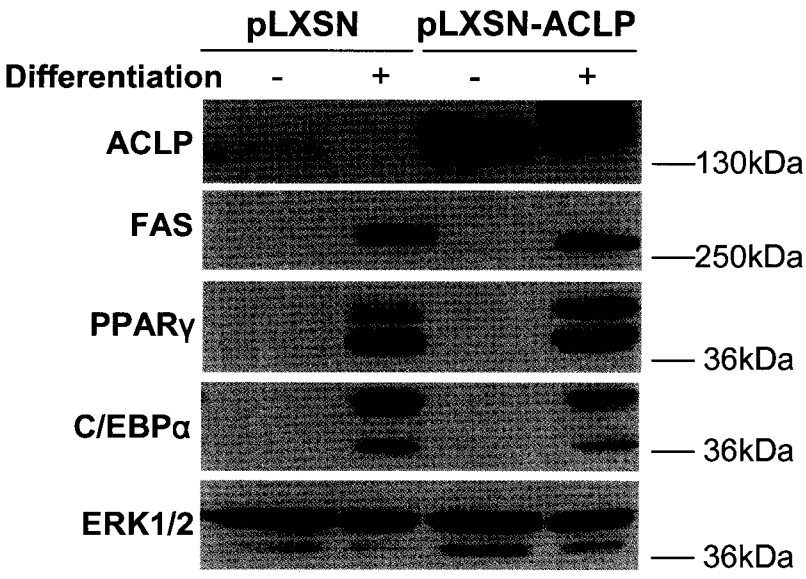
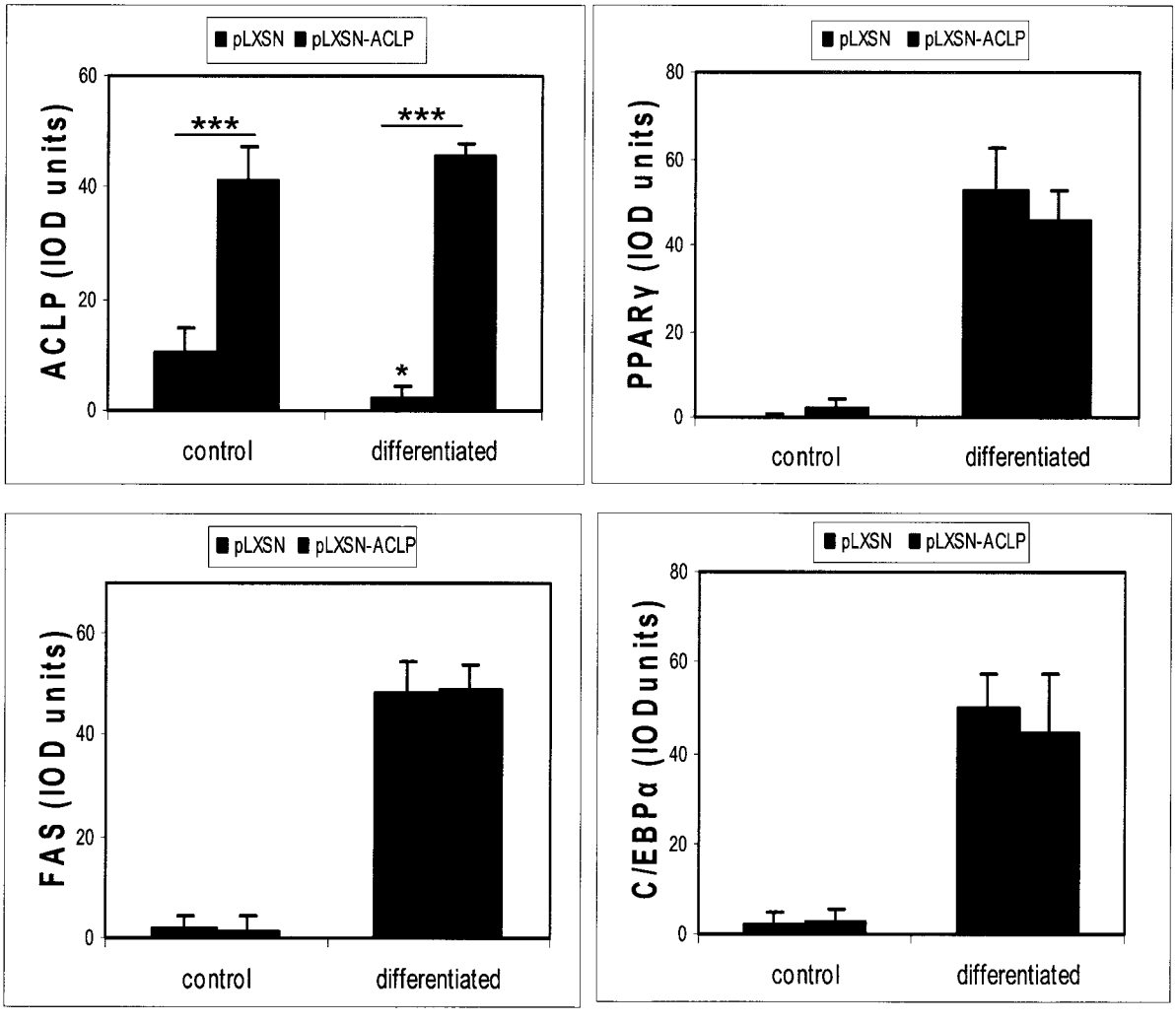

pLXSN
pLXSN-ACLP
800
600
400
200
0
TG (µg/mg protein)
***
***
control
differentiated

Figure 7: Sustained overexpression of ACLP during 3T3-L1 adipogenesis on standard dishes does not affect the expression of differentiation markers. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were grown to confluence. Cells were induced to differentiate or kept in control medium for 8 days as described. On day 8 cells were lysed and equal amounts of solubilized protein were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with the indicated antibodies. ERK1/2 is used as a loading control. A. Representative immunoblots are shown. B. The average densitometric analysis (IOD = Integrated optical density) of four independent experiments ± SD are shown. Statistical analysis was performed using ANOVA. There was a significant upregulation of FAS, PPARγ and C/EBPα with differentiation. *** $p<0.001$ vs. respective pLXSN controls. * $p<0.05$ vs. respective un-differentiated preadipocytes.

A.


pLXSN
pLXSN-ACLP
Differentiation - + - +
ACLP
FAS
PPARγ
C/EBPα
ERK1/2
—130kDa
—250kDa
— 36kDa
— 36kDa
— 36kDa

B.


pLXSN pLXSN-ACLP
ACLP (IOD units)
PPARγ (IOD units)
FAS (IOD units)
C/EBPα (IODunits)
***
***
*
control
differentiated
0
20
40
60
80

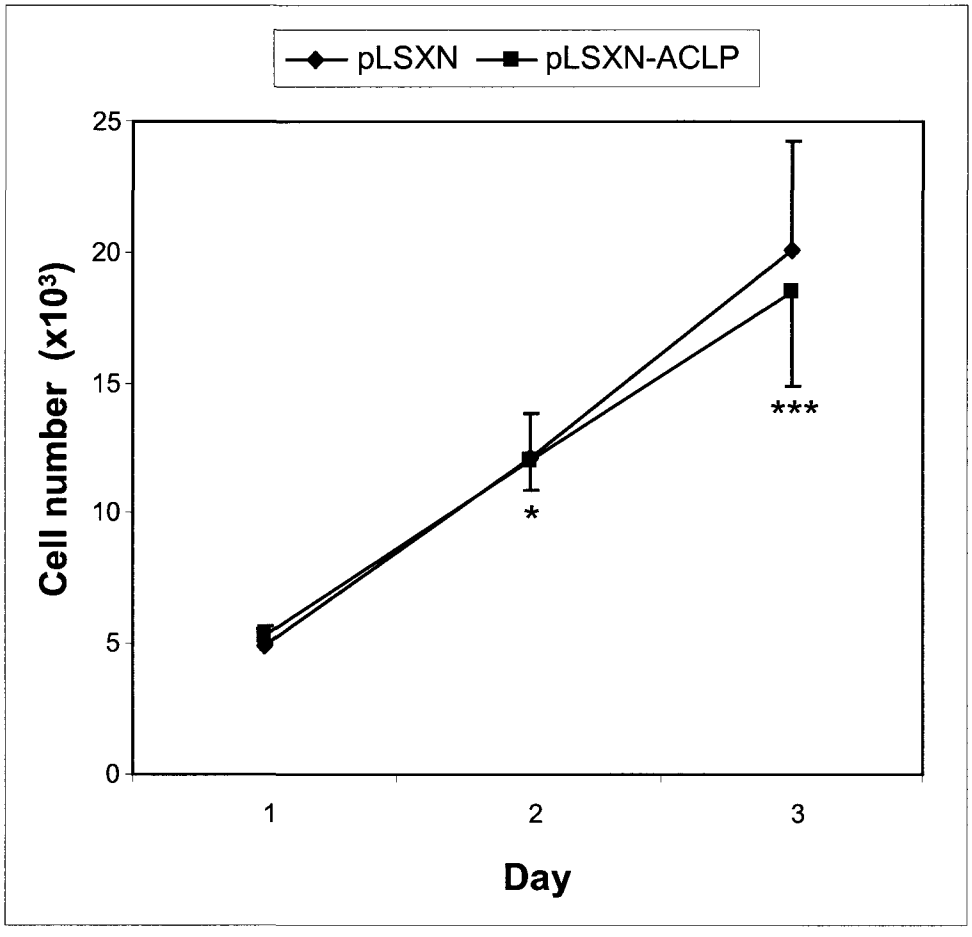
into adipocytes. However, no difference was noted between the expression of the three differentiation markers in pLXSN versus pLXSN-ACLP adipocytes, showing that sustained ACLP overexpression on standard dishes does not affect the expression of key differentiation markers in 3T3-L1 preadipocytes or those induced to differentiate into adipocytes.

As previously noted, the expression of ACLP is reduced with differentiation of pLXSN preadipocytes to adipocytes (4.3 fold reduction). There was a 3.9-fold overexpression of ACLP in pLXSN-ACLP preadipocytes compared to pLXSN preadipocytes. This was sustained during the differentiation of pLXSN-ACLP adipocytes. This resulted in an 18.9 fold overexpression of ACLP in pLXSN-ACLP adipocytes compared to pLXSN adipocytes. Therefore, the sustained ACLP overexpression did not affect 3T3-L1 adipogenesis on standard dishes, as assessed by morphology, TG accumulation and the expression of three key differentiation markers.

Assessing 3T3-L1 subconfluent proliferation and cell death

Since sustained ACLP overexpression did not affect 3T3-L1 adipogenesis, initial experiments were done to determine if overexpression of ACLP might affect other 3T3-L1 preadipocyte functions. Layne's group showed that ACLP knockout mice exhibit reduced proliferation of isolated embryonic dermal fibroblasts (68). Hence, the role of ACLP overexpression in subconfluent proliferation of 3T3-L1 preadipocytes was examined. pLXSN or pLXSN-ACLP cells were plated at a density of $2.5x10^3$ cells/cm^2 on day 0, and the number of adherent cells present after 24, 48 and 72 hours were counted. The number of pLXSN preadipocytes doubled every 24 hours (Fig.8). Similar numbers of cells were

Figure 8: ACLP overexpression does not affect subconfluent 3T3-L1 preadipocyte proliferation. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were plated at a density of $2.5x10^3$ cells/cm^2 (day 0). On indicated days, cells were trypsinized and enumerated in duplicate. Results are the mean ± SD of five independent experiments, each performed in triplicate. Statistical analysis was performed using ANOVA. * $p<0.05$, *** $p<0.001$ vs. day 0.


pLSXN
pLSXN-ACLP
Cell number (x10³)
25
20
15
10
5
0
1
2
3
Day
*
***

counted for pLXSN-ACLP preadipocytes at all time points, showing that ACLP overexpression does not affect subconfluent proliferation of 3T3-L1 preadipocytes.

Next I examined if sustained ACLP expression affects 3T3-L1 preadipocyte cell death. Apoptotic death of serum-supplemented 3T3-L1 preadipocytes was assessed by Hoescht dye staining of the nuclei. Apoptic cells have nuclei that consist of more condensed DNA than viable cells, and their nuclei are distinguishable as smaller, brighter spots when stained with Hoescht (see arrow Fig.9). There were 1.6% apoptotic cells in pLXSN preadipocytes, and 1.6% apoptic cells in pLXSN-ACLP preadipocyte. Hence, no significant difference in the amount of apoptotic cells as a result of ACLP overexpression in 3T3-L1 preadipocytes was noted.

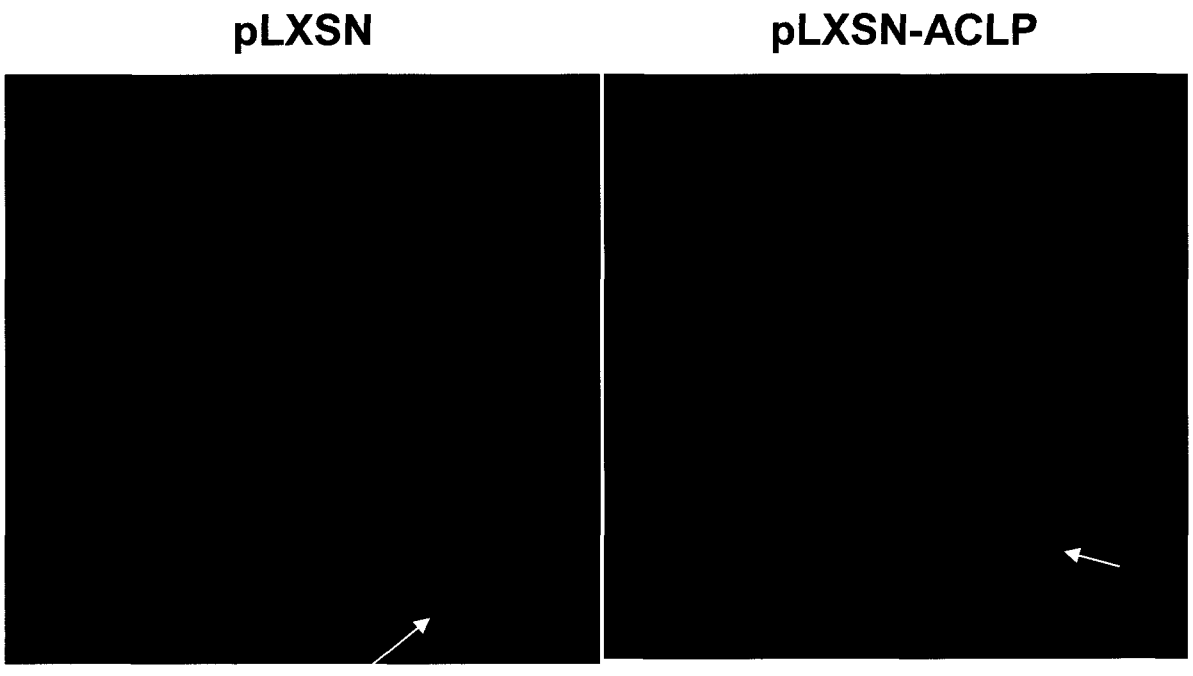
Subsequently, I wanted to determine whether ACLP overexpression affects 3T3-L1 cell death when challenged with serum-free medium. pLXSN and pLXSN-ACLP confluent preadipocytes were either placed in serum-free medium or serum-supplemented medium for 16 hours, at which time the number of remaining adherent cells were counted. Serum deprivation led to the death of 50% of both pLXSN and pLXSN-ACLP preadipocytes. Additionally, the same amount of cells were counted for pLXSN and pLXSN-ACLP preadipocytes in serum-supplemented state. (Fig.10). Hence, ACLP overexpression does not affect serum-deprived cell death of 3T3-L1 preadipocytes.

Assessing collagen I/III expression and gelatinase activity of MMP-2/MMP-9

As previously mentioned, ACLP is hypothesized to interact with ECM proteins, and specifically a collagen–ACLP interaction has been proposed. The effect of sustained

Figure 9: ACLP overexpression does not affect the apoptosis of serum-supplemented confluent 3T3-L1 preadipocytes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were plated on coverslips, grown to confluence, then fixed and stained with Hoechst dye. A. Cells were visualized using fluorescence microscope. Arrows show apoptotic cells. B. Total cells, as well as apoptotic cells were counted in 10 fields per coverslip. Results represent the mean ± SD of three independent experiments, each performed in triplicate.

A.


pLXSN
pLXSN-ACLP

B.

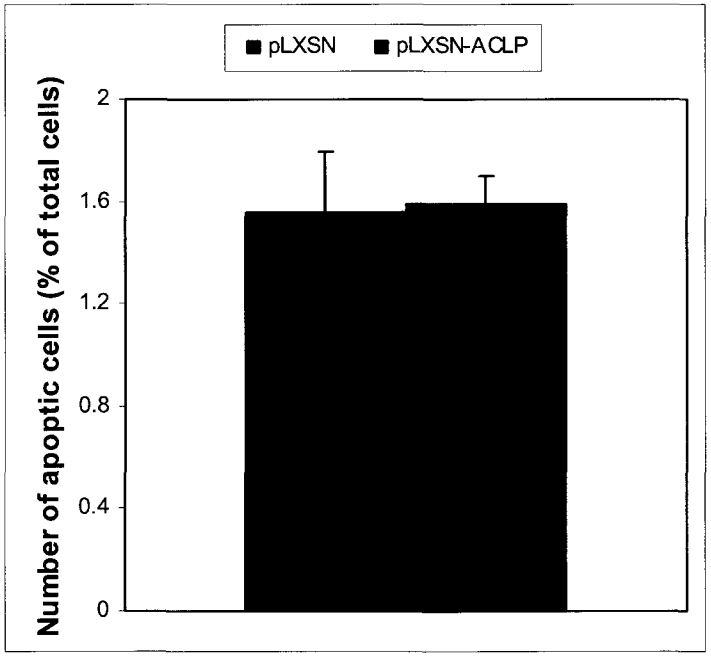
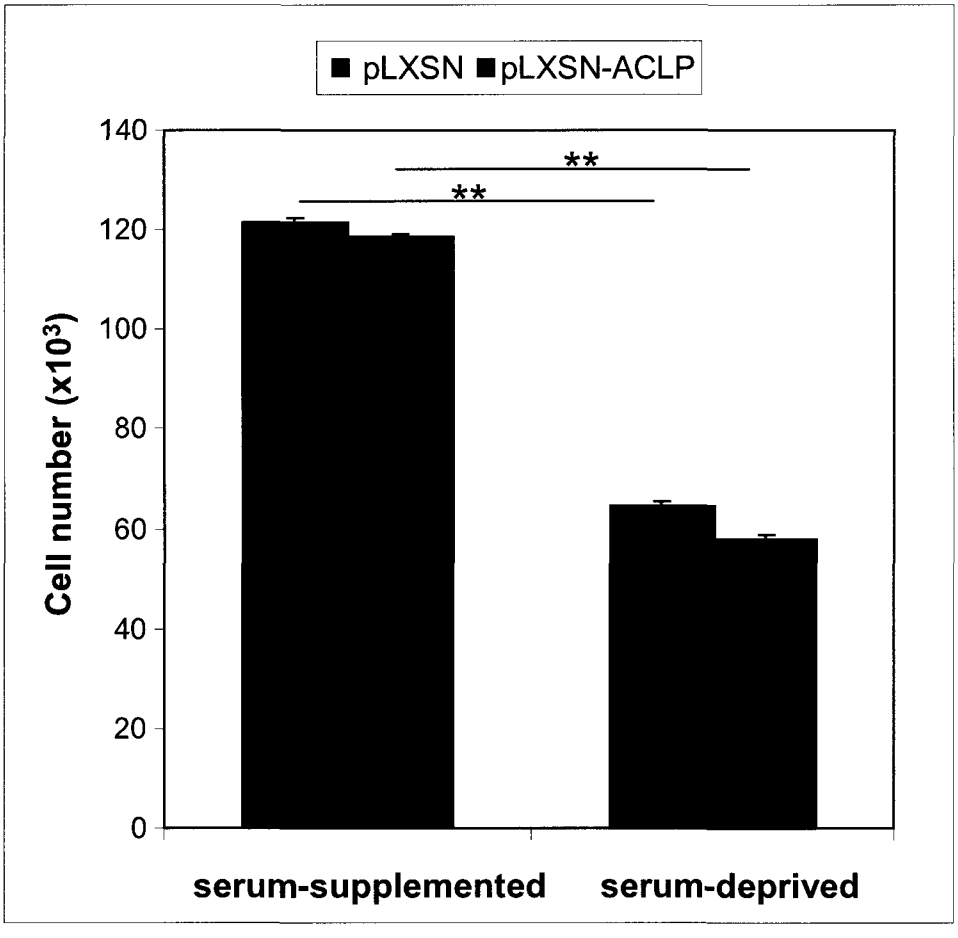

pLXSN
pLXSN-ACLP
Number of apoptic cells (% of total cells)
2
1.6
1.2
0.8
0.4
0

Figure 10: ACLP overexpression does not affect serum-deprived cell death in confluent 3T3-L1 preadipocytes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were grown. Upon confluence cells were either kept in regular medium (DMEM/10%CS/1%PS) or starvation medium (DMEM/1%PS) as described. After 16 hours cells were trypsinized and enumerated in duplicate per cm^2. Results represent the mean ± SD of three independent experiments, each performed in duplicate. Statistical analysis was performed using ANOVA. ** $p<0.01$ vs. respective serum supplemented controls.


■ pLXSN ■ pLXSN-ACLP
140
120
100
80
60
40
20
0
Cell number (x10³)
**
**
serum-supplemented
serum-deprived

ACLP overexpression on the levels of collagen I and III in 3T3-L1 preadipocytes and adipocytes was examined. Confluent pLXSN and pLXSN-ACLP preadipocytes were exposed to differentiation medium or control medium for 8 days. On day 8 the level of collagen I and III was measured in both preadipocytes and adipocytes by immunoblotting. No significant difference in the total amount of collagen I and III (Fig. 11) protein present in pLXSN preadipocytes versus adipocytes was noted. Additionally, no significant difference in the total amount of collagen I and III protein present in pLXSN versus pLXSN-ACLP preadipocytes or adipocytes was evident.

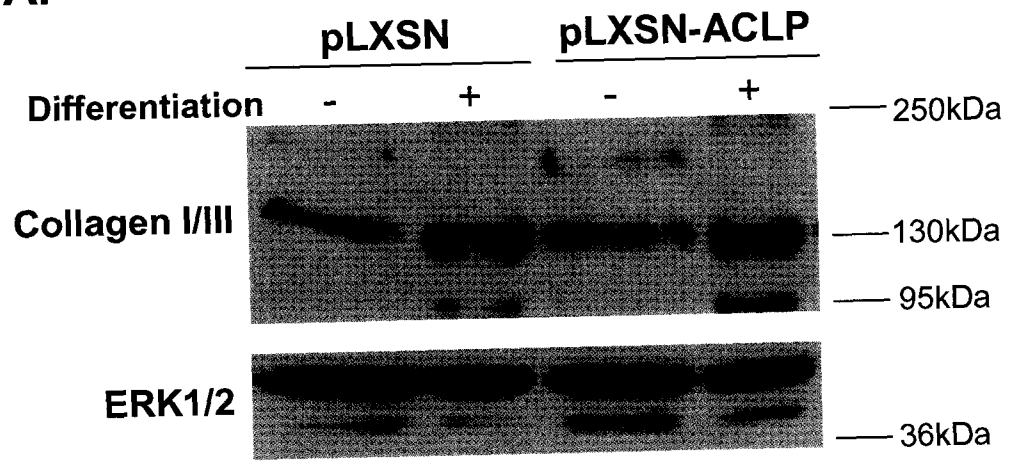
Proteins that are crucial for ECM remodeling during differentiation of 3T3-L1 preadipocytes are extracellular degrading enzymes. Metalloproteinase 2 and 9 (MMP-2 and MMP-9) are known to preferentially degrade collagen, and thus were of interest. Using zymography, it was noted that the gelatinase activity of MMP-9 is about 2 fold higher than that of MMP-2 in both pLXSN preadipocytes and adipocytes (Fig.12). Additionally, the gelatinase activity of MMP-2 decreased about 3 fold with pLXSN differentiation, while the gelatinase activity of MMP-9 decreased about 1.5 fold with pLXSN differentiation. However, no significant difference in the gelatinase activity of MMP-2 or MMP-9 between pLXSN and pLXSN-ACLP preadipocytes or adipocytes was present.

Assessing the effect of ACLP overexpression on adipogenesis using collagen-I coated dishes

Sustained ACLP overexpression in 3T3-L1 preadipocytes cultured in standard dishes showed no effect on adipogenesis. A recent paper suggested an ACLP – collagen-I

Figure 11: Sustained ACLP overexpression does not affect the expression of collagen I and III in 3T3-L1 preadipocytes or adipocytes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were grown to confluence. Cells were induced to differentiate or kept in control medium for 8 days as described. On day 8 cells were lysed and equal amounts of solubilized protein were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with the collagen I/III antibody. ERK1/2 is used as a loading control. A. Representative immunoblots are shown. B. The average densitometric analysis (IOD = Integrated optical density) of three independent experiments ± SD are shown. The multiple bands obtained in each lane were grouped together when performing densitometry.

A.


pLXSN
pLXSN-ACLP
Differentiation
-
+
-
+
250kDa
Collagen I/III
130kDa
95kDa
ERK1/2
36kDa

B.

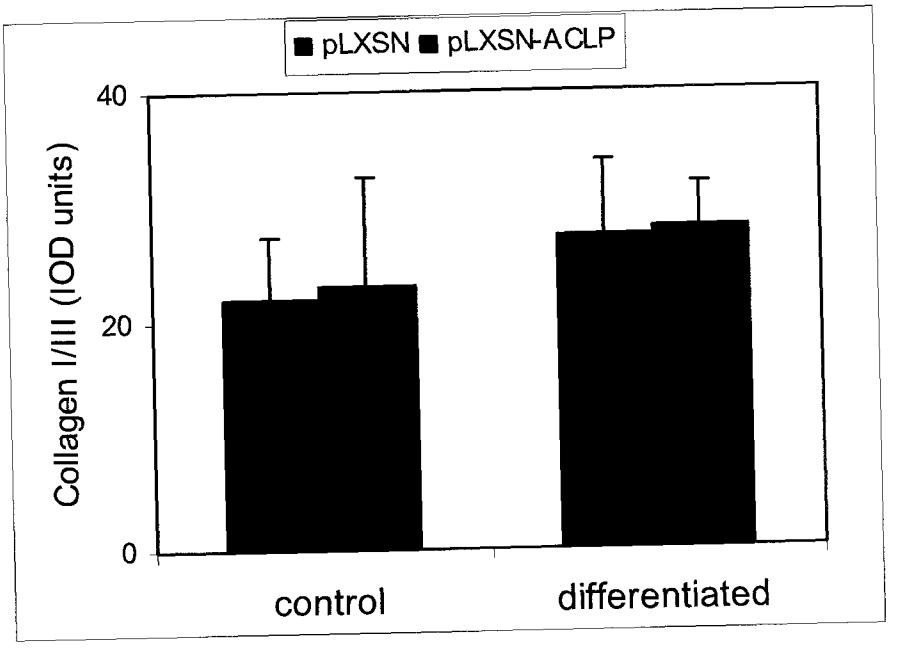
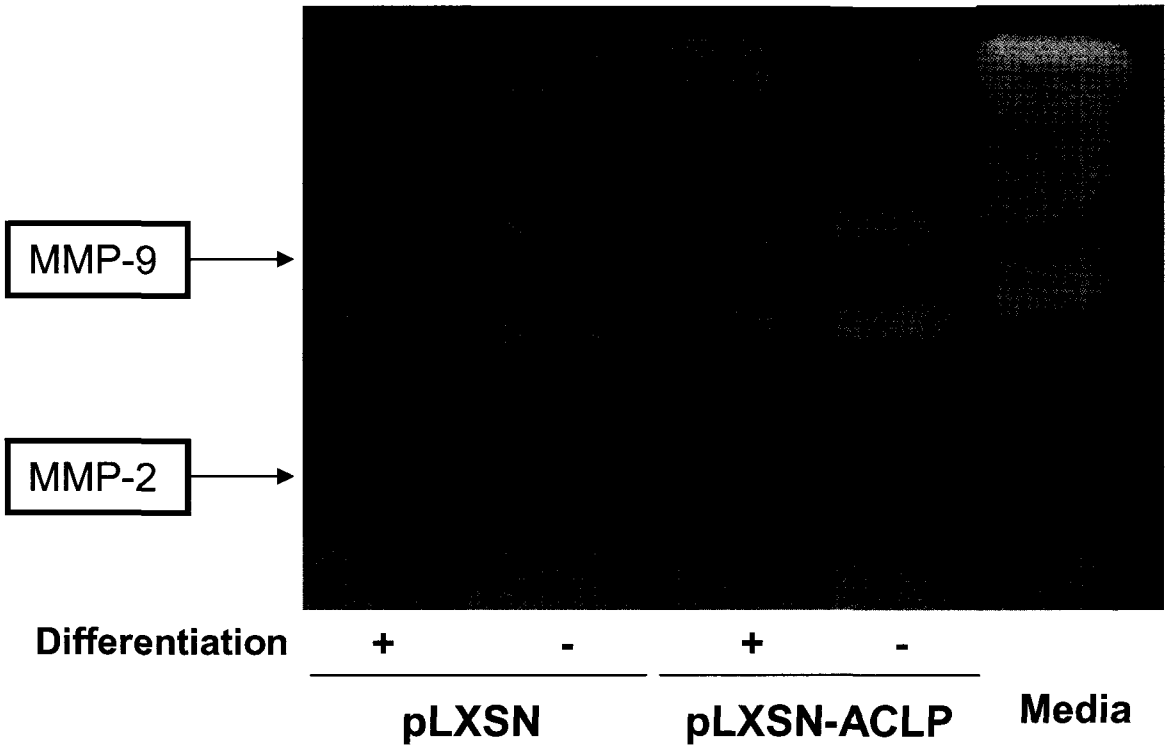
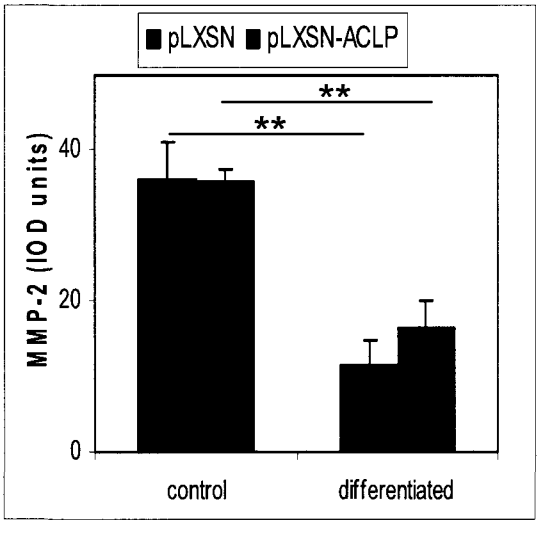

pLXSN
pLXSN-ACLP
40
20
0
Collagen I/III (IOD units)
control
differentiated

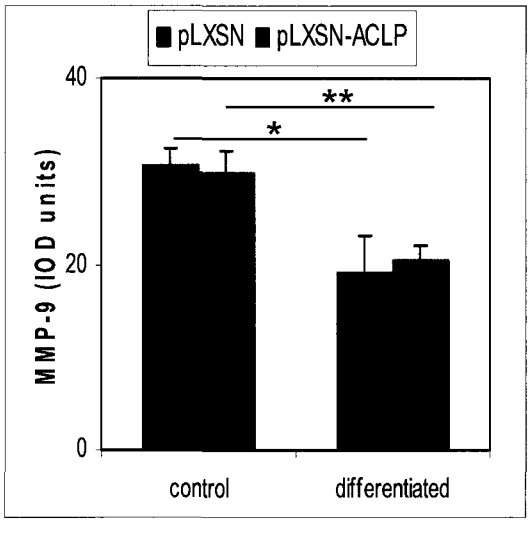
Figure 12: Sustained ACLP overexpression does not affect the gelatinase activity of extracellular degrading enzymes, MMP-2 and MMP-9, in 3T3-L1 preadipocytes or adipocytes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were grown to confluence. Cells were induced to differentiate with or kept in control medium for 8 days as described. On day 8, cells were starved in DMEM/0.5%BSA/1%PS for 3 hours as described. Culture medium were collected, and run in a non-reducing SDS-PAGE gel co-polymerized with gelatin. Degradation of gelatin was visualized with Coomassie Brilliant Blue. A. A zymograph from a single experiment is shown. B. The average densitometric analysis (IOD = Integrated optical density) of three independent experiments ± SD are shown. Statistical analysis was performed using ANOVA. * $p<0.05$, ** $p<0.01$, vs. respective undifferentiated controls.

A.


MMP-9
MMP-2
Differentiation
+
-
+
-
pLXSN
pLXSN-ACLP
Media

B.


pLXSN
pLXSN-ACLP
MMP-2 (IOD units)
40
20
0
**
**
control
differentiated


pLXSN
pLXSN-ACLP
MMP-9 (IOD units)
40
20
0
**
*
control
differentiated

interaction for lung fibroblasts plated on collagen-I matrix (78). Hence, it is possible that a similar collagen-I enriched environment might reveal an effect of sustained ACLP overexpression on 3T3-L1 adipogenesis. pLXSN and pLXSN-ACLP preadipocytes were seeded on standard and collagen-I coated dishes, and kept in either control medium or differentiation medium for 8 days. On day 8, the differentiation of pLXSN and pLXSN-ACLP adipocytes was assessed through morphology, TG accumulation and the expression of the three key differentiation markers.

Using the standard differentiation medium, no significant difference in the differentiation of pLXSN versus pLXSN-ACLP preadipocytes on regular or collagen-I coated dishes was noted (data not shown). Similarly, no significant difference in the differentiation of pLXSN preadipocytes on regular versus collagen-I coated dishes was present. I next examined the possibility that any potential difference in the differentiation response for cells on collagen-I coated dishes may have been overcome by the potency of the differentiation medium. Hence, a modified differentiation protocol with CS instead of FBS and lower concentrations of the adipogenic stimulants was used.

The differentiation of pLXSN and pLXSN-ACLP preadipocytes, using the modified differentiation protocol, in regular dishes versus collagen-I coated dishes was assessed. As previously noted, pLXSN preadipocytes grown on standard dishes were elongated and become rounded and lipid laden with differentiation into adipocytes (Fig.13). The same trend was observed with pLXSN preadipocytes differentiated on collagen-I coated dishes, showing that collagen-I coating does not affect the morphology of 3T3-L1 preadipocytes or those differentiated into adipocytes. The morphology of pLXSN-ACLP preadipocytes and adipocytes grown on both standard and collagen-I coated dishes was similar to that of

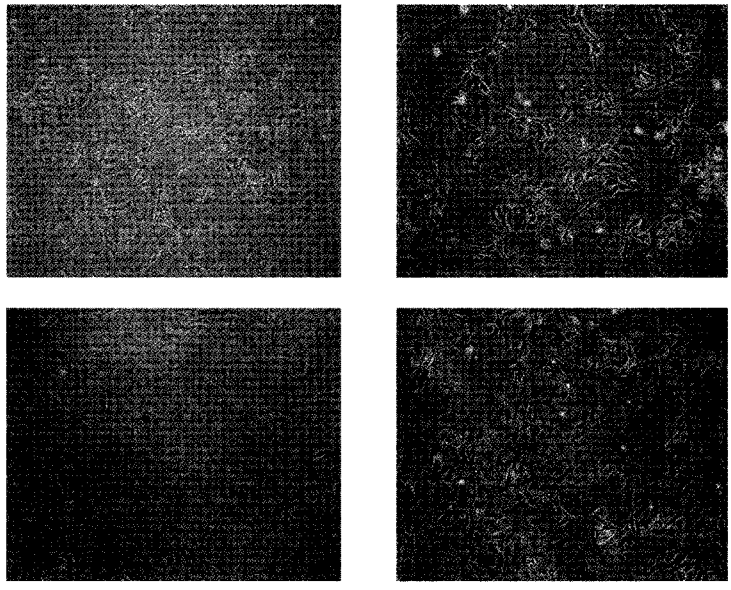
Figure 13: Sustained ACLP overexpression does not affect the morphology 3T3-L1 preadipocytes or adipocytes grown on collagen I-coated dishes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were seeded on either standard dishes or collagen-I coated dishes. These cells were either kept in control medium or induced to differentiate in the presence of modified differentiation cocktail as described. Photomicrographs (100X) of the various cultures on day 8, from one experiment, representative of five independent experiments are shown.

Control

Collagen-I dish - +

pLXSN

pLXSN-ACLP

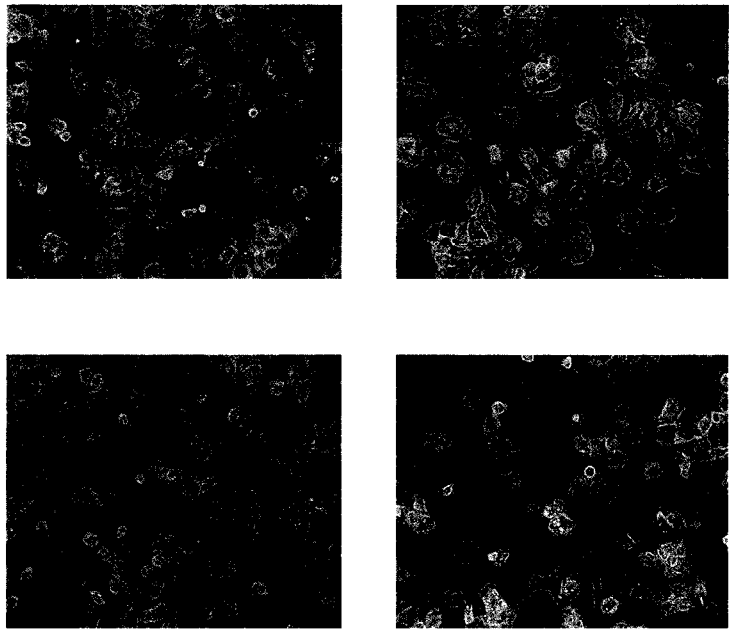


Differentiated

Collagen-I dish - +

pLXSN

pLXSN-ACLP



pLXSN preadipocyte or adipocytes, respectively. This showed that sustained ACLP overexpression does not affect the morphology of 3T3-L1 preadipocytes or adipocytes grown on standard or collagen-I coated dishes.

TG level, with the modified differentiation protocol, was 2.5 fold higher in pLXSN adipocytes than in pLXSN preadipocytes grown on standard dishes (Fig.14). Collagen-I coated dishes did not affect the amount of TG that pLXSN adipocytes accumulated with differentiation, showing that collagen-I coating does not affect the TG accumulation of 3T3-L1 adipocytes. As shown before, on the standard dishes, sustained ACLP overexpression in 3T3-L1 adipocytes did not affect the amount of TG accumulated during differentiation. Finally, sustained ACLP overexpression on collagen-I coated dishes did not affect TG accumulation of 3T3-L1 preadipocytes differentiated into adipocytes.

The effect of collagen-I coated dishes on the expression of three key differentiation markers in pLXSN and pLXSN-ACLP adipocytes were assessed. As shown previously, the expression of key differentiation markers was significantly increased with pLXSN preadipocyte differentiation into adipocytes on standard dishes (Fig.15). I noted that collagen-I coated dishes, in comparison to standard dishes, increased the expression of the three key differentiation markers in pLXSN adipocytes. FAS protein expression was increased 1.6 fold whereas the expression of C/EBPα and PPARγ was each increased 1.4 fold. In contrast, the expression of key differentiation markers in pLXSN-ACLP adipocytes was not significantly different when cells were grown on collagen-I coated versus standard dishes. Thus, collagen-I coated dishes, compared to standard dishes, enhance the expression of key differentiation markers in pLXSN adipocytes but not those of pLXSN-ACLP adipocytes.

Figure 14: Sustained ACLP overexpression during 3T3-L1 adipogenesis on collagen-I coated dishes does not affect triacylglycerol accumulation. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were seeded on either standard dishes or collagen I-coated dishes. These cells were either kept in control medium or induced to differentiate in the presence of modified differentiation cocktail as described. On day 8 TG was extracted, solubilized and quantified. Total TG is normalized per total protein present in the dish. Results are the mean ± SD of three independent experiments. Statistical analysis was performed using ANOVA. *** $p<0.001$ vs. respective undifferentiated controls.

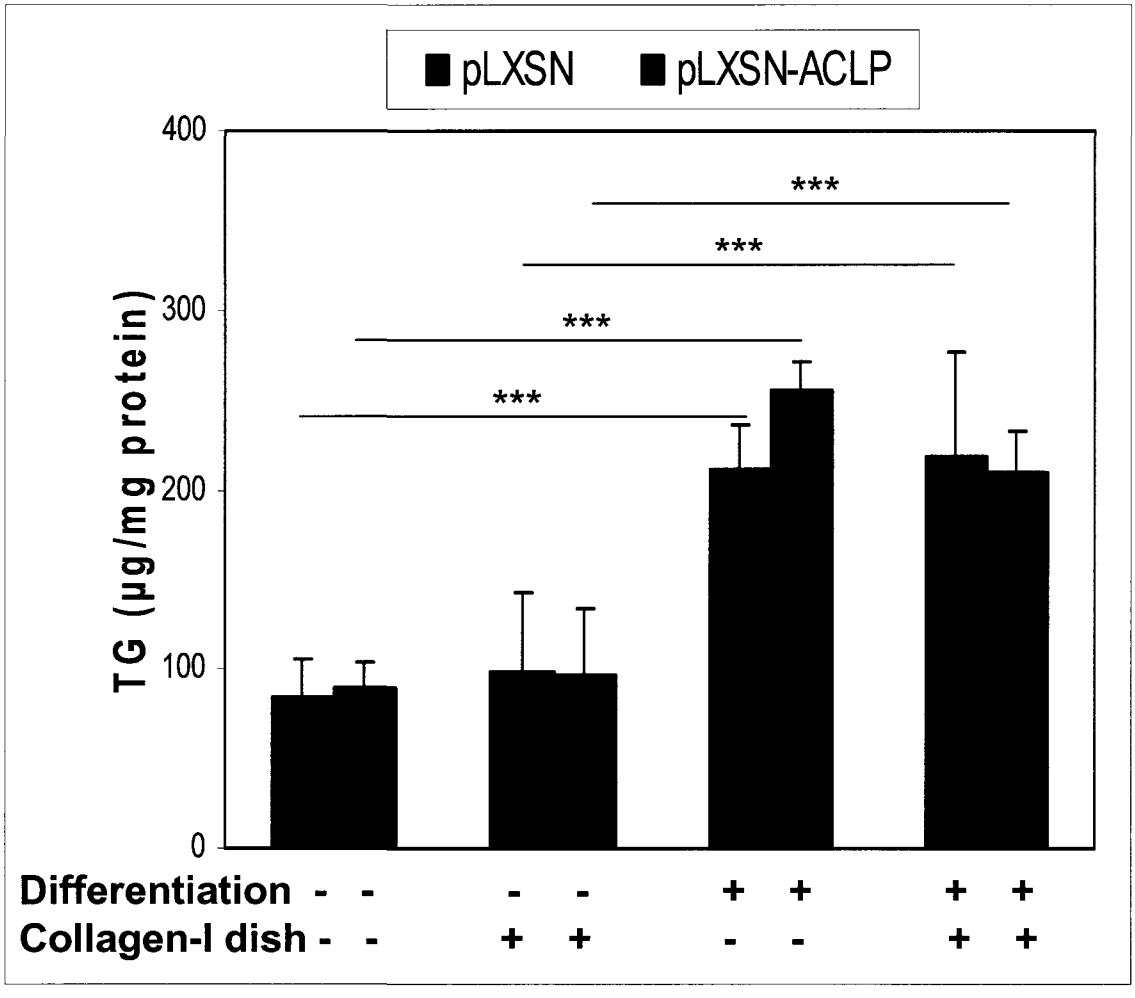
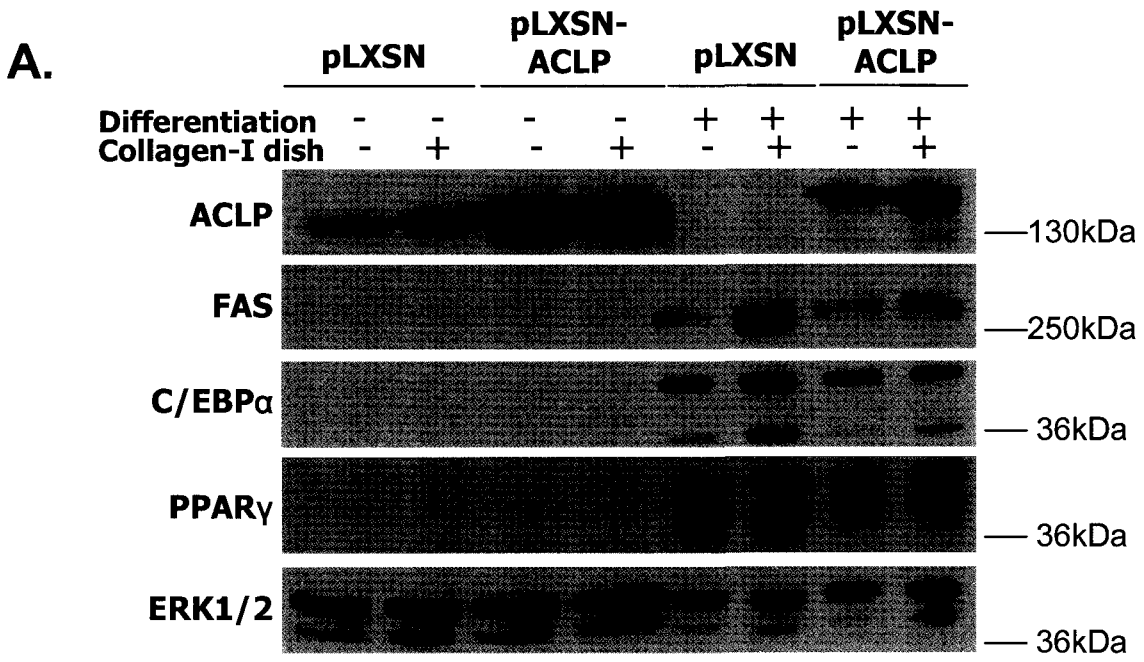
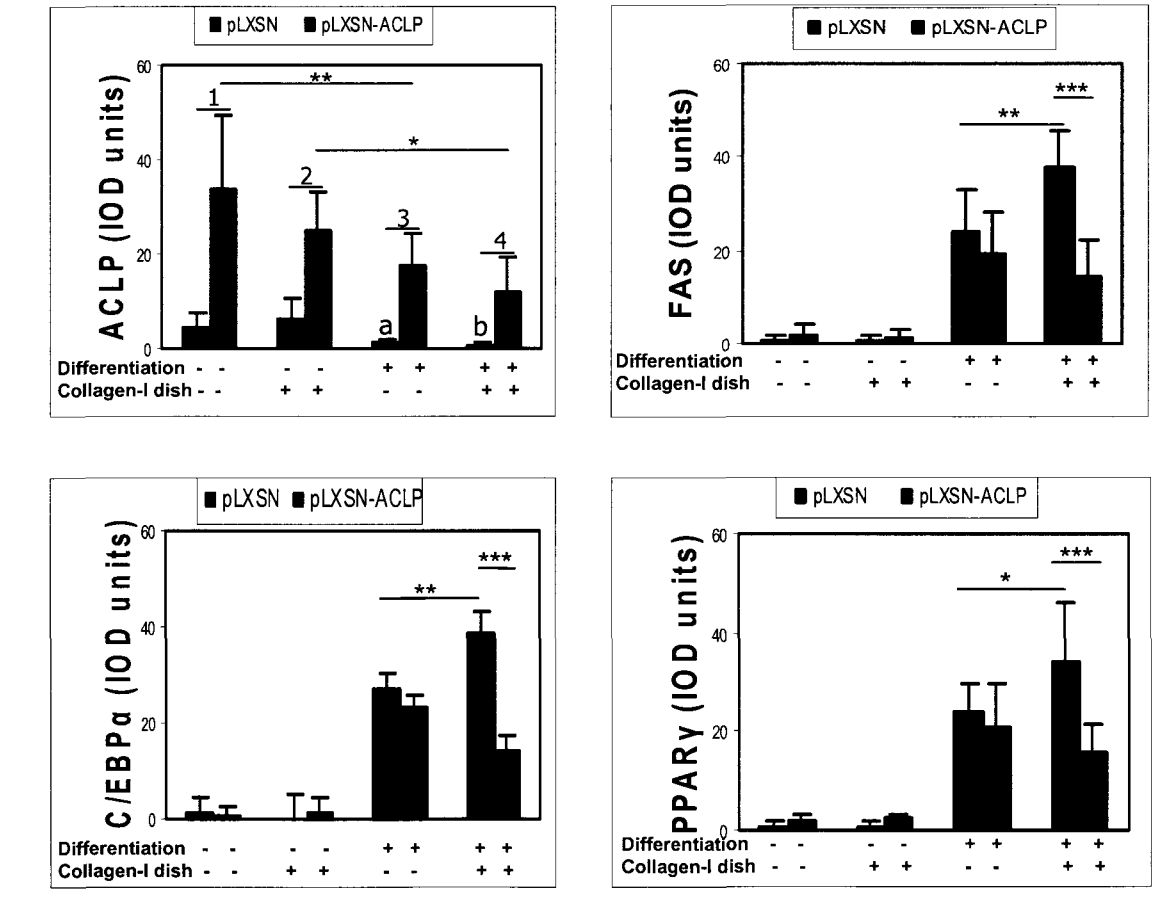

pLXSN
pLXSN-ACLP
400
300
200
100
0
TG (µg/mg protein)
***
***
***
***
Differentiation - - - - + + + +
Collagen-I dish - - + + - - + +

Figure 15: Sustained ACLP overexpression reduces the expression of three key differentiation markers in 3T3-L1 adipocytes grown on collagen-I coated dishes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were seeded on either standard dishes or collagen-I coated dishes. These cells were either kept in control medium or induced to differentiate in the presence of modified differentiation cocktail as described. On day 8 equal amounts of solubilized protein were separated by SDS-PAGE, transferred and immunoblotted using the indicated antibody. ERK1/2 is used as a loading control. A. Representative immunoblots are shown. B. The average densitometric analysis (IOD = Integrated optical density) of five independent experiments ± SD are shown. Statistical analysis was performed using ANOVA. There was a significant upregulation of FAS, PPARγ and C/EBPα with differentiation. 1 $p<0.001$, 2 $p<0.01$, 3 $p<0.05$, 4 $p<0.05$ vs. respective pLXSN controls. a $p<0.05$, b $p<0.05$, vs. respective undifferentiated controls. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.


A.
pLXSN
pLXSN-ACLP
pLXSN
pLXSN-ACLP
Differentiation - - - - + + + +
Collagen-I dish - + - + - + - +
ACLP
FAS
C/EBPα
PPARγ
ERK1/2
130kDa
250kDa
36kDa
36kDa
36kDa


B.
pLXSN
pLXSN-ACLP
ACLP (IOD units)
FAS (IOD units)
C/EBPα (IOD units)
PPARγ (IOD units)
Differentiation - - - - + + + +
Collagen-I dish - - + + - - + +

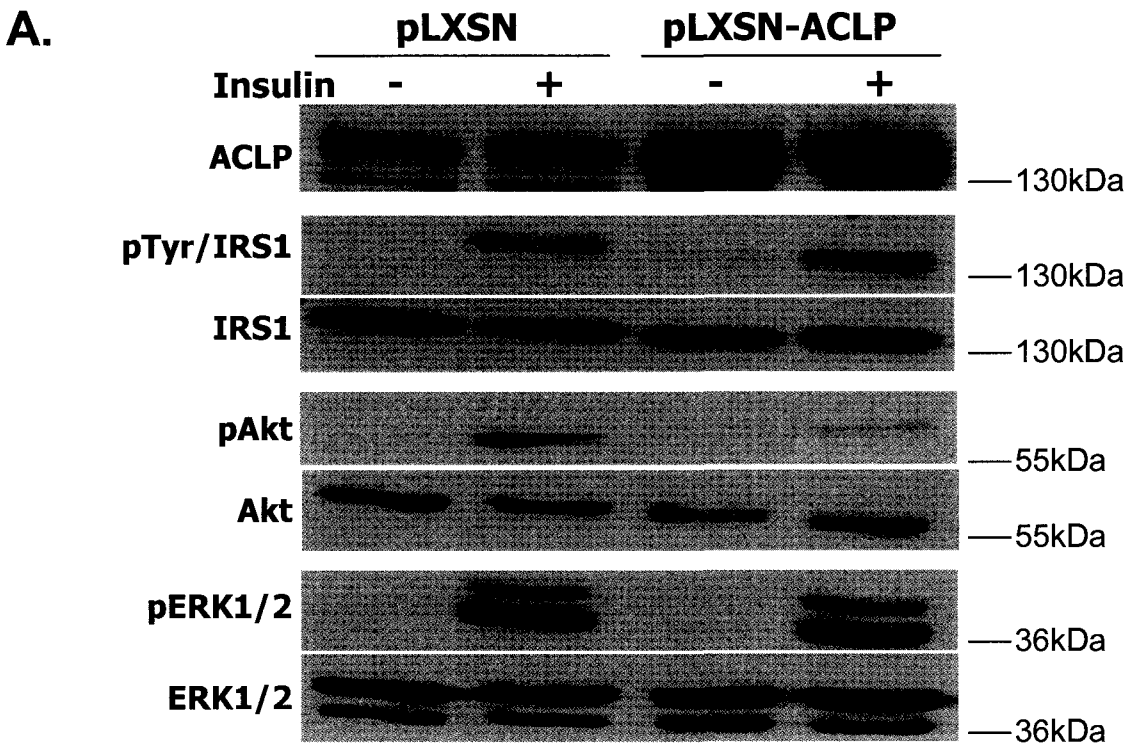
Additionally, the effect of sustained ACLP overexpression on the expression of three key differentiation markers in 3T3-L1 adipocytes on both collagen-I coated and standard dishes were assessed. As before, sustained ACLP overexpression in 3T3-L1 preadipocytes differentiated on standard dishes had no effect on the expression of key differentiation makers. However, sustained ACLP overexpression on collagen-I coated dishes significantly reduced the expression of key differentiation markers. FAS protein was decreased 2.6 fold, C/EBPα was decreased 2.1 fold and PPARγ was decreased 2.7 fold, respectively. Hence, sustained ACLP expression on collagen-I coated dishes diminishes the expression of the three key differentiation markers in 3T3-L1 preadipocytes.

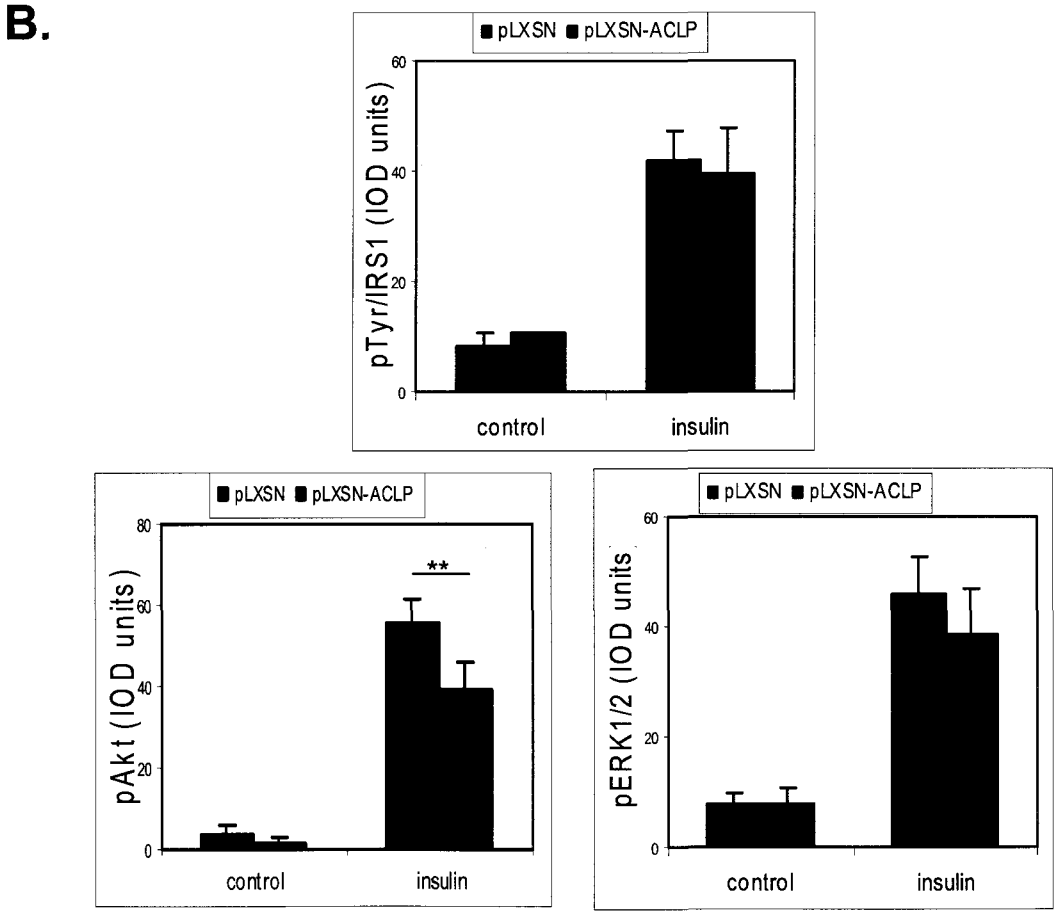
Assessing insulin signaling in preadipocytes grown on collagen-I coated dishes

Sustained overexpression of ACLP in 3T3-L1 preadipocytes grown on collagen-I coated dishes inhibits the expression of three key differentiation markers. A possible mechanism for this could be through inhibited insulin stimulated pro-adipogenic signaling in preadipocytes. Insulin causes the sequential phosphorylation of a number of signaling proteins, and this was assessed by measuring the phospho-content of IRS-1, Akt, and ERK1/2 in post-confluent preadipocytes seeded on collagen-I coated dishes. The level of the proteins themselves remained constant with insulin stimulation of pLXSN preadipocytes, whereas the levels of the phosphorylated forms increased ~5 fold for IRS-1, ~13 fold for Akt and ~6 fold for ERK1/2 (Fig 16).

There was no significant difference in the insulin-stimulated phosphorylation of IRS-1 or ERK1/2 between pLXSN versus pLXSN-ACLP preadipocytes. However, insulin-stimulated Akt phosphorylation was 27% lower in pLXSN-ACLP preadipocytes compared

Figure 16: ACLP overexpression reduces Akt mediated insulin signaling in 3T3-L1 preadipocytes grown on collagen-I coated dishes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were seeded on collagen I-coated dishes. Two days post-confluence cells were starved for 16 hours with DMEM/1%CS/1%PS before stimulation with either 10nM insulin or vehicle (KRH) for 5 minutes. Cells were lysed and equal amounts of solubilized protein were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with the indicated antibodies. A. Representative immunoblots are shown. B. The average densitometric analysis (IOD = Integrated optical density) of three independent experiments ± SD are shown. Statistical analysis was performed using ANOVA. There was a significant increase in the expression of pTyr/IRS1, pAkt and pERK1/2 with insulin stimulation. ** $p<0.01$.


A.
pLXSN
pLXSN-ACLP
Insulin - + - +
ACLP
130kDa
pTyr/IRS1
130kDa
IRS1
130kDa
pAkt
55kDa
Akt
55kDa
pERK1/2
36kDa
ERK1/2
36kDa


B.
pLXSN pLXSN-ACLP
pTyr/IRS1 (IOD units)
control
insulin
pLXSN pLXSN-ACLP
pAkt (IOD units)
**
control
insulin
pLXSN pLXSN-ACLP
pERK1/2 (IOD units
control
insulin

to that in pLXSN preadipocytes. Hence, ACLP overexpression in 3T3-L1 preadipocytes grown on collagen-I coated dishes inhibits insulin stimulated Akt phosphorylation in 3T3-L1 preadipocytes.

Assessing adipocyte function

Sustained ACLP expression in 3T3-L1 preadipocytes grown on collagen-I dishes showed an inhibition of the three key differentiation markers, but no effect on morphology or TG accumulation. Though these adipocytes accumulated similar amounts of TG, it is possible that other adipocyte functions were affected. The following experiments were done on standard dishes, but in the future it would be interesting to look at these adipocyte functions on collagen-I coated dishes.

pLXSN and pLXSN-ACLP preadipocytes were differentiated for 8 days and three adipocyte functions were assessed: lipolysis, insulin-stimulated glucose uptake and insulin signaling. A beta-adrenergic agonist, isoproterenol, caused a dose dependent increase in pLXSN lipoysis (Fig.17). However, there was no significant difference in basal or isoproterenol-induced lipolysis between pLXSN and pLXSN-ACLP adipocytes.

Insulin-stimulated glucose uptake was 2 fold higher than basal glucose uptake in pLXSN adipocytes, based on the labeled ^{3}H-deoxyglucose assay (Fig.18). However, there was no significant difference in basal or insulin stimulated glucose uptake between pLXSN and pLXSN-ACLP adipocytes.

Insulin signaling was done in adipocytes, as before for the preadipocytes, by assessing the insulin-stimulated phosphorylation of IRS-1, Akt and ERK1/2. The level of the proteins themselves remained constant with insulin stimulation, whereas the level of the

Figure 17: Sustained ACLP overexpression does not affect isoproterenol-induced lipolysis in 3T3-L1 adipocytes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were grown to confluence. Cells were induced to differentiate as described. On day 8 cells were treated with the indicated concentration of isoproterenol for 4 hours. Culture medium was collected and the rate of TG breakdown was determined by measuring the amount of glycerol released. Total glycerol is normalized per total TG. Results are the mean ± SD of three independent experiments, each performed in triplicate. Statistical analysis was performed using ANOVA. * $p<0.05$, **$p<0.01$, vs. respective vehicle stimulated controls (0nm isoproterenol).

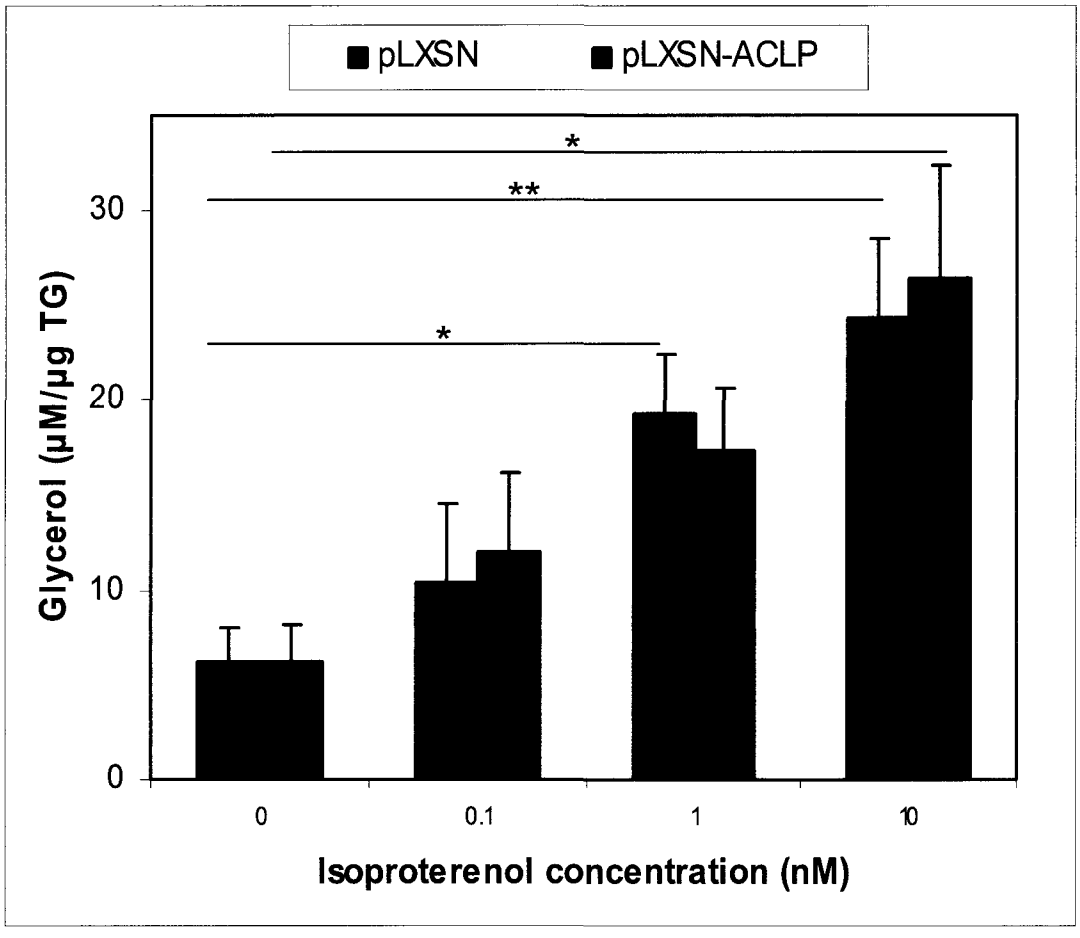
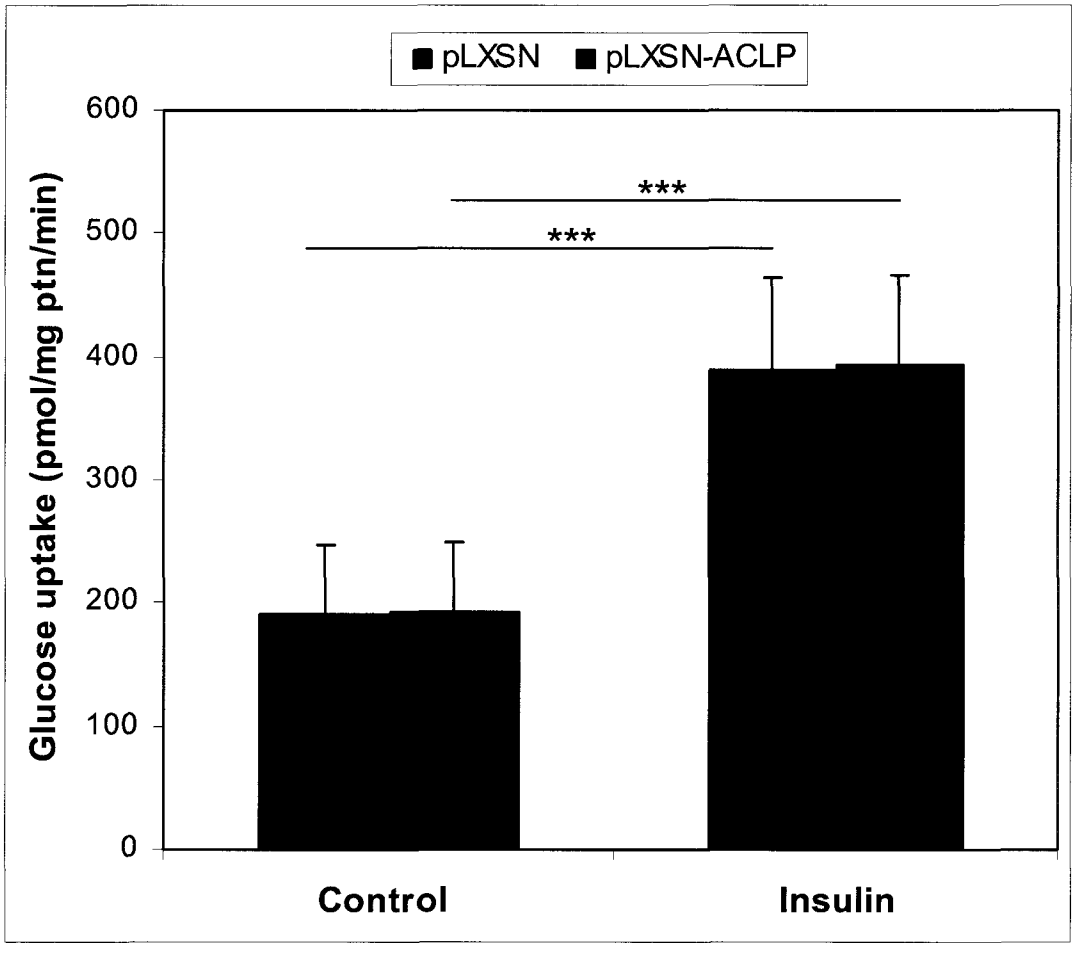

pLXSN
pLXSN-ACLP
*
**
*
30
20
10
0
Glycerol (µM/µg TG)
0
0.1
1
10
Isoproterenol concentration (nM)

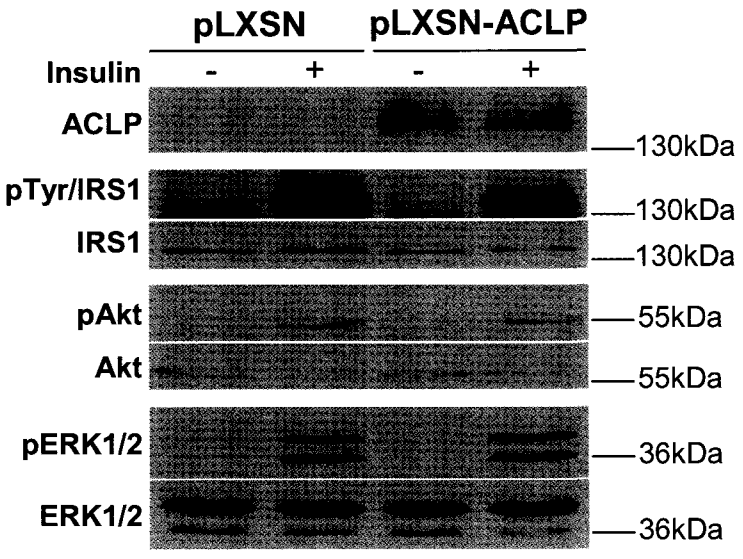
Figure 18: Sustained ACLP overexpression does not affect insulin dependent glucose uptake in 3T3-L1 adipocytes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were grown to confluence. Cells were induced to differentiate as described. Cells were starved for 2 hours with DMEM/1%PS before stimulation with 10nM insulin or vehicle (α-MEM) for 30 minutes. $HgCl_2$ was added to selected cells to determine baseline glucose uptake. Cells were radiolabeled with 0.2mCi/mL ^3H deoxyglucose and scintillation counts were done to determine the amount of radiolabeled deoxyglucose present. Glucose uptake is expressed as total amount of radiolabeled deoxyglucose detected in the cells per total protein present. Results are the mean ± SD of three independent experiments, each performed in triplicate. Statistical analysis was performed using ANOVA. *** $p<0.001$ vs. vehicle stimulated controls.


pLXSN
pLXSN-ACLP
Glucose uptake (pmol/mg ptn/min)
600
500
400
300
200
100
0
***
***
Control
Insulin

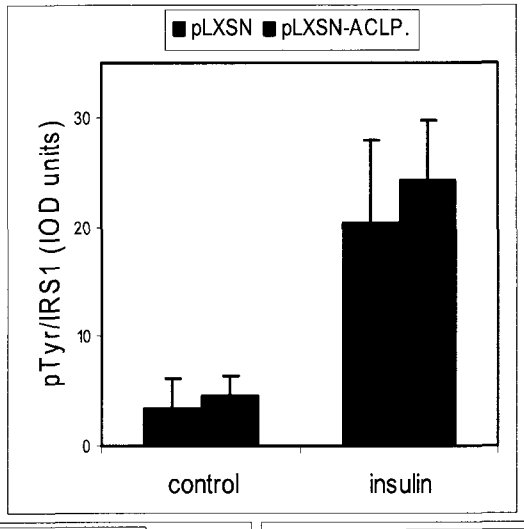
phosphorylated forms increased significantly (Fig 19). However, there was no significant difference between pLXSN and pLXSN-ACLP adipocytes with respect to the phosphorylation of these three proteins, in response to insulin.

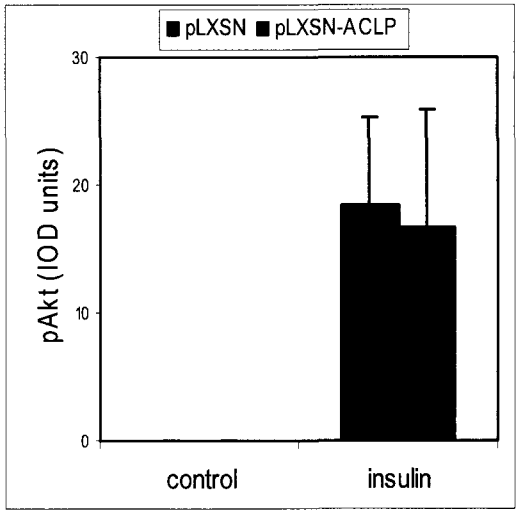
Figure 19: Sustained ACLP overexpression does not affect insulin signaling in 3T3-L1 adipocytes. 3T3-L1 preadipocyte stable clones that either overexpress ACLP (pLXSN-ACLP) or act as controls (pLXSN) were grown to confluence. Cells were induced to differentiate for 8 days as described. On day 8 cells were starved for 16 hours with DMEM/1%CS/1%PS before stimulation with either 10nM insulin or vehicle (BSA/KRH) for 5 minutes. Cells were lysed and equal amounts of solubilized protein were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with the indicated antibodies. ERK1/2 is used as a loading control. A. Representative immunoblots are shown. B. The average densitometric analysis (IOD = Integrated optical density) of four independent experiments ± SD are shown. Statistical analysis was performed using ANOVA. There was a significant increase in the expression of pTyr/IRS1, pAkt and pERK1/2 with insulin stimulation.

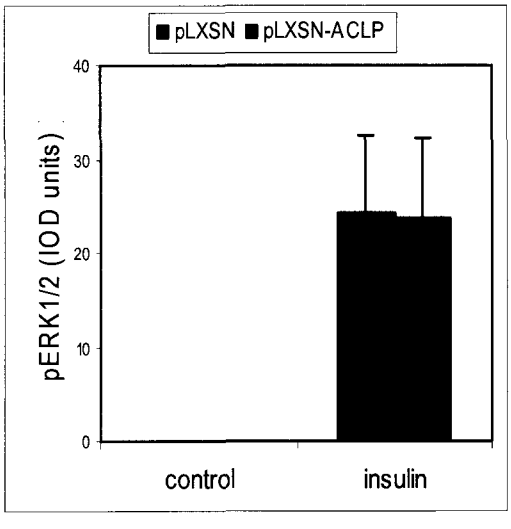
A.


pLXSN
pLXSN-ACLP
Insulin
- + - +
ACLP
130kDa
pTyr/IRS1
130kDa
IRS1
130kDa
pAkt
55kDa
Akt
55kDa
pERK1/2
36kDa
ERK1/2
36kDa

B.


pLXSN pLXSN-ACLP.
pTyr/IRS1 (IOD units)
0
10
20
30
control
insulin


pLXSN pLXSN-ACLP
pAkt (IOD units)
0
10
20
30
control
insulin


pLXSN pLXSN-ACLP
pERK1/2 (IOD units)
0
10
20
30
40
control
insulin

DISCUSSION

Obesity has become a major health hazard, predisposing humans to type 2 diabetes, cardiovascular disease and cancer (1,2). Fat storage in adipose tissue involves adipocyte enlargement as well as the differentiation of fibroblastic preadipocytes into lipid laden rounded adipocytes; this latter process is termed adipogenesis. Differentiation requires extensive changes in the extracellular matrix, including decreased expression of fibrillar collagens and fibronectin, and increased expression of laminin and entactin (48-50). ECM remodeling during adipogenesis allows preadipocytes to assume a more rounded shape to optimize lipid accumulation. Preventing ECM remodeling, e.g. prevention of fibronectin downregulation during 3T3-F442A preadipocyte differentiation, inhibits adipogenesis (51). Hence, ECM remodeling is crucial for normal adipogenesis to occur.

Confirmation that sustained ACLP overexpression does not affect 3T3-L1 adipogenesis on standard dishes

ACLP expression was downregulated during 3T3-L1 preadipocyte differentiation (Fig. 3), suggesting a role for ACLP in adipogenesis. However, the role of ACLP in adipose tissue is controversial. Our group has found that sustained ACLP overexpression in 3T3-L1 or 3T3-F442A preadipocytes does not affect adipogenesis on standard dishes (69, 70), whereas Amri's group found that it inhibits 3T3-F442A adipogenesis and also leads to trans-differentiation into smooth muscle cells (79). My study confirmed that sustained ACLP overexpression does not inhibit 3T3-L1 preadipocyte differentiation on standard dishes, as

assessed by morphology (Fig. 5), TG accumulation (Fig. 6) and the expression of three key differentiation markers, C/EBPα, PPARγ and FAS (Fig. 7).

The inhibition observed by Amri's group could be due possibly to the c-myc tag that they attached to the ACLP construct (79). c-myc tag is an 11 amino acid tag that has been shown to be effective in detecting the expression of recombinant proteins (92). Amri's group attached the c-myc tag to the full length ACLP cDNA, as well as to a truncated form of ACLP cDNA that corresponds to AEBP1 cDNA. They noted that overexpression of c-myc tagged ACLP, but not c-myc tagged AEBP1, inhibits 3T3-F442A adipogenesis. It is likely that the c-myc tag alone had no effect on 3T3-F442A adipogenesis. However, the overexpression of the c-myc tag in combination with overexpression of ACLP, rather than the sustained ACLP overexpression, might have affected 3T3-F442A adipogenesis.

I also confirmed that the lack of effect by ACLP overexpression on 3T3-L1 adipogenesis is not due to a problem with secretion of ACLP. ACLP overexpressing (pLXSN-ACLP) preadipocytes had 3.2 fold more ACLP protein present in their medium than did empty vector (pLXSN) preadipocytes (Fig. 4). This is lower than the 4.9 fold increase in ACLP protein expression in pLXSN-ACLP preadipocyte lysate compared to pLXSN preadipocyte lysate. The rate of ACLP protein expression versus secretion is not known and variation between these processes could account for the difference. It is also possible that ACLP is degraded slightly in the medium. It is likely that ACLP secretion in pLXSN-ACLP adipocytes is sustained at the same level as in preadipocytes, as ACLP overexpression in lysates is sustained with differentiation (Fig.7), but further experiments are needed to confirm this.

Amri's group obtained a 2 fold overexpression in 3T3-F442A preadipocytes with their c-myc tagged ACLP construct. However, they did not show that the overexpressed ACLP is secreted. It is possible that the c-myc tagged ACLP was not secreted in Amri's 3T3-F442A cells, causing a build up of c-myc tagged ACLP in the secretory compartments of the cell and an inhibition of adipogenesis as a result of such an artifact.

ACLP overexpression does not affect 3T3-L1 subconfluent proliferation or cell death

Since ACLP expression is downregulated with differentiation, I speculated that ACLP overexpression might alter preadipocyte responses. Important preadipocyte functions are proliferation and cell death. Layne's group showed that embryonic dermal fibroblasts from ACLP knockout mice exhibit reduced proliferation (68). Layne's group also showed that lung fibroblasts from ACLP knockout mice exhibit a defect in cell proliferation (78). Hence, I predicted that ACLP overexpression would enhance cell proliferation.

The results show that ACLP overexpression in 3T3-L1 preadipocyte had no effect on subconfluent proliferation over the time period tested (Fig.8). Proliferation was only assessed until 72 hours, where ~$2x10^4$ cells/cm^2 were counted but confluence is not expected until the 35mm dish used reaches $1.2x10^5$ cells/cm^2. It is therefore possible that a difference in subconfluent proliferation of pLXSN versus pLXSN-ACLP preadipocytes might be noted if proliferation was continued to longer time points.

In comparison to Layne's ACLP-KO study, ACLP overexpression could be expected to increase subconfluent proliferation. However, overexpression studies do not always provide an opposite phenotype to knockout/knockdown studies. Cells may need a certain amount of ACLP protein to proliferate normally, and an ACLP knockdown could hinder cell

proliferation. However, further ACLP expression beyond the normal level might be unnecessary, thus not affecting cell proliferation. Additionally, the fact that I did not observe the expected phenotype could be because Layne's group used isolated primary dermal and lung fibroblasts, whereas I used the 3T3-L1 immortalized cell line.

I examined if ACLP overexpression causes programmed cell death in 3T3-L1 confluent preadipocytes in serum-supplemented medium. pLXSN-ACLP preadipocytes had the same rate of apoptosis as pLXSN preadipocytes, showing that ACLP overexpression does not affect the apoptosis of 3T3-L1 preadipocytes (Fig.9). I also investigated whether ACLP overexpression affects susceptibility to cell death under conditions of serum deprivation. I noted that serum deprivation for 16 hours reduced 50% of viable cells in both pLXSN and pLXSN-ACLP confluent preadipocytes (Fig.10). This showed that ACLP overexpression has no effect on serum deprived cells death of 3T3-L1 preadipocytes.

Sustained ACLP overexpression does not affect collagen I/III expression or MMP-2/9 gelatinase activity

Since ACLP is a secreted protein, I hypothesized that its role in adipose tissue may be connected to other ECM proteins. Collagen is known to interact with proteins that contain discoidin domains, such as DDR (60-62). Since ACLP contains a discoidin domain similar to that of DDR, it is possible that ACLP binds to collagen. The mRNA expression of fibrillar collagens I and III is decreased with adipogenesis (50, 93, 94), similar to that of ACLP expression (63) further hinting at possible interactions between these proteins.

The effect of sustained ACLP overexpression on the expression of collagen I and III protein was assessed. Though the different forms of processed collagen changed with

differentiation, the total expression of collagen I and III protein in cell lysates of pLXSN preadipocytes did not change significantly with differentiation (Fig. 11). The same expression of collagen I and III was found during the differentiation of pLXSN-ACLP preadipocytes, showing that sustained ACLP overexpression does not affect the expression of collagen I and III protein in lysates of 3T3-L1 preadipocytes or adipocytes.

The collagen I and III protein that is measured from the cell lysates is thought to be mostly intracellular, rather than from the extracellular matrix. This is because the extraction of extracellular matrix proteins, including collagen, is difficult due to their large size and insolubility, thus requiring stringent conditions for isolation (95-97). Although a small amount of extracellular matrix collagen I and III protein could have been scraped of the dish, most of collagen I and III protein I detected from the cell lysates is likely to be intracellular.

The synthesis of mature fibrillar collagens is achieved by the N and C-terminal cleaving of procollagen, which occurs in the extracellular matrix (54). Since most of the collagen I and III protein detected in cell lysates is expected to be intracellular, the collagen I and III detected is also expected to be mostly procollagen. Examining the immunoblots of collagen I and III protein in pLXSN and pLXSN-ACLP cell lysates, the most prominent bands are the three bands of ~95kDa, ~130kDa and ~200kDa. There is no consensus in the literature on the size at which the alpha chains of collagen I and III run on an SDS-PAGE gel (98-100), so further experiments need to be done to identify the three bands I obtained for collagen I and III.

The expression of collagen I and III mRNA decreases with 3T3-L1 adipogenesis (60-62). However, there are no reports in the literature showing the expression of collagen I and III protein in cell lysates during differentiation. The secretion of collagen I and III in the

medium during 3T3-L1 adipogenesis has been assessed in two studies. Whereas one study found that collagen I protein, in the medium of 3T3-L1 preadipocytes, is decreased consistently throughout the 10 day differentiation (94), another study found that it is initially downregulated (day 0-4) but then goes back up to preadipocyte level (day 4-12) (55). It is possible that post-translational regulations occur, so that mRNA levels of collagen I and III do not correspond to the protein levels produced. Similarly, it is possible that preadipocytes secrete much more collagen I and III than adipocytes, but the amount of intracellular collagen I and III protein remains constant. It would be interesting to see whether the secretion of collagen I and III in the medium of my model of 3T3-L1 preadipocyte changes with differentiation or with sustained ACLP overexpression.

Collagen and other members of the ECM are remodeled during adipogenesis. Degradation of early ECM proteins to allow for the formation of new proteins is performed by extracellular degrading enzymes. MMP-2 and MMP-9 are extracellular degrading enzymes that preferentially degrade collagen. The inhibition of MMP-2 and MMP-9 has been shown to be detrimental for murine 3T3-F442A (101) and 3T3-L1 adipogenesis (102). I assessed the effect of ACLP overexpression on the gelatinase activity of MMP-2 and MMP-9. The gelatinase activity of MMP-2 and MMP-9 was decreased during pLXSN preadipocyte differentiation (Fig. 10). The same amount of gelatinase activity was noted during the differentiation of pLXSN-ACLP preadipocytes, demonstrating that sustained ACLP overexpression does not significantly influence the gelatinase activity of MMP-2 or MMP-9 in 3T3-L1 preadipocytes or adipocytes (Fig. 12).

My review of the literature reveals there is a lack of consensus regarding the expression and gelatinase activity of MMP-2 and MMP-9 during adipogenesis. One group

found that the mRNA expression, secretion and gelatinase activity of MMP-2 and MMP-9 is increased with 3T3-F442A adipogenesis (101). In contrast, another group found that the gelatinase activity of MMP-2 and MMP-9 is unchanged with 3T3-L1 adipogenesis (102). A third group found that the mRNA expression of MMP-2 and MMP-9 is decreased with 3T3-L1 adipogenesis (103). The decrease in gelatinase activity of MMP-2 and MMP-9 that I observed during 3T3-L1 adipogenesis could be due to the difference in time of exposure to serum-free conditions before medium was collected. I exposed my cells to serum-free conditions for 3 hours, whereas other groups exposed their cells to serum-free condition for 9-24 hours.

Sustained ACLP overexpression inhibits adipogenic signaling on collagen-I coated dishes

Sustained ACLP overexpression did not have an affect on the differentiation of 3T3-L1 preadipocytes on standard dishes. However, it is possible that any effect that ACLP has on adipogenesis is mediated by another protein. Chun's group attempted to determine the role of a matrix metalloproteinase, MT1-MMP, on adipogenesis by engineering a MT1-MMP knockout and looking at the corresponding primary isolated preadipocytes. No affect on adipogenesis was noted when MT1-MMP knockout preadipocytes were grown on standard dishes. However, when MT1-MMP knockout adipocytes were grown on a 3D collagen-I matrix, Chun's group saw a significant inhibition of adipogenesis (104). Since ACLP may interact with collagen, it is possible that a collagen-I environment is needed to mediate the effect of sustained ACLP overexpression on 3T3-L1 adipogenesis.

Growth and differentiation of pLXSN and pLXSN-ACLP preadipocyte was induced as before, but on collagen-I coated dishes instead of standard dishes, and adipogenesis was assessed. In comparison to pLXSN adipocytes, pLXSN-ACLP adipocyte expression of FAS, C/EBPα, and PPARγ, was decreased significantly (Fig.15).

Since sustained ACLP expression in 3T3-L1 preadipocytes grown on collagen-I coated dishes inhibited the expression of differentiation markers, upstream signaling mechanisms were examined. During 3T3-L1 adipogenesis, the expression of C/EBPβ and C/EBPδ occurs within the first hour of adipogenic induction and in turn activate the expression of C/EBPα and PPARγ (105). It would be interesting to see whether the inhibited expression of C/EBPα and PPARγ is the result of inhibited expression of C/EBPβ and C/EBPδ.

Insulin signaling has also been shown to activate adipogenesis, including the expression of adipogenic genes (105,106). Hence, the effect of ACLP overexpression on insulin signaling in 3T3-L1 preadipocytes grown on collagen-I coated dishes was assessed. It was determined that ACLP overexpression in 3T3-L1 preadipocytes grown on collagen-I coated dishes does not affect the phosphorylation of IRS1 or ERK1/2, but significantly inhibits the phosphorylation of Akt (Fig.16). The Akt pathway of insulin signaling involves the activation of number of proteins/phospholipids between IRS-1 and Akt (37). It is possible that the effect of sustained ACLP overexpression on collagen-I coated dishes acts downstream of IRS1 but upstream of Akt, by affecting the activation of PI3-kinase. It would be interesting to see whether sustained ACLP overexpression on collagen-I coated dishes inhibits the activation of PI 3-kinase, and this results in the inhibited phosphorylation of Akt.

Insulin stimulated phosphorylation of Akt leads to phosphorylation of a number of target proteins. Two targets of Akt are Ser253 of FOXO1 (Forkhead box O 1) proteins and Ser401 of GATA2 (GATA binding protein 2). Both FOXO1 and GATA2 are inhibitors of adipogenesis, and the serine phosphorylation by Akt inactivates them, promoting adipogenesis including the expression of key differentiation markers (106-109). It is possible that the inhibition of insulin stimulated Akt phosphorylation inhibited phosphorylation of FOXO1 and GATA2, preventing their inactivation and therefore hindering adipogenesis and the expression of the adipogenic genes that were assessed.

Sustained ACLP overexpression in 3T3-L1 preadipocytes grown on standard dishes did not affect adipogenesis, but when they were grown on collagen-I coated dishes I noted an inhibition in the expression of differentiation markers and insulin stimulated Akt phosphorylation. ACLP may interact with collagen-I on collagen-I coated dishes, which in turn inhibits intracellular insulin-stimulated Akt signaling. Collagen-I can alter intracellular signaling through a number of membrane receptors including integrins, discoidin domain receptors (DDRs), glycoprotein IV, LAIR-1, manose receptor family and leukocyte-associated IG-like reccptor-1. Most effects of collagen through these membrane receptor are specific to the immune system, with the exception of DDR's and integrins. Thus, DDR's and integrins are the most likely candidates for ACLP - collagen-I mediated intracellular signaling (58).

DDR's are a family of tyrosine kinases receptors composed of two members, DDR1 and DDR2. DDR1 and DDR2 are both activated by collagen-I (60-62). DDR activation results in phosphorylation of proteins that contain Src homology 2 (SH2) and phosphotyrosine binding (PTB) such as ShcA, Shp-2 and Nck2 (111-114). Further

downstream targets have not been identified, but a PTB domain is present in IRS1 so it is possible that DDR can phosphorylate IRS1. I determined that sustained ACLP expression on collagen-I coated dishes does not effect IRS1 phosphorylation, so the possible collagen-DDR phosphorylation of IRS1 does not fit in my model.

Integrins are the second group of collagen membrane receptors whose effect is not specific to the immune system. There are 4 integrins that have been shown to interact with collagen, namely integrin $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$. Integrins $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$are recently discovered so their role in collagen - integrin signaling has not been well assessed (115). From the two remaining integrins, integrin $\alpha_1\beta_1$ has been shown to preferentially interact with basement membrane collagens, while integrin $\alpha_2\beta_1$ has been shown to preferentially interact with fibrillar collagens including collagen-I (116-118). β_1 domain of integrins has been shown to selectively regulate Akt/PKB signaling via PI3-kinase (119). The effect of collagen - integrin $\alpha_2\beta_1$1 on adipogenic signaling has not been examined, but on the human osteosarcoma Saos-2 cells it has been shown to affect p38 MAPK signaling (120), as well as to inhibit phosphorylation of Akt through protein serine/threonine phosphatase 2A (PP2A) (121). I determined that sustained ACLP expression on collagen-I coated dishes inhibits Akt phosphorylation, so the possible collagen-1 - integrin $\alpha_2\beta_1$ inhibiton of Akt phosphorylation is consistent with my model.

Sustained ACLP overexpression does not affect the morphology or TG levels of 3T3-L1 adipocytes differentiated on collagen-I coated dishes

Sustained ACLP overexpression in 3T3-L1 preadipocytes differentiated on collagen-I dishes showed an inhibition of differentiation markers (Fig.15), but no effect on

morphology (Fig.13) or TG accumulation (Fig.14). The expression of FAS, the enzyme that catalyzes the synthesis of fatty acids (40) is expected to affect lipid accumulation. A decrease in the expression of FAS mRNA, using siRNA as well as pharmacological FAS inhibitors, was shown to inhibit lipid droplet accumulation during the differentiation of 3T3-L1 preadipocytes (122, 123). In my study the sustained ACLP overexpression in 3T3-L1 preadipocytes grown on collagen-I coated dishes decreased the expression of FAS (Fig.13) but did not affect cell morphology (Fig.11) or TG accumulation (Fig.12).

Though I expected inhibited expression of differentiation markers to be coupled to inhibited lipid accumulation and change in morphology, this was not the case. Although sustained ACLP overexpression in 3T3-L1 preadipocytes differentiated on collagen-I coated dishes inhibited the expression of differentiation markers, it is possible that the expression of these differentiation markers was still at a sufficient level to induce normal levels of TG synthesis.

The decreased expression of differentiation markers in pLXSN-ACLP adipocytes compared to pLXSN adipocytes might have caused decreased TG accumulation on days 4-7 of differentiation, due to slower rate of TG accumulation. However, while pLXSN adipocytes would have accumulated maximal TG levels on days 4-7 of differentiation, pLXSN-ACLP adipocytes would have continued to accumulate TG until day 8 of differentiation. Hence, on day 8 of differentiation TG levels would be equal for pLXSN and pLXSN-ACLP adipocytes. This could be analyzed by assessing the level of TG accumulation of pLXSN and pLXSN-ACLP preadipocytes on days 4, and 6, as well as day 8 of differentiation.

Finally, culturing the preadipocytes on a 3D collagen-I matrix might result in a more potent inhibitory effect of ACLP overexpression, compared to the collagen-I coated dishes used here.

The effect of sustained ACLP overexpression on 3T3-L1 adipocyte functions was further analyzed. It would have been interesting to see whether these adipocyte functions were affected by sustained ACLP expression in 3T3-L1 preadipocytes grown on collagen-I coated dishes. Even though no effect was seen with sustained ACLP overexpression on differentiation induced 3T3-L1 TG synthesis or morphology, it is possible that the inhibition of differentiation markers had an effect on mature adipocyte functions. However, initial experiments were performed on standard dishes. Looking at the different adipocyte functions, it was determined that sustained ACLP overexpression does not affect the breakdown of TG into glycerol and fatty acids (lipolysis) (Fig.17), insulin stimulated glucose uptake (Fig.18) or insulin signaling of mature adipocytes (Fig.19).

The effect of collagen-I coated dishes on 3T3-L1 adipogenesis

The assessment of empty vector 3T3-L1 preadipocytes (pLXSN) adipogenesis on collagen-I coated dishes, showed a significant upregulation in the expression of differentiation markers (Fig.15). However, collagen-I coated dishes had no effect on the pLXSN morphology (Fig.13) or TG accumulation (Fig.14).

The effect of collagen-I coating on 3T3-L1 adipogenesis, and especially on the expression of the key differentiation markers during adipogenesis has not been assessed in other studies. A few studies examining the effect of collagen-I coating on adipogenesis have been done on primary stromal vascular (SV) cells, which contain preadipocytes as well as

blood and endothelial cells. It was found that differentiating SV cells on collagen-I coated dishes does not affect their morphology (124), adipocyte and lipid droplet number (125), or total amount of lipid staining by Oil Red O (126). The differentiation of 3T3-F442A cells on collagen-I coated dishes did not cause a significant change in lipids stained by Oil Red O (127) but an increase in the activities of two enzymes involved in adipogenesis, lipoprotein lipase and glycerophosphate dehydrogenase (128). Thus, other than my study, there is at least one study showing that collagen-I coating enhances the expression of adipogenic genes (128) but does not affect lipid levels (124-126, 127).

It was not determined whether collagen-I coating enhances Akt phosphorylation. Since inhibited expression of differentiation markers with ACLP overexpression in 3T3-L1 preadipocytes is coupled to inhibited insulin signaling, it is possible that enhanced expression of key differentiation markers with collagen-I coating is coupled to enhanced insulin signaling. As previously mentioned, DDR has been shown to phosphorylate proteins that contain PTB domains. Hence, it is possible that collagen-I coating on pLXSN preadipocytes interacts with DDR to induce insulin stimulated IRS1 phosphorylation through IRS1 PTB domain. This would lead to enhanced pro-adipogenic insulin signaling and in turn enhance the expression of key differentiation markers.

Conclusion/Proposed model

Sustained ACLP overexpression on collagen-I coated dishes inhibits the expression of differentiation markers, C/EBPα, PPARγ, and FAS, as well as insulin stimulated Akt phosphorylation. The mechanism has not been investigated but it can be suggested based on current literature in the model I propose.

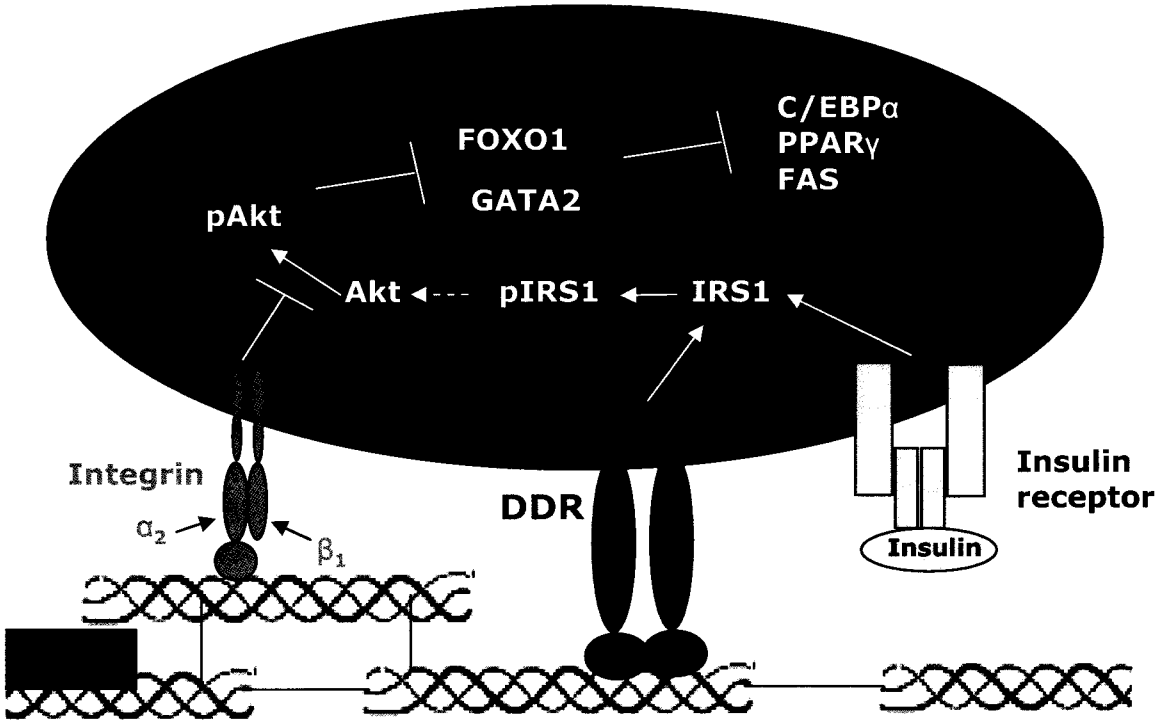
In pLXSN preadipocytes, collagen-I from collagen-I coated dishes interacts with DDR and integrins. Interaction of collagen-I with DDR occurs through DDR's discoidin domain, inducing DDR to phosphorylate the PTB domain of IRS1. This leads to enhanced insulin signaling by sequential activation of PI3-kinase and Akt. Enhanced insulin signaling, in turn inactivates FOXO1 and GATA2, activating the expression of C/EBPα, PPARγ, and FAS. Additionally, interaction of collagen-I with integrin $\alpha_2\beta_1$ leads to an inhibition of insulin-stimulated Akt phosphorylation, and inhibited expression of C/EBPα, PPARγ, and FAS. For an unknown reason, the interaction of collagen with DDR prevails over the interaction of collagen with integrin $\alpha_2\beta_1$1 in pLXSN preadipocytes (Fig.20).

In pLXSN-ACLP preadipocytes, the overexpression of ACLP leads to competition of ACLP with DDR for discoidin domain binding spot of collagen-I. Hence, the collagen-I - DDR interaction is disrupted, thus hindering the collagen-I - DDR pathway of insulin stimulated IRS1 phosphorylation and downstream enhancement of C/EBPα, PPARγ, and FAS. Since integrin $\alpha_2\beta_1$ does not contain a discoidin domain, it is possible that it binds to a different region of collagen-I than ACLP does, allowing collagen-I to bind to both overexpressed ACLP and integrin $\alpha_2\beta_1$. This allows for collagen – integrin $\alpha_2\beta_1$1 interaction to predominate over the collagen – DDR interaction. The collagen – integrin $\alpha_2\beta_1$1 interaction leads to an inhibition of insulin stimulated Akt phosphorylation. Inhibited insulin signaling, in turn prevents the inactivation of FOXO1 and GATA2, which inhibit the normal expression of C/EBPα, PPARγ, and FAS (Fig.21).

Figure 20: Hypothetical model for the mechanism by which collagen-I coated dishes enhance the expression of differentiation markers in pLXSN adipocytes. In 3T3-L1 preadipocytes that stably express the empty pLXSN vector, collagen-I from collagen-I coated dishes interacts with DDR and integrins. Interaction of collagen-I leads to enhanced expression of C/EBPα, PPARγ, and FAS. Interaction of collagen-I with integrin $\alpha_2\beta_1$ leads to an inhibited expression of C/EBPα, PPARγ, and FAS. For an unknown reason, the interaction of collagen-I with DDR prevails over the interaction of collagen with integrin $\alpha_2\beta_1$ in pLXSN preadipocytes, leading to enhanced expression of C/EBPα, PPARγ, and FAS, in the cell.

Hypothetical model

pLXSN preadipocytes



Collagen-I coated dish

Vogel, et al. 2006. *Cell Signalling.* 18:1108-1116.

Figure 21: Hypothetical model for the mechanism by which sustained ACLP overexpression in 3T3-L1 preadipocytes differentiated on collagen-I coated dishes reduces the expression of differentiation markers. In 3T3-L1 preadipocytes that stably overexpress ACLP (pLXSN-ACLP), collagen-I from collagen-I coated dishes interacts with DDR and integrins. Interaction of collagen-I leads to enhanced expression of C/EBPα, PPARγ, and FAS. Interaction of collagen - I with integrin $\alpha_2\beta_1$ leads to an inhibited expression of C/EBPα, PPARγ, and FAS. The discoidin domain of ACLP competes with that of DDR for collagen-I binding spot, reducing the total amount of DDR - collagen-I interactions. The interaction of collagen-I with integrin $\alpha_2\beta_1$ prevails due to a different binding spot from ACLP, leading to reduced expression of C/EBPα, PPARγ, and FAS, in the cell.

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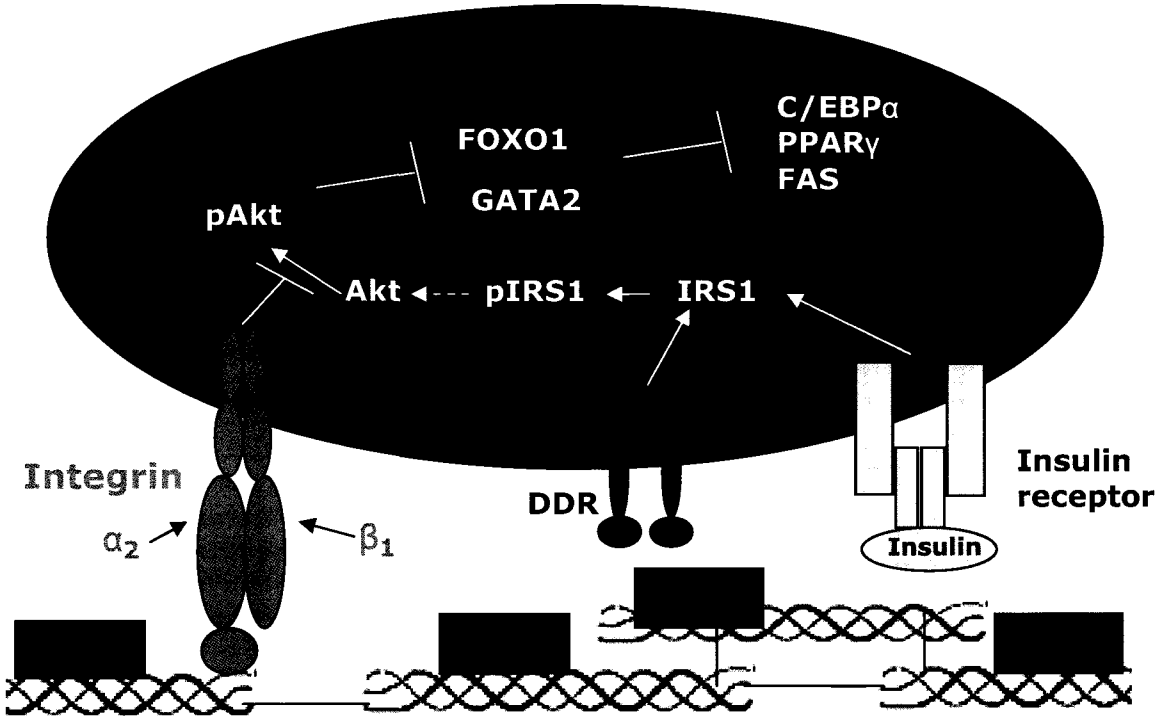
Hypothetical model

pLXSN-ACLP preadipocytes



Collagen-I coated dish

Vogel, et al. 2006. *Cell Signalling.* 18:1108-1116.

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The pLXSN and the pLXSN-ACLP murine constructs were obtained from Dr. Matthew Layne. Additionally, Anne Landry performed one of the three independent experiments for insulin-stimulated glucose uptake. Seham Rabba performed one of the two counts for the apoptosis study.

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CONFERENCES ATTENDED:

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