2013

The role of aortic carboxypeptidase-like protein in adipose-derived mesenchymal stem cell adipogenesis and fibrosis

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http://hdl.handle.net/2144/12193

Boston University
BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

THE ROLE OF AORTIC CARBOXYPEPTIDASE-LIKE PROTEIN IN ADIPOSE-
DERIVED MESENCHYMAL STEM CELL ADIPOGENESIS AND FIBROSIS

by

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Submitted in partial fulfillment of the
requirements for the degree of
Master of Arts
2013
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ACKNOWLEDGEMENTS

This thesis would not have been possible without the help of Dr. Matthew Layne, whose guidance has been invaluable to my research. I am also deeply indebted to my fellow lab mates, Mike Jager, Kathleen Tumelty, and Dahai Wang, for their unfailing patience and scientific advice. I am particularly grateful for Kathleen, whose gracious production of ACLP gave me the means to seek answers to my questions.
THE ROLE OF AORTIC CARBOXYPEPTIDASE-LIKE PROTEIN IN ADIPOSE-DERIVED MESENCHYMAL STEM CELL ADIPOGENESIS AND FIBROSIS

ANKUR PRASAD
Boston University School of Medicine, 2013
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ABSTRACT

The prevalence of obesity and obesity related diseases are increasing worldwide. Obesity is characterized by the pathological expansion of white adipose tissue. Previous studies on white adipose tissue of obese individuals have detected inflammation and fibrosis. These conditions may cause dysregulation of the tissue, leading to negative outcomes, including type II diabetes and metabolic syndrome.

Aortic carboxypeptidase-like protein (ACLP) is a secreted extracellular matrix protein that is upregulated in fibrotic lung tissue. Importantly ACLP knockout mice are protected from experimentally induced lung fibrosis. ACLP is expressed in adipose tissue and is downregulated as stem cells undergo adipogenesis. Its overexpression increases α smooth muscle actin expression
and impairs adipogenesis in preadipocyte lines; however, its role in white adipose tissue fibrosis has not been fully explored.

The studies presented in this thesis aimed to investigate the hypothesis that ACLP overexpression in fibrotic white adipose tissue would promote a fibroblast to myofibroblast transition and repress adipogenesis. To determine if ACLP promotes a fibroblast to myofibroblast transition, we tested the capacity of ACLP to induce α smooth muscle actin and collagen I protein expression and increase contractility of primary stromal vascular cells. To assess the effects of ACLP on adipogenesis, we tested the ability of 10T1/2 fibroblasts and stromal vascular cells to undergo adipogenesis in collagen I gels under ACLP treatment.

Results presented herein demonstrate ACLP is a potent inhibitor of adipogenesis and induces an upward trend in myofibroblast proteins and RNA expression. Significantly, these studies used murine adipose-derived cells to show the effects of ACLP, suggesting these results might be reflected in adipose tissue. These experiments support a model where ACLP potentiates adipose tissue fibrosis by inhibiting adipogenesis, resulting in fewer developing adipocytes, and stimulating myofibroblast differentiation, resulting in further collagen deposition and tissue compaction. This contribution to adipose tissue dysfunction also gives ACLP a possible role in the development of obesity related diseases, including diabetes and metabolic syndrome, identifying it as a possible target for therapeutics.
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<tbody>
<tr>
<td>αSMA</td>
<td>α Smooth Muscle Actin</td>
</tr>
<tr>
<td>ACLP</td>
<td>Aortic Carboxypeptidase-Like Protein</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CAAT/Enhancer-Binding Protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMI</td>
<td>0.25 µM Dexamethasone, 0.5 mM IBMX, and 8 nM Insulin in 10% FBS DMEM</td>
</tr>
<tr>
<td>DMII</td>
<td>5 nM Dexamethasone, 0.5 mM IBMX, 4 nM Insulin, and 12.5 µM Indomethacin in 10% FBS DMEM</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>FABP4</td>
<td>Fatty Acid Binding Protein 4</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator-Activated Receptor</td>
</tr>
<tr>
<td>PSG</td>
<td>100 U/ml Penicillin, 100 µg/ml Streptomycin, and 2 mM L-Glutamine</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real Time-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted Protein Acidic and Rich in Cysteine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SVF</td>
<td>Stromal Vascular Fraction</td>
</tr>
<tr>
<td>TBST</td>
<td>25 mM Tris pH 8, 125 mM Sodium Chloride, 0.1% Tween 20</td>
</tr>
<tr>
<td>TBST-Milk</td>
<td>TBST and 4% Non-Fat Dry Milk</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>WAT</td>
<td>White Adipose Tissue</td>
</tr>
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</table>
INTRODUCTION

Obesity and Metabolic Syndrome

Obesity is a disease whose global prevalence is increasing at an alarming rate (Ogden et al., 2006; Rennie and Jebb, 2005; Svacina, 2012). White adipose tissue (WAT) plays a central role in the development of obesity. WAT acts as a repository for the storage of lipids and expands when there is a positive energy balance, i.e. when energy intake exceeds energy expenditure (Sethi and Vidal-Puig, 2007). Obesity represents a state of prolonged positive energy balance resulting in pathological adipose tissue expansion, medically defined as a body mass index of 30 or higher (Flegal et al., 2013).

In obesity, WAT displays increased inflammation and fibrosis, resulting in dysregulation of the tissue (Trayhurn, 2013). These factors contribute to the development of metabolic syndrome, a grouping of several metabolic abnormalities, including high blood pressure, elevated triglycerides, and insulin insensitivity, which together increase the risk of cardiovascular disease and diabetes (Gorden et al., 2012; Maury and Brichard, 2010; Safar et al., 2013).

While the regulatory mechanisms of WAT are becoming better understood, there are still many unanswered questions about its function in fibrosis and disease. In order to understand the role of WAT in the development of obesity related pathological conditions, it is necessary to understand normal WAT function and its dysregulation in obesity.
Adipose Tissue

WAT is composed of numerous cell types, including adipocytes, fibroblasts, and endothelial and smooth muscle cells of blood vessels. Adipocytes are differentiated cells that are responsible for the lipid storage and metabolic activity of WAT. They contain large lipid droplets that fill the majority of the cell, with a smaller volume occupied by the nucleus, mitochondria, and other essential cell components. These cells are potent regulators of energy homeostasis in the body. In times when energy intake is greater than energy expenditure, adipocytes store lipids for future use. In times of energy deficit, intracellular lipids are catabolized and released for use by other organs (Wronska and Kmiec, 2012). Through secretion of hormones (adipokines), including leptin and adiponectin, adipocytes also have an anti-inflammatory role and regulate both appetite and angiogenesis (Adya et al., 2012; Campfield et al., 1996).

The other cells of adipose tissue, which can be separated from adipocytes for further study, are designated the stromal-vascular fraction (SVF). The SVF includes a multitude of cell types that together support adipocytes. Among the many cell types are endothelial cells, fibroblasts, smooth muscle cells, immune cells, and stem cells (Peinado et al., 2012). Endothelial cells line blood vessels that carry oxygen and nutrients to adipocytes (Ouchi et al., 2011); immune cells defend against pathogens and clear dead cell debris (Cinti et al., 2005); and stem cells, given the proper stimulus, can differentiate into adipocytes (Ong and Sugii, 2013; Zuk et al., 2001, 2002).
**Adipogenesis**

Adipogenesis is a complex process that is still not clearly understood. In general, fibroblastic stem cells change from their spindly fibroblastic shape to a spherical one and accumulate lipid droplets. The origin of the mesenchymal stem cell (MSC) that gives rise to the preadipocyte may be from vascular pericytes in adipose tissue (Tang et al., 2008). However, it should be noted that not all pericyte-derived MSCs differentiate into adipocytes (Diaz-Flores et al., 1990; Diaz-Flores et al., 1991, 1992). Depending on tissue localization, molecular cues, and transcriptional regulators, MSCs can differentiate into a variety of other cell types (Collett and Canfield, 2005; Paul et al., 2012).

Adipogenic induction is still not fully understood; however, two transcriptional factors, CAAT/enhancer-binding protein (C/EBP)α and peroxisome proliferator-activated receptor (PPAR)γ are generally considered to be master regulators of the adipogenic program (Lane et al., 1996; Tontonoz et al., 1995). During adipogenesis, several genes are differentially regulated as part of a transcriptional regulatory cascade; however, induction of C/EBPs and PPARγ by upstream stimulators of adipogenesis occurs relatively early in the process (Brun et al., 1996).

C/EBPα is a transcriptional factor that has been widely studied for its role in adipogenesis. It is not an adipocyte specific protein; however, it is significantly upregulated just before many adipocyte specific genes are activated (Brun et al., 1996; Mandrup and Lane, 1997). It forms homo- or heterodimers and binds to the
promoters of essential adipocyte genes, including those that code for the insulin receptor and the insulin-regulated glucose transporter, to induce their expression (Kaestner et al., 1990; McKeon and Pham, 1991). C/EBPα null mice fail to develop WAT (Wang et al., 1995), showing C/EBPα to be necessary for the development of mature adipocytes.

Expression of the transcription factor PPARγ is dramatically increased prior to the induction of several adipocyte-specific genes (Tontonoz et al., 1994). Its expression is required for the differentiation of adipocytes in vitro and in vivo (Barak et al., 1999; Rosen et al., 1999, 2002). PPARγ heterodimerizes with retinoic acid receptors or retinoid X receptors which in turn can bind DNA sequences termed PPAR response elements (Schoonjans et al., 1996a). PPARγ responds to a variety of ligands, including fatty acids and eicosanoids (Yu et al., 1995), and once activated, induces several important adipocyte genes such as FABP4 that codes for fatty acid binding protein 4 (FABP4) (Tontonoz et al., 1994) and LPL, that codes for lipoprotein lipase (Schoonjans et al., 1996b). PPARγ is a potent stimulator of adipogenesis and is sufficient to induce transdifferentiation of other cell types, including myoblasts, into adipocytes (Teboul et al., 1995).

Fully differentiated adipocytes express and secrete a variety of components, including those essential for normal adipocyte function and energy homeostasis (Wronska and Kmiec, 2012). Adiponectin is a protein expressed and secreted by adipocytes (Hu et al., 1996; Scherer et al., 1995). Elevated circulating adiponectin levels correlate with greater insulin sensitivity and lower
body mass index (Hu et al., 1996; Yamauchi et al., 2001). Adiponectin may act by increasing β-oxidation of fatty acids in various tissues, including muscle (Bruce et al., 2005; Fruebis et al., 2001; Yamauchi et al., 2001). Adiponectin knockout mice exhibit several phenotypes, including hypertension, hyperlipidemia, and reduced insulin sensitivity (Kubota et al., 2002; Maeda et al., 2002; Ouchi et al., 2003), highlighting adiponectin secretion as an important function of the mature adipocyte.

FABP4 is an intracellular lipid binding protein. FABP4 complexes with hormone-sensitive lipase (Gillilan et al., 2007; Smith et al., 2007) and acts as an intracellular fatty acid trafficking protein. Mice with FABP4 null mutations show decreased lipolytic activity and increased intracellular lipid accumulation (Coe et al., 1999; Hertzel et al., 2006); however, although they are still susceptible to diet-induced obesity, they do not develop the insulin resistance or cardiovascular disease normally associated with the obese phenotype (Hotamisligil et al., 1996; Layne et al., 2001a; Lehmann et al., 2004).

The development of mature adipocytes in WAT plays an important role in energy homeostasis and metabolic function. Therefore, disturbances in adipogenesis or WAT function by disease or injury can have severe consequences, including the development of cardiovascular disease and insulin insensitivity (Gorden et al., 2012; Safar et al., 2013).
Fibrosis

Tissue fibrosis is a condition characterized by the excessive deposition of extracellular matrix (ECM) proteins, such as collagens, by cells including fibroblasts and myofibroblasts. Buildup of fibrotic tissue in an organ can lead to dysfunction and ultimately, organ failure (Weber, 1997). Fibrosis is believed to be caused by chronic inflammatory responses induced by a variety of factors, such as chronic infections or tissue injury (Wynn, 2008; Wynn and Ramalingam, 2012). Hypoxia is among the causes of inflammation related fibrosis in adipose tissue (Halberg et al., 2009; Trayhurn, 2013). Hypoxia is a state in which cells or tissues are supplied with insufficient oxygen to meet their metabolic needs. In adipose tissue, this can occur when adipocytes grow rapidly in times of excessive energy intake (obesity) and the vasculature fails to supply the expanded tissue and cells with oxygen due to diffusion limits. Hypoxia can result in cell and tissue necrosis, attracting leukocytes in an inflammatory response (Halberg et al., 2009; Trayhurn, 2013). The inflammatory response results in the local release of several “profibrotic” factors, particularly transforming growth factor β (TGF-β), that, over time, result in tissue fibrosis (Wynn and Ramalingam, 2012).

Resident tissue fibroblasts can undergo a process called fibroblast to myofibroblast transition. Stimulated by growth factors, such as TGF-β, which is increased in obese murine adipose tissue (Samad et al., 1997), fibroblasts increase the expression of pro-contractile cytoskeletal elements, such as α
smooth muscle actin (αSMA), and the secretion of ECM proteins (Desmoulière et al., 1993; Shukla et al., 2013). In the context of wound healing, this activity helps to give structure and compact the damaged tissue, aiding the healing process (Hinz, 2010); however, improper regulation of this process can lead to an excessive amount of ECM deposition and non-functional tissue, i.e. fibrosis (Thiery, 2003).

**Adipose Tissue ECM**

Adipocyte growth, including during obesity, requires remodeling of the ECM through several pathways (Chavey et al., 2003; Maquoi et al., 2002). Knockdown of MT1-MMP, a membrane anchored collagenase, results in smaller, improperly differentiated, multi-locular adipocytes (Chun et al., 2006). Decreased collagen I in fat pads of secreted protein acidic and rich in cysteine (SPARC)-null mice results in increased adipocyte size and number without increased overall body mass (Bradshaw et al., 2003). These findings underscore the importance of ECM structure and remodeling in adipocyte formation and growth.

A recent study using collagen VI knockout mice demonstrated the effect of ECM proteins on adipocyte development and expansion (Khan et al., 2009). The study showed that without collagen VI, the fat pads of mice fed a high fat diet expanded, resulting in improved insulin sensitivity and reduced inflammation. Khan et al. proposed that the improved phenotype is an effect of the reduction in ECM rigidity through the knockout of an ECM constituent, suggesting that the
rigidity of the ECM restricts the expansion of adipocytes. The authors also suggest a possible mechanism for obesity related inflammation and fibrosis (Khan et al., 2009). Due to an excessive caloric intake and metabolic stress, adipocytes grow at a rapid rate. In response to the stress of such a rapid expansion in a restrictive ECM, increased necrosis of stressed adipocytes induces an immunologically stressed state in the tissue, resulting in adipose fibrosis. This suggests that the rigidity of the ECM may restrict rapidly growing adipocytes in obesity and may be a significant factor in promoting a fibrotic state.

The emerging role of the ECM in WAT fibrosis provides significant insight into the cause and mechanism of disease; however, further understanding of how adipocytes and cells of the SVF interact with the components of the ECM is still necessary.

**Aortic Carboxypeptidase-Like Protein (ACLP)**

ACLP is a secreted protein consisting of a signal peptide, lysine and proline rich extensin domain, a discoidin domain, and a catalytically inactive metallocarboxypeptidase domain at its C-terminus (Layne et al., 1998). The functions of most of the domains are not yet clearly understood; however, the discoidin domain has been shown to associate with the ECM (Schissel et al., 2009), most likely through collagen binding. ACLP expression was originally noted in the vascular smooth muscle cells of adult murine aorta (Layne et al., 1998). ACLP is important for organogenesis and murine fibroblasts lacking ACLP
are less proliferative and correlated with deficient wound healing (Layne et al., 2001b). These findings support a role for ACLP in fibroblast proliferation and differentiation into active myofibroblasts. This fibroblast to myofibroblast transition is a classic characteristic of fibrotic tissue and indicative of a possible role for ACLP in tissue fibrosis. ACLP is highly upregulated in bleomycin-induced lung fibrosis (Schissel et al., 2009) and is histologically associated with areas expressing significant amounts of collagen. ACLP knockout mice exhibited a 4.8 fold decrease in αSMA staining, greatly decreased collagen staining, and 28% less hydroxyproline content (an indicator of collagen content). This indicates that ACLP knockout mice were protected from severe fibrosis and myofibroblast accumulation, supporting the proposed role of ACLP in aggravating tissue fibrosis.

ACLP is expressed at low levels in adipose tissue, primarily in the SVF (Abderrahim-Ferkoune et al., 2004). Given the evidence that ACLP is secreted from the micro-vasculature (Hocking et al., 2010) and adipocyte precursors may arise from pericytes (Tang et al., 2008), it is possible that ACLP interacts with these precursors, either at the pericyte level or as MSCs in the stroma, and may be part of the adipogenic regulatory mechanism.

*In vitro* studies using 3T3 preadipocytes have shown that ACLP expression is regulated during the adipogenic process (Gagnon et al., 2002, 2005; Rodriguez et al., 2004), such that it is downregulated in the early stages of normal 3T3 preadipocyte differentiation. However, if ACLP is overexpressed in
3T3 cells, adipogenesis is inhibited and a transdifferentiation to smooth muscle-like cells is induced (Abderrahim-Ferkoune et al., 2004).

**Cell Culture Substrata and 3 Dimensional Cell Culture**

Traditional plastic culture is useful for creating *in vitro* models to study cellular processes and interactions; however, it has its limitations. Growing cells on plastic can induce morphological changes and alter protein expression patterns (Lee et al., 2013; Petersen et al., 1992). This can generate significant differences in the way cells behave compared to their true physiological behavior due to the altered stiffness of the environment. For example, 3T3-L1 adipocytes show reduced insulin sensitivity on culture surfaces with greater rigidity than that of adipose tissue (Li et al., 2009). Many studies have shown advantages to using ECM proteins to support adipogenesis, such as increases in the number and size of differentiating cells (Hausman et al., 1996; Hilliou et al., 1988; O’Connor et al., 2003).

Additionally, in cell culture conditions where cells are plated on top of any culture surface, cells will attach only in a single layer (monolayer). Such a model deprives cells of many possible 3-dimensional ECM contacts formed in tissue. This is particularly problematic for cells, such as adipocytes, that require the structural support and modification of an ECM for proper differentiation or growth, (Chun et al., 2006; Stacey et al., 2009) and for proteins that interact with the ECM, such as ACLP (Schissel et al., 2009). To address this problem, a 3-
dimensional model can be used. For example, a native conformation collagen I gel can be used as a cellular lattice, thereby generating a simplified ECM. Embedding cells within such a matrix allows both cells and proteins that interact with the ECM to better mimic their in vivo 3-dimensional environments (Cukierman et al., 2002; Griffith and Swartz, 2006).

**Goals of Thesis Research**

Considering the elevated expression of ACLP in lung fibrosis, a similar increase in ACLP expression could be expected in adipose tissue fibrosis. Given the structure of ACLP, the collagen binding discoidin region in particular, it could be expected to localize and bind to collagen rich regions throughout the fibrotic tissue and exert its effects. **The goal of the studies presented in this thesis is to test the hypothesis that ACLP overexpression in fibrotic WAT would promote a fibroblast to myofibroblast transition and repress adipogenesis.**
METHODS

Recombinant Expression of ACLP

Recombinant ACLP was prepared in our laboratory by Kathleen Tumelty. Briefly, 293 cells were stably transfected with a plasmid encoding the BM40 signal peptide-mouse ACLP followed by a myc-His tag. Cells were grown in SFM4HEK293 media. The spent medium was centrifuged at 3,220xg for 15 minutes and the supernatant was retained and dialyzed using 100,000 molecular weight cutoff dialysis tubing for 36 hours into dialysis buffer (300 mM KCl, 50 mM KH₂PO₄, pH 8). Protein was purified using chromatography by passing dialyzed media through a Bio-Scale Mini Profinity IMAC Cartridge (Bio-Rad) using a Bio-Rad Duo Flow and washed extensively using a 100 mM sodium carbonate buffer (pH 10.8). Protein was eluted with a 250 mM imidazole elution buffer. Eluted protein was concentrated and dialyzed into Phosphate Buffered Saline with Calcium and Magnesium (Mediatech). Protein concentration was determined using a Bradford assay and purity was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Coomasie blue staining.

Cell Isolation and Culture

SVF cells were isolated from discarded inguinal and epididymal adipose depots from male and female C57BL/6 mice 6-16 weeks of age using the following procedure. Fat pads were removed, minced, and digested with 100
μg/ml dispase (Becton Dickinson), 1 mg/ml collagenase (Worthington Biochemical) and 3 U/ml DNase (Worthington Biochemical). The digestion cocktail was neutralized using Dulbecco's modified Eagle medium (DMEM, Mediatech) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (PSG, Mediatech), and 4% bovine serum albumin (American Bioanalytical). Digested tissue was passed through a 100 µm cell strainer and centrifuged at 200xg for 10 minutes. The SVF pellet was resuspended in DMEM supplemented with 10% FBS and PSG (10% FBS DMEM). SVF cells were cultured at low passage (3 or less) in 10% FBS DMEM. 10T1/2 cells (American Type Culture Collection) were cultured in 10% FBS DMEM. All experiments used 10% FBS DMEM unless otherwise indicated. All ACLP treatments used 30 nM recombinant ACLP and all TGF-β treatments used 1 nM recombinant human TGF-β (R&D Systems). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Cell Contraction Assay

Passaged SVF cells were grown in 100 mm diameter plastic cell culture dishes (BD Falcon) and treated with 10% FBS DMEM (Control), 30 nM ACLP in 10% FBS DMEM (ACLP), or 1 nM TGF-β in 10% FBS DMEM (TGF-β). After 3 days, cells were released from dishes using 0.25% trypsin EDTA (Mediatech) and counted using a hemocytometer. Cell suspensions were pelleted by centrifugation at 200xg for 10 minutes, resuspended in DMEM supplemented
with 0.5% FBS and PSG, and mixed with rat tail Collagen I (BD) and Phosphate Buffered Saline (PBS, Mediatech) to achieve a final concentration of 400,000 cells/ml and 1 mg/ml Collagen I. Polymerization of the mixture was initiated using 23 µl 1N sodium hydroxide/ml volume of Collagen I used (regardless of concentration). The mixture was then plated into a 24-well cell culture plate (BD Falcon) in triplicate at 500 µl/well and allowed to set at 37°C for 30 minutes. Gels were covered with 10% FBS DMEM, gently separated from the sides of the well using a p200 pipet tip, and photographed at intervals. The area of wells and gels were measured using ImageJ. Percent contraction represents the difference between the area of the well and the area of the gel at the given time point.

10T1/2 Adipogenesis in Collagen Gels

10T1/2 cells were grown in 100 mm diameter plastic cell culture dishes in 10% FBS DMEM and treated at 80-90% confluence with 10% FBS DMEM (Control), 30 nM ACLP in 10% FBS DMEM (ACLP), and 1 nM TGF-β in 10% FBS DMEM (TGF-β). Media were changed daily for four days and cells reached confluence after two days. Cells were released from dishes using 0.25% trypsin EDTA and counted using a hemocytometer. The cell suspension was pelleted by centrifugation at 200xg for 10 minutes and resuspended in 0.5% FBS DMEM. The cell suspension was mixed with rat tail Collagen I and PBS to achieve a final concentration of 200,000 cells/ml and 1 mg/ml Collagen I. Polymerization and setting of the mixture was performed as described above. Cells were treated
immediately with an adipogenesis induction cocktail of 5 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 4 nM insulin, and 12.5 µM indomethacin in 10% FBS DMEM (DMII) and experimental treatments: DMII (Control), 30 nM ACLP in DMII (ACLP), and 1 nM TGF-β in DMII (TGF-β). Every two days post-induction, media were replaced with 1 nM insulin in 10% FBS DMEM (Control), 30 nM ACLP and 1 nM insulin in 10% FBS DMEM (ACLP), and 1 nM TGF-β and 1 nM insulin in 10% FBS DMEM (TGF-β). Protein samples were harvested 6 days post-induction.

**SVF Adipogenesis in Collagen Gels**

Isolated SVF cells were cultured in 150 mm diameter plastic cell culture dishes in 10% FBS DMEM for 2 days. Cells were released from dishes using 0.25% Trypsin EDTA, counted, and set in collagen I gels as described above at a final concentration of 200,000 cells/ml and 1 mg/ml Collagen I. Cells were treated immediately with an adipogenesis induction cocktail of 0.25 µM dexamethasone, 0.5 mM IBMX, and 8 nM insulin in 10% FBS DMEM (DMI) and experimental treatments: DMI (Control) and 30 nM ACLP in DMI. Two days post-induction, media were replaced with 1 nM insulin in 10% FBS DMEM (Control) and 30 nM ACLP and 1 nM insulin in 10% FBS DMEM (ACLP). RNA samples were harvested four days post-induction.
Protein Isolation and Western Blot Analysis

For protein lysates from cells cultured on plastic, cells were washed twice with ice cold PBS and scraped on ice in Western extraction buffer (25 mM Tris pH 7.4, 50 mM sodium chloride, 0.5% sodium-deoxycholate, 2% IGEPAL CA-630 octylphenyl-polyethylene glycol, 0.2% sodium dodecyl sulfate) with cOmplete mini protease and PhosSTOP phosphatase inhibitors (Roche). Lysates were clarified by centrifugation at 12,000xg for 10 minutes at 4°C.

For cell lysates from cells embedded in gel, gels were washed twice with ice cold PBS, released from wells and placed in Pierce snap-cap spin columns (Thermo Scientific). Columns were filled with ice cold PBS and spun at 100xg for 30 seconds repeatedly while discarding the flow through until it no longer changed color (usually within 3-5 washes). Western extraction buffer was added to the columns and the columns were sealed using Parafilm “M” (Pechiney). Columns were subjected to nutation overnight at 4°C then centrifuged at 6,000xg for 1 minute to collect extracted cell lysates.

Quantification of protein was performed using the Pierce BCA Protein Assay Kit (Thermo Scientific), comparing cell lysates to a standard curve. Total protein from lysates was resolved on 4%-20% tris-glycine gels (Biorad) and transferred to a Protran nitrocellulose membrane (Whatman). Membranes were blocked using a solution of TBST (25 mM tris pH 8, 125 mM sodium chloride, 0.1% tween 20) and 4% non-fat dry milk (LabScientific) (TBST-Milk). Membranes were incubated in primary antibodies overnight at 4°C. Primary antibodies used
include rabbit polyclonal ACLP antibody diluted 1:4000 (Layne et al., 1998), rabbit collagen I antibody (Rockland) diluted 1:4000, murine αSMA antibody (Sigma) diluted 1:2000, murine pan-actin antibody (Millipore) diluted 1:4000, rabbit adiponectin antibody (Affinity BioReagents) diluted 1:3000, rabbit FABP4 antibody (Cell Signaling Technologies) diluted 1:1000, and rabbit PPARγ antibody (SantaCruz) diluted 1:200. After primary antibody incubation, membranes were washed briefly in TBST-Milk and incubated in horseradish peroxidase (HRP)-linked secondary antibodies (General Electric Healthcare) diluted 1:2500 for one hour. Membranes were washed briefly and secondary antibody was visualized using SuperSignal West Dura Extended Substrate (Thermo Scientific) or SuperSignal West Femto (Thermo Scientific) and imaged on a ChemiDoc XRS+ System (Bio-Rad). Some membranes were stripped following visualization using Western Stripping Buffer (Thermo Scientific) to allow for further blotting. All washes and incubations were performed with constant shaking.

**RNA Isolation, Reverse Transcription, and Real Time-Polymerase Chain Reaction (RT-PCR) Analysis**

RNA was isolated from SVF cells in collagen I gels using the RNeasy Mini Kit (Qiagen). In general, gels were washed twice with ice cold PBS, released from the wells, and dissolved in the RNA extraction buffer by overnight nutation at 4°C. RNA was isolated using the protocol for isolation of RNA from mammalian
cells as per manufacturer’s instruction. RNA was quantified using the Take3 plate and Gen5 System (Biotek) as per manufacturer’s instruction. The Fermentas Maxima Reverse Transcriptase Kit (Thermo Scientific) and the Fermentas Maxima Probe Kit (Thermo Scientific) were used to reverse transcribe and amplify 500 ng of RNA as per manufacturer’s instruction. Primers used are indicated in Table 1. Samples were run on a 7300 Real Time PCR System (Applied Biosystems). Analysis was done using the delta-delta C$_t$ method.

**Statistical Analysis**

Data are represented as mean ± standard deviation. Statistical significance was determined using either a two-way analysis of variance or two-tailed Student’s t-test as indicated and defined p<0.05 as significant.
### Table 1. Primers Used for RT-PCR

Primers used for RT-PCR were obtained from Invitrogen

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s</td>
<td>CGGCTACCACATCCAAGGAA</td>
<td>TTTTCGTCACTACCTCCCCCG</td>
</tr>
<tr>
<td>Col1a2</td>
<td>GTAACCTTCGTGCCTAGCAACA</td>
<td>CTTTTGTCAGAATACTGAGCAGC</td>
</tr>
<tr>
<td>Col6a1</td>
<td>GATGAGGGTGAAAGTGGGAGA</td>
<td>CAGCAGGAGGGATGTCAAA</td>
</tr>
<tr>
<td>Cebpa</td>
<td>AAAGCCAAAGAAGTCGGTGACGA</td>
<td>CTTCTCTTGTCGTCTCCACGTT</td>
</tr>
<tr>
<td>FABP4</td>
<td>AAGGTGAAGAGCATCATAACCTCCT</td>
<td>TCACGGCTTTTCATAACATTTCC</td>
</tr>
<tr>
<td>Pparg</td>
<td>GCATGCTGCTCCCTCGCTGA</td>
<td>TGGCATCTCTGTGTCAACCATG</td>
</tr>
<tr>
<td>Acta2</td>
<td>TGACGCTGAAGTATCCGATAGA</td>
<td>GTACGTCCAGAGGCATAGG</td>
</tr>
</tbody>
</table>
RESULTS

**ACLP Increases Myofibroblast Protein Expression in SVF Cells**

The fibroblast to myofibroblast transition is characterized by several changes in protein expression, most notably the induction of cytoskeletal and ECM protein expression, particularly αSMA and collagen I (Liu et al., 2012). These protein changes are accompanied by increased cellular contractility (Phan, 2008).

In fibrotic lung tissue, ACLP is associated with increased expression of αSMA and myofibroblast contractility and its knockout results in fewer αSMA positive fibroblasts and reduced fibrosis (Schissel et al., 2009). This suggests an ACLP-induced fibroblast to myofibroblast transition in lung fibrosis. The role of ACLP in adipose tissue fibrosis has not been well explored. Adipose tissue expression of ACLP may induce a fibroblast to myofibroblast transition in the resident fibroblasts of the tissue, promoting a “pro-fibrotic” phenotype.

To test this hypothesis, murine adipose tissue SVF cells were isolated, passaged to separate clusters, and treated with ACLP daily. Protein was harvested 2 and 3 days from initial treatment, resolved by Western blot, and analyzed for expression of αSMA and collagen I, markers of the myofibroblast phenotype (Figure 1). TGF-β is known to stimulate the fibroblast to myofibroblast transition and was used as a positive control.
Figure 1. ACLP Induces Myofibroblastic Protein Expression in SVF Cells

WAT fat pads were excised from wild-type mice and SVF cells were isolated, passaged, and plated into a 24-well cell culture plate. SVF cells were treated near confluence with 10% FBS DMEM (Control), 30 nM ACLP in 10% FBS DMEM (ACLP), and 1 nM TGF-β in 10% FBS DMEM (TGF-β). Media were replaced daily and cellular protein was harvested at 2 and 3 days from the initial treatment. Total protein (12 µg/lane) was run on a 4-20% tris-glycine gel, transferred to a nitrocellulose membrane, and probed with antibodies against ACLP, collagen I, αSMA, and pan-actin. These results are representative of data observed in three separate experiments using three separate SVF extractions.
ACLP expression was not affected by treatments at 2 or 3 days. ACLP treatment slightly induced collagen I expression at 2 and 3 days. TGF-β had no effect on collagen I expression at 2 days, but slightly increased expression at 3 days. Expression of αSMA was slightly induced by both ACLP and TGF-β at 2 and 3 days. Results were reproducible across three separate experiments using cells from three separate extractions.

**ACLP Increases Contractility of SVF Cells Embedded in Collagen I Gels**

Myofibroblasts show increased contractility, presumably due to the increase in cytoskeletal elements and matrix binding proteins (Hinz et al., 2001a, 2001b; Tomasek et al., 2002). Given the upward trend in αSMA expression in SVF cells exposed to ACLP, some increase in cell contractility was a likely outcome of ACLP treatment. An ACLP gain-of-function model increasing cell contractility would be consistent with findings that ACLP loss-of-function fibroblasts exhibit reduced contractility on collagen I gels (Schissel et al., 2009).

Several studies have assessed the contractility of various cells using collagen I contraction assays (Daniels and Khaw, 2000; Umino et al., 2000). To study ACLP-induced contractility, nearly confluent SVF cells were cultured on plastic and treated with ACLP or TGF-β. After 3 days of treatment, cells were embedded in a 1 mg/ml collagen I gel and released. Gel contractions were imaged at the indicated time points (Figure 2a) and analyzed to determine differences in cell contractility (Figure 2b).
Figure 2a. ACLP Increases Contractility of SVF Cells Embedded in Collagen I Gels

WAT fat pads were excised from wild-type mice and SVF cells were isolated, passaged, and cultured in 100 mm diameter plastic cell culture dishes. SVF cells were treated near confluence with 10% FBS DMEM (Control), 30 nM ACLP in 10% FBS DMEM (ACLP), or 1 nM TGF-β in 10% FBS DMEM (TGF-β) for 3 days. After 3 days, cells were plated in 1 mg/ml collagen I gels in triplicate at a concentration of 400,000 cells/ml. Gels were gently released and photographed every hour from 2-8 hours and again at 24 hours post-release. These results are representative of data observed in two independent experiments using two separate SVF extractions.
Figure 2b. ACLP Increases Contractility of SVF Cells Embedded in Collagen I Gels

ACLP and TGF-β treated cells were embedded in collagen I gels and allowed to contract as described in Figure 2a. Gels were photographed every hour from 2-8 hours and again at 24 hours post-release. Contracted gel images were analyzed using ImageJ. Measurements of well and gel areas were taken in arbitrary units for each image and compared. Percent contraction represents the difference between the area of the well (the original area of the gel) and the area of the gel at the given time point. Results represent average data for conditions in triplicate. At the earliest measured time point (2 Hours), gels with ACLP treated cells were 156±3% more contracted than gels with control cells. Gels with TGF-β treated cells were 205±1% more contracted than gels with control cells. Both ACLP and TGF-β significantly increase SVF cell contractility in the first 24 hours post-release as determined by a two-way analysis of variance (**p<0.001).
Treatment with ACLP and TGF-β showed statistically significant increases in the contractility of SVF cells. At 2 hours post-release, ACLP and TGF-β treated cells exhibited 156±3% (p<0.001) and 205±1% (p<0.001) higher contraction, respectively, than control cells. The degree of gel contraction achieved by ACLP and TGF-β treated cells was consistently higher than that achieved by control cells in the first 24 hours post-release. These results are representative of two independent experiments using two separate SVF extractions.

**ACLP Decreases Expression of Differentiated Adipocyte Proteins in 10T1/2 Cells**

Having investigated the effects of ACLP on SVF cells, we next investigated the effects of ACLP on another component of adipose tissue, developing adipocytes. ACLP overexpression in 3T3 preadipocytes cultured on plastic has previously been shown to inhibit adipocyte differentiation and induce transdifferentiation to a smooth muscle cell phenotype (Abderrahim-Ferkoune et al., 2004). In contrast, other studies have shown that ACLP overexpression does not decrease adipocyte differentiation markers or affect morphology of 3T3-L1 preadipocytes when induced to differentiate on plastic (Gagnon et al., 2005; Gusinjac et al., 2011). Furthermore 3T3-L1 preadipocytes overexpressing ACLP induced to undergo adipogenesis on a collagen I gel show decreased expression of fatty acid synthase, C/EBPα, and PPARγ compared to control cells (Gusinjac et al., 2011). Given the evidence that ACLP has a collagen binding discoidin
domain and associates with the ECM in fibrosis (Schissel et al., 2009), it is possible that the effects of ACLP on differentiating adipocytes may be, in part, mediated by ACLP binding to collagen and as a consequence, being presented to cells at a greater concentration.

Earlier in this study, ACLP treatment was shown to increase αSMA expression (Figure 1) and contractility (Figure 2) of SVF cells. This suggests a possible ACLP induced fibroblast to myofibroblast transition. Given this functional change and the evidence that αSMA expression is suppressed in cells committed to adipogenesis (Okada et al., 2012), it is possible that ACLP exposure before and during adipogenesis may promote a myofibroblastic phenotype in adipocyte precursors and prevent adipogenesis. Noting that ACLP overexpression impairs adipogenesis of preadipocytes cultured on top of prepared collagen I gels and the possibility that the effects of ACLP on adipogenesis may be mediated by ECM binding, we hypothesized ACLP would hinder adipogenesis of cells embedded in a collagen I gel.

To test this hypothesis, 10T1/2 cells were incubated in ACLP or TGF-β for four days, embedded in a 1 mg/ml collagen I gel, and induced to undergo adipogenesis as described in the methods section. TGF-β is a known inhibitor of adipogenesis and is expressed in obese adipose tissue (Choy and Derynck, 2003; Samad et al., 1997). Protein samples were harvested 6 days post-induction and analyzed by Western blot. The blot was probed with antibodies
Figure 3. Pre-Induction ACLP Treatment Results in Decreased Expression of Adiponectin, FABP4, and PPARγ in Differentiating 10T1/2 Cells

10T1/2 cells were cultured in 100 mm diameter plastic cell culture dishes and treated near confluence with 10% FBS DMEM (Control), 30 nM ACLP in 10% FBS DMEM (ACLP), and 1 nM TGF-β in 10% FBS DMEM (TGF-β). Media were changed daily for four days, at which time cells were plated in a 1 mg/ml collagen I gel and treated immediately with 5 nM dexamethasone, 0.5 mM IBMX, 4 nM insulin, and 12.5 µM indomethacin in 10% FBS DMEM (DMII) and treatments: DMII (Control), 30 nM ACLP in DMII (ACLP), and 1 nM TGF-β in DMII (TGF-β). Every two days post-induction, media were replaced with 1 nM insulin in 10% FBS DMEM (Control), 30 nM ACLP and 1 nM insulin in 10% FBS DMEM (ACLP), and 1 nM TGF-β and 1 nM insulin in 10% FBS DMEM (TGF-β). Protein samples were harvested 6 days post-induction. Total protein (16 µg/lane) was resolved by Western blot and probed with antibodies against adiponectin, FABP4, PPARγ, αSMA, and pan-actin. Results represent a single preliminary experiment.

<table>
<thead>
<tr>
<th>Control</th>
<th>ACLP</th>
<th>TGF-β</th>
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<tbody>
<tr>
<td>Adiponectin</td>
<td></td>
<td></td>
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<tr>
<td>FABP4</td>
<td></td>
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<td>PPARγ</td>
<td></td>
<td></td>
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<tr>
<td>αSMA</td>
<td></td>
<td></td>
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<tr>
<td>Pan-actin</td>
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</table>
against adiponectin, FABP4, and PPARγ, markers of differentiated adipocytes, as well as αSMA (Figure 3).

It should be noted the extraction of cellular protein from within a collagen I gel requires the extraction of FBS components that accumulate within the gel and are not removed by media changes or simple washes of the gel. Resolution of protein samples with significant FBS content results in protein smears as observed by Coomasie blue and non-specific antibody staining (data not shown). To obtain clear bands by Western blot and Coomasie blue staining, centrifugation in spin columns at low speeds and at 4°C was used to force some of the media containing FBS out of the gel. By placing the gel in PBS for each centrifugation, more media washed out of the gel, as evidenced by the color of the flow-through. Once flow-through no longer changed color, resolution consistently gave clear bands by Western blot and Coomasie blue staining (data not shown).

Cells treated with ACLP and TGF-β exhibited drastically decreased expression of adiponectin, FABP4, and PPARγ expression. Expression of αSMA was similar for treatments and controls. Inspection of the cells before harvest under a microscope showed visible adipocytes among control cells, but few/none in the ACLP and TGF-β treated cells (data not shown). Results represent a single preliminary experiment.
ACLP Induces Acta2 and Collagen Gene Expression and Reduces Expression of Adipogenic Genes in Differentiating SVF cells

ACLP slightly increased αSMA and collagen I expression in SVF cells (Figure 1), indicative of a possible fibroblast to myofibroblast transition. ACLP also impaired 10T1/2 cell adipogenesis (Figure 3). This inhibition may be mediated by changes in RNA levels. TGF-β inhibits adipogenesis by repressing C/EBP transcriptional activity through Smad signaling (Choy and Derynck, 2003). ACLP may promote a fibroblast to myofibroblast transition and inhibit adipogenesis at a transcriptional level by increasing transcription of genes associated with myofibroblasts and decreasing transcription of genes associated with adipocytes.

To test this hypothesis, isolated SVF cells were cultured for 2 days on plastic, then embedded in 1 mg/ml collagen I gels. Cells were induced to differentiate and treated with ACLP in quadruplicate (see methods section). RNA samples were harvested four days post-induction to observe levels of Pparg and Cebpa, which are upregulated early in adipogenesis. Total RNA was subjected to reverse transcription and RT-PCR (Figures 4 and 5).

Treatment with ACLP increased Acta2 (the gene for αSMA) RNA levels to 132.29% and Col6a1 to 139.03% compared to controls (Figure 4); however neither of these differences were statistically significant (p=0.46 and p=0.08, respectively). Treatment significantly increased expression of Col1a2 RNA to 194.44% compared to controls (Figure 4). Treatment with ACLP significantly
Isolated SVF cells were cultured on 150 mm diameter plastic cell culture dishes for 2 days then embedded in 1 mg/ml collagen I gels in quadruplicate. Cells were treated immediately with 0.25 μM dexamethasone, 0.5 mM IBMX, and 8 nM insulin in 10% FBS DMEM (DMI) (Control) or 30 nM ACLP in DMI (ACLP). Two days post-induction, media were replaced with 8 nM insulin in 10% FBS DMEM (Control) or 30 nM ACLP and 8 nM insulin in 10% FBS DMEM (ACLP). RNA was harvested four days post-induction (see methods). Total RNA (500 ng) was subjected to reverse transcription and RT-PCR. Treatment with ACLP increased expression of Acta2, Col1a2, and Col6a1 to 132.29%, 194.44%, and 139.03%, respectively. Statistical significance was determined using a two-tailed Student’s t-test (*p<0.05). Results represent a single preliminary study.
Fig 5. ACLP Suppresses Adipogenic Gene Expression in Differentiating SVF cells

Isolated SVF cells were treated and harvested for RNA as in Figure 4. Total RNA (500 ng) was subjected to reverse transcription and RT-PCR. Treatment with ACLP significantly decreased expression of Cebpa, FABP4, and Pparg RNA to 44.93%, 2.78%, and 0.82%, respectively. Statistical significance was determined using a two-tailed Student’s t-test (*p<0.05, **p<0.001). Results represent a single preliminary study.
decreased expression of Cebpα, FABP4, and Pparg RNA to 44.93%, 2.78%, and 0.82%, respectively, compared to controls (Figure 5). These results represent a single preliminary study.

The preliminary studies presented in these results demonstrate that ACLP treatment 1) induces an upward trend in the expression of proteins and RNA characteristic of myofibroblasts and 2) significantly suppresses adipogenesis at both the protein and RNA level.
DISCUSSION

This study set out to determine the effects of ACLP on SVF cells and differentiating adipocytes, testing the hypothesis that ACLP would induce a fibroblast to myofibroblast transition in SVF cells and inhibit adipogenesis. The results presented in this study suggest ACLP may be capable of inducing a myofibroblastic phenotype in SVF cells. These studies also indicate that ACLP is a potent inhibitor of adipogenesis. These findings may serve as the basis for further experiments determining the role of ACLP in WAT fibrosis and suggest a possible target for improving or preventing obesity-induced WAT fibrosis and its consequent disease symptoms.

ACLP Induced Fibroblast to Myofibroblast Transition

ACLP plays a significant role in the development of bleomycin-induced lung fibrosis (Schissel et al., 2009). In fibrotic lung tissue, elevated ACLP expression was associated with areas of high collagen deposition. ACLP knockout mice were protected from severe lung fibrosis and exhibited decreased collagen I deposition and αSMA expression. ACLP knockout fibroblasts also showed decreased contractile activity. This suggests ACLP expression is necessary for the induction or activation of these fibroblasts to contractile, collagen secreting myofibroblasts. In a 3T3 preadipocyte model, ACLP overexpressing cells have shown the capacity to differentiate into αSMA expressing cells (Abderrahim-
This study expands these findings to SVF cells from murine adipose tissue. It also adds the ability of ACLP to slightly induce collagen I expression and significantly increase cell contractility (Figures 1 and 2). These are hallmarks of activated myofibroblasts (Phan, 2008) and are similar to the effects of ACLP observed in fibrotic lung tissue.

The upward trend in myofibroblast proteins and RNA levels with ACLP treatment support the idea that ACLP may be inducing a fibroblast to myofibroblast transition. It is possible that under different experimental conditions, a greater difference in expression might be seen. For example, cell culture surface rigidity induces αSMA expression in cells (Huang et al., 2012; Schlunck et al., 2008). Culturing cells on softer surfaces throughout experiments might reduce non-treatment induction of αSMA, highlighting the difference in αSMA expression between control and treatment conditions. Further studies are needed to determine the efficacy of ACLP-induced myofibroblast differentiation.

In any case, this finding in adipose stromal cells certainly suggests that ACLP-induced fibroblast to myofibroblast transition may occur in WAT and play a significant role in WAT fibrosis. In parallel with fibrotic lung tissue, increased ACLP expression associated with the increased collagen I deposition can be expected in fibrotic WAT tissue. Increased ACLP localization with collagen I throughout fibrotic tissue is most likely an effect of its collagen I binding discoidin domain (Schissel et al., 2009). It is possible that continuous binding to the collagen I ECM would create an increase in local ACLP concentration,
compounding its effects. In fibrotic WAT, this might create a concentrated or localized region of ACLP expression which is different from its normal expression in the microvasculature (Hocking et al., 2010). This irregular exposure of ACLP in tissue may result in greatly increased myofibroblast differentiation in the stroma. The increased number of myofibroblasts would alter tissue structure in two ways. Firstly, the increase in ACLP-induced myofibroblasts would result in increased deposition of collagen I in the ECM. Secondly, through increased contractility, these cells would compact the tissue. Both of these methods have the effect of increasing the ECM density, the amount of ECM proteins in a given area.

Adipocyte hypertrophy, like that seen in obesity, is normally associated with inflammatory responses as a result of increased adipocyte necrosis (Cinti et al., 2005). Chronic inflammatory responses to this necrosis exacerbate adipose tissue inflammation (Wynn, 2008; Wynn and Ramalingam, 2012). Knockout of collagen VI or SPARC results in mice with increased adiposity without the associated inflammation (Bradshaw et al., 2003; Khan et al., 2009). This suggests increased ECM density is more restrictive to adipocyte growth and may be inversely correlated with expansion related inflammation and fibrosis. Given the evidence that ACLP may increase ECM density and its knockout protects tissue from severe fibrosis, ACLP may aggravate fibrosis and inflammation in WAT.
ACLP Impairs Adipogenesis

ACLP inhibits adipogenesis through suppression of the key regulators, PPARγ and C/EBPα. Results in this study show ACLP exposure causes a drastic decrease in PPARγ and FABP4 protein expression in differentiating preadipocytes (Figure 3). Importantly, ACLP caused reductions in Pparg, Cebpa, and FABP4 RNA expression in SVF cells induced to undergo adipogenesis (Figure 5). This demonstrates that ACLP is able to inhibit adipogenesis in murine adipose-derived cells. Evidence in the literature on the effects of ACLP overexpression in preadipocytes is conflicting. Abderrahim-Ferkoune et al. have shown ACLP overexpression to decrease PPARγ2 and FABP4 RNA levels, resulting in an inability to differentiate into adipocytes. This is consistent with the suppression seen in our studies. Interestingly, Gusinjac et al. reported no effects of ACLP overexpression on cells induced to differentiate on tissue culture plastic, but a suppression of C/EBPα, PPARγ, and fatty acid synthase was observed in ACLP overexpressing cells induced on a collagen I coated surface. However, unpublished studies from our lab show that ACLP treatment is indeed inhibitory to cells induced on plastic as measured by decreased Oil Red O staining and drastically decreased PPARγ and FABP4 protein expression. This difference could be explained by different concentrations of ACLP in the experiments. Our lab used a recombinant form of ACLP at 30 nM, whereas the concentration of ACLP from overexpressing 3T3 cells was not reported, but may have been lower considering the lack of effect in cells cultured on plastic. In their studies, the
observed effects of ACLP overexpression on cells cultured on a collagen surface may have been mediated by ACLP binding the collagen. As described above, this may increase local concentration of ACLP and prevent its removal from culture through media changes. Therefore, the cells on the collagen surface would be exposed to a greater local concentration of ACLP, resulting in stronger anti-adipogenic effects.

Increased ability of ACLP to inhibit adipogenesis through collagen I binding suggests that ACLP binding in fibrotic tissue would result in greater local concentrations, exerting an anti-adipogenic effect in areas that might normally promote adipogenesis. This effect may be compounded by continued ACLP binding in the course of fibrotic disease.

This observation has significant impact for obesity-induced WAT fibrosis. Considering the increased necrotic rate in obese WAT (Cinti et al., 2005), an increased adipogenic rate would be needed to generate new adipocytes in order to compensate for adipocytes lost to necrosis. In heavily fibrotic adipose tissue, collagen-bound ACLP would inhibit adipogenesis, preventing such compensation. This would correspond to greater decreases in circulation of adipokines, such as adiponectin, and greater increases in circulating free fatty acids, which have been linked to several disease symptoms, including insulin resistance, type II diabetes mellitus, and heart disease (Jensen, 2006; Weyer et al., 2001; Yamauchi et al., 2001).
Adipocyte and Myofibroblast Differentiation

MSCs of adipose tissue are capable of differentiating into a variety of cell types, including adipocytes and myofibroblasts (Gimble and Guilak, 2003; Zuk et al., 2001, 2002). However, evidence in the literature suggests that the stimulants of adipogenesis are inhibitory to myofibroblast differentiation and vice versa (Hong et al., 2007). PPAR\(\gamma\) ligands inhibit TGF-\(\beta\)-induced myofibroblast differentiation through the blockade of several pathways, some of which are even PPAR\(\gamma\) independent (Ferguson et al., 2009; Kuriyan et al., 2012). Similarly, TGF-\(\beta\) stimulation inhibits the adipogenic program through the impairment of PPAR\(\gamma\) activity as well as the expression and nuclear localization of C/EBP\(\alpha\) (Choy and Derynck, 2003; Kumar et al., 2012). Differentiated myofibroblasts are highly contractile cells as a function of increased \(\alpha\)SMA expression, induced as part of their differentiation program (Phan, 2008). Adipocytes, on the other hand, specifically decrease their expression of \(\alpha\)SMA as part of the adipogenic program (Okada et al., 2012). This suggests that the pathways leading from precursors to adipocytes and myofibroblasts are conflicting and unlikely to be activated and followed simultaneously.

Results from this study support this model. ACLP was shown to induce an upward trend in the expression of myofibroblast genes and proteins. It was also demonstrated to strongly inhibit expression of adipocyte genes and proteins. This suggests ACLP inhibits adipogenesis and may simultaneously promote myofibroblast differentiation.
Seemingly contrasting with the idea that ACLP induces myofibroblast differentiation and inhibits adipogenesis, αSMA expression was similar between control and ACLP treated conditions (Figure 3), indicative of equivalent myofibroblast presence in both ACLP and control conditions. This result is confusing however, considering the difference in adipocyte protein expression between control and ACLP conditions. This may be explained by the development of a heterogeneous mixture of cells, only some of which retained adipogenic potential. It is possible that four days of culture on rigid plastic surfaces may have induced significant αSMA expression (Huang et al., 2012; Schlunck et al., 2008), reflecting ACLP-independent myofibroblast differentiation, across control and treatment conditions. In this case, total αSMA expression in this particular experiment may not be useful for determining the myofibroblast inducing effects of ACLP, as the change in αSMA induced by ACLP may have been small or negligible compared to the degree of overall αSMA expression. However, the drastic decrease in adipocyte proteins in the ACLP condition indicates that ACLP inhibited adipogenesis of the cells that retained adipogenic potential.

Immunohistochemistry and fluorescence imaging studies could be used to test the hypothesis that these two pathways are mutually exclusive and result in a heterogeneous population of cells by determining if the localization of αSMA and FABP4 are primarily in different cells.
Targeting ACLP in Obesity-Induced WAT Fibrosis

Obesity-induced WAT fibrosis and inflammation are serious conditions linked with several disease states, most notably insulin insensitivity and type II diabetes (Jensen, 2006; Weyer et al., 2001; Yamauchi et al., 2001). Considering the significant and detrimental effects of WAT fibrosis, understanding the mechanism of this disease and identifying key pathways will give insight into possible treatments and cures and perhaps even a way to prevent it.

Evidence presented in this study suggests ACLP may play a role in aggravating tissue fibrosis and subsequent disease states in obesity-induced WAT, identifying it as a possible therapeutic target. ACLP's mechanism of action is not yet well defined; however, responses to ACLP treatment are similar to those observed from TGF-β treatment (Figures 1-3). In fact, TGF-β regulates ACLP expression in 3T3 cells (Gagnon et al., 2005; Schissel et al., 2009). Though further studies will be necessary to elucidate ACLP mechanisms of action, similarities between responses to TGF-β and ACLP indicate some possibilities.

1) ACLP and TGF-β may have an overlapping pathway, sharing some of the same signaling molecules.
2) ACLP may activate the TGF-β receptor.
3) ACLP may enhance TGF-β binding to its receptor.
4) ACLP may increase expression of TGF-β through enhanced transcription or repressed degradation.
Figure 6. Proposed Model of ACLP Aggravation of Fibrosis

ACLP is hypothesized to aggravate fibrosis by (A) inhibiting adipogenesis and inducing myofibroblast differentiation of stromal precursor cells. (B) Increased ACLP expression, associated with the increased collagen I deposition in fibrosis, induces myofibroblast differentiation of stromal cells. The increased number of myofibroblasts causes increased collagen deposition and tissue compaction, resulting in adipocyte necrosis. Adipocyte necrosis induces an immune response, which in turn stimulates further tissue fibrosis.
CONCLUSIONS

This study has presented preliminary findings indicating ACLP may induce the expression of myofibroblastic proteins and inhibits the expression of adipogenic genes in murine adipose derived cells. Inhibition of adipogenic gene expression by ACLP was supported by protein data from 10T1/2 cells. These results suggest a role for ACLP in aggravating obesity-induced WAT fibrosis and indicate it as a possible therapeutic target. Further investigations will elucidate the function of ACLP in fibrotic WAT and the mechanisms of ACLP action and may lead to treatments for adipose tissue dysfunction.
REFERENCES


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- Assisted in the isolation, concentration, and analysis of azurin and
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- Supervised by Dr. Todd Krauss

OU Health Sciences Center, Urology Dept.              Oklahoma City, OK
Volunteer Researcher                                 06/08 – 08/08

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  *Enterococcus*-infected human cells using RT-PCR analysis
- Supervised by Dr. Robert Hurst