LIPOCALIN 2 DEFICIENCY INFLUENCES TRANSFORMING GROWTH FACTOR-β EFFECT ON INFLAMMATION AND EXTRACELLULAR MATRIX REMODELING IN INGUINAL ADIPOCYTES

A THESIS
SUBMITTED TO THE FACULTY OF THE
UNIVERSITY OF MINNESOTA
BY

AMBRIA PFARR

IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

ADVISOR: XIAOLI CHEN, MD, PhD
CO-ADVISOR: LINDA BRADY, PhD

SEPTEMBER 2015

© Ambria Pfarr 2015

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Xiaoli Chen, for her patience, guidance, and support throughout my graduate school journey. Her commitment to excellence and ability to provide help in any situation has truly shaped my graduate experience. To my lab colleagues: Dr. Hong Guo, Jessica Deis, Ming Zhao, Jingyi Li, and Xiaoxue (Cheryl) Qui for answering questions, providing assistance, and guiding me in all of my work and learning. To my committee members, Dr. Linda Brady and Dr. A. Saari Csallany for their time and support and suggestions towards my research project. Lastly, to my friends, fiancé, and family who have been extremely supportive in my time spent focused on school and the journey to learn and grow; without all of you I wouldn’t have made it where I am today.

Ambria Pfarr wrote chapter 1. The manuscript authored by Ambria Pfarr and Xiaoli Chen is to be submitted. Ambria Pfarr performed all the experiments and wrote chapter 2.

ABSTRACT

Chronic low-grade inflammation present in hypertrophic obesity has the ability to cause remodeling of the adipose tissue due to the increased presence of macrophages and pro-inflammatory cytokines. Transforming Growth Factor-β (TGF- β) is a cytokine released from macrophages that is increased in obesity and plays a major role in extracellular matrix (ECM) remodeling of tissues. Lipocalin 2 (Lcn2), a cytokine expressed in adipose tissue, is related to inflammation and ECM remodeling. Due to the qualities that both adipokines possess the role in extracellular matrix remodeling and inflammation, we looked into the TGF- β effect on the regulation of ECM and inflammatory cytokines in Lcn2 deficient adipocytes to acquire more information about the function of Lcn2 under inflammatory stimuli and the association with metabolic diseases. Therefore, we performed experiments to address how Lcn2 knock out (KO) influences the response of adipocytes to TGF- β in the production of pro-inflammatory and anti-inflammatory cytokines and ECM proteins. Our results demonstrate that TGF- β down-regulates Lcn2 expression at both the mRNA and protein levels in inguinal adipocytes. Lcn2 KO adipocytes have lower levels of TGF- β expression, but normal levels of p70S6K phosphorylation and normal response to TGF- β stimulation in mammalian target of rapamycin complex 1 (mTORC1) signaling activation. However, the inhibitory effect of rapamycin on TGF- β expression is attenuated in Lcn2 KO adipocytes. Moreover, Lcn2 KO impairs the rapamycin potentiation of TGF- β effect on the expression of ECM proteins, but shows little effect on dysregulation of cytokines.

TABLE OF CONTENTS

I. List of Figures…………………………………………………………...…iv

Chapter 1

Literature Review……..………………………………………………………...…1

- I. Obesity and metabolic diseases…………………..……………………2
  - a. Prevalence of obesity……………………………………………….2
  - b. Mechanisms for obesity………………………………………...…3
- II. Adipose tissue in obesity and diabetes ……………………………...…4
  - a. White adipose tissue (WAT) …………………………………..……4
  - b. Functions of WAT……………………………………………………5
    - i. Lipid storage………………………………………………….5
    - ii. Metabolic function and secretion………………………………6
      - 1. Inflammation and ECM remodeling ……………..……7
      - 2. Macrophage infiltration and inflammation……………8
- III. Transforming growth factor β…………………………………………..10
  - a. The TGF-β signaling pathway and metabolic functions…………...10
  - b. TGF- β function in adipocyte differentiation..………………………12
  - c. TGF- β and cancer…………………………………………………13
  - d. TGF-β in obesity and other metabolic diseases……………………14
  - e. Role of TGF- β in fibrosis…………………………………..………14
- IV. Lipocalin 2 (Lcn2) ……………..……………………………………..15
  - a. Lcn2 background……..……………………………………………..15
  - b. Lcn2 and inflammation……………..………………………………16
  - c. Lcn2 and obesity……………..……………………………………...17
  - d. Lcn2 and cancer ………..……………………………………………..19
  - e. TGF- β and Lcn2 in activation of mTOR signaling …..……………19
- V. Summary and objectives…………………………………………….21

Chapter 2

- I. Introduction ……………………………………………………………….26
- II. Results……………………………………………………………………30
  - a. Lcn2 expression in inguinal adipocytes is regulated by TGF- β….30
  - b. TGF- β expression in inguinal adipocytes is decreased, but TGF- β action on mTOR signaling activation is unaffected in Lcn2 KO inguinal adipocytes…………………………………………31
  - c. The effect of TGF- β and rapamycin on inflammation in Lcn2 KO adipocytes…………………………………………………………34
  - d. The effect of TGF- β and rapamycin on extracellular matrix remodeling in Lcn2 KO adipocytes…………..……………………36
- III. Discussion and Summary ………………………………………………39
- IV. Materials and Methods…………………………………………………..47
- V. Bibliography ……………………………………………………………….52
- VI. Appendices………………………………………………………………..60

LIST OF FIGURES

Chapter 1

Figure 1A Proposed pathway for TGF- β activation of mTOR signaling…..……24

Chapter 2

Figure 1A Lcn2 gene expression in response to TGF- β and rapamycin……….60

Figure 1B Lcn2 protein expression in response to TGF- β and rapamycin……..60

Figure 1C The effect of TGF- β on Lcn2 protein expression in WT adipocytes...60

Figure 1D Lcn2 secretion was down-regulated by TGF- β in WT adipocytes…..60

Figure 2A TGF- β gene expression in response to rapamycin treatment……….61

Figure 2B TGF- β stimulated p70S6k expression in inguinal adipocytes……….61

Figure 3A TGF- β stimulated COX-2 expression in inguinal adipocytes………...62

Figure 3B The effect of TGF- β and rapamycin on p70S6K protein
expression in WT and Lcn2 KO inguinal adipocytes………………..…….62

Figure 4A MCP-1 gene expression in response to TGF- β and rapamycin…..…63

Figure 4B TNFα gene expression in response to TGF- β and rapamycin………63

Figure 4C IL-6 gene expression in response to TGF- β and rapamycin…………63

Figure 4D MRC-1 gene expression in response to TGF- β and rapamycin……..63

Figure 4E Arg1 gene expression in response to TGF- β and rapamycin………..63

Figure 5A Col3A1 gene expression in response to TGF- β and rapamycin…….64

Figure 5B MMP9 gene expression in response to TGF- β and rapamycin…..…64

Figure 5C Col3A1 gene expression in response to TGF- β and rapamycin…….64

Figure 5D The effect of TGF- β on MMP9 protein expression in inguinal
adipocytes…………………………………………………………………...64

CHAPTER 1

LITERATURE REVIEW

I. Obesity and metabolic diseases

a. Prevalence of obesity

Overweight and obesity are a growing worldwide epidemic linked to poor health outcomes and increased risk for other chronic diseases. Defined as excess body fat causing altered health status and characterized by low-grade, systemic inflammation, overweight and obesity pose threat to normal metabolic function ([1]). A body mass index (BMI) of 25 kg/m^2 or greater is considered overweight, whereas a BMI of 30 kg/m^2 or greater classifies an individual as obese. According to the World Health Organization (WHO), 68% of Americans are either overweight or obese; 35.1% of adults in the United States and 13% (600 million) of adults worldwide over 20 years of age are obese. This disease also affects approximately 18% of children ages 6-19 in the United States and 42 million children under the age of 5 worldwide, increasing disease risk for the upcoming generations. Causes of obesity are commonly associated with increased intake versus expenditure or caloric surplus, genetics, metabolic stress, aging, and medications, although obesity is considered a preventable disease ([2,3]).

Obesity, often associated with excess adipose storage in the visceral depot, is highly comorbid with chronic diseases such as type II diabetes mellitus (DMII), insulin resistance (IR), cancers, non-alcoholic fatty liver disease, metabolic disease, sleep apnea, atherosclerosis, and heart disease and its related complications such as dyslipidemia, ischemia,

hypertension, and death ([4,5,6]). Obesity is also correlated with a shorter life span and higher economic consequences due to healthcare costs ([7]).

b. Mechanisms for the development of obesity

Energy release from the adipose tissue comes from the dispersion of fatty acids into circulation. The fatty acids present within the adipocyte are generated by the breakdown of triacylglycerols, the primary storage form of fat within the adipocyte, and released when the primary fuel, glucose, is not readily available ([8,9]). Each lipid droplet is composed of a phospholipid monolayer that surrounds a lipid core. In a fed state, surplus energy is stored in the form of triacylglycerol in a lipid droplet within the adipocyte; this core is protected from lipases by perilipin 1, which is released upon feeding. In a fasting state, triacylglycerols are hydrolyzed by hormone-sensitive lipase and adipose triglyceride lipase to release fatty acids for energy ([9]). The imbalance created between energy intake and expenditure is the major contributor to the accumulation of excess fat mass, leading to the development of obesity.

II. Adipose tissue in obesity and diabetes

a. White adipose tissue (WAT)

WAT serves as a site for energy storage in the form of triacylglycerols and as an endocrine organ, working to regulate energy, maintain weight homeostasis, control inflammatory response, and to regulate the action of insulin. Adipose tissue also has mechanical function to protect the heart and other major organs from damage by serving as insulation or cushion ([2]).

The WAT is composed of mature adipocytes and the stromal-vascular component consisting of immune cells– such as T cells and macrophages, blood vessels, pre-adipocytes, extracellular matrix (ECM), and fibroblasts. The fibroblasts support and synthesize the collagen, which makes up the ECM ([2]).

All of the functions of adipose tissue are depot dependent. In humans, adipose tissue can be found in two major depots, the subcutaneous and visceral adipose tissue, but can also be located intramuscularly ([4,8,10]). Visceral adipose tissue is known to have higher turnover rates than subcutaneous adipose tissue ([11]). Studies indicate that fat distribution can be controlled by multiple factors, one of the major factors being sex, due to differences in hormone secretion; men tend to accumulate fat viscerally and women, subcutaneously. Age and physical activity level are also linked to depot-specific adiposity, which increased age and decreased activity levels contribute to visceral adiposity, respectively ([12]).

Subcutaneous adipose tissue development typically occurs before visceral adipose tissue, starting in the second trimester in utero ([13]).

The subcutaneous depot is associated with fat storage beneath the skin, whereas the visceral depot is located around the organs and intra-abdominally. These depots are differential in terms of their adipogenic capability, secretory function, and inflammatory response ([10]). Visceral adipose tissue is often related to a higher lipogenic activity, whereas subcutaneous depots have a lower lipid turnover ([11,12]).

Studies support evidence that increased visceral adipose tissue is linked to higher morbidity rates for type II diabetes mellitus and cardiovascular disease due to its direct relationship with release of free fatty acids (FFA) into portal circulation to the liver ([4,8,12]). The increase in FFA increases lipogenesis and gluconeogenesis in the liver, causing insulin resistance and exacerbating the affects of increased adiposity such as glucose intolerance, hypertension, and atherosclerosis ([12]).

Mice also have differing depots that support different metabolic functions. Mice have four abdominal adipose depots, perigonadal (in male mice), perirenal, mesenteric, and omental depots. The two superficial depots are the inguinal and subscapular (brown) depots. These depots also show regional differences in many attributes such as cell differentiation, gene expression, protein abundance, and growth, causing overall differences in function ([10]). Research found that oxidative metabolism is higher in specific depots in mice as it is in human adipose

tissue; the female periovarian depot is higher in oxidative metabolism than the inguinal adipose tissue, but the interspacular brown adipose is significantly higher than both of the mentioned depots ([11]).

b. Functions of WAT

i. Lipid storage

The primary role of WAT is energy storage. There are two different forms of storage in the functional adipocyte, hypertrophy, which describes an increase in cell size, versus hyperplasia, which is an increase in number of adipocytes. Adipocytes have a rapid response to nutrient balance—in a surplus, the adipocyte will increase in size, in a deficit, the adipocyte with decrease in size to maintain energy homeostasis ([2]).

Both forms of storage, hyperplasia and hypertrophy, increase the total adipose tissue, which increases the event of apoptosis, dysregulation of fatty acid homeostasis, hypoxia, and cytokine secretion; hypertrophy is more often related to increased inflammation and hypoxia due to the changes in metabolic function and secretion within the cells ([4,14]). Additionally, hypertrophy poses a higher risk because it is more prevalent in obesity that hyperplasia ([14]). Research indicates that mice fed a chronic high-fat diet have larger fat pads that have fewer adipocytes present, suggesting high-fat diets aid in reducing adipogenesis and promote hypertrophy ([6]).

ii. Metabolic function and secretion

Another major function of WAT is secretion of adipokines and cytokines ([5]). A normal, metabolically stable adipose tissue displays characteristics of proper vascularization and normal secretion of adipokines and cytokines to aid in immune regulation, energy metabolism, and insulin sensitivity regulation. Adipocyte dysfunction associated with obesity is present when cells are hypertrophic, hypoxic, and/or have increased inflammation ([4]).

It is in fully dysfunctional adipose tissue where inflammation is present, increased secretion of pro-inflammatory cytokines, recruitment of macrophages, and T-helper cells occurs. With any dysfunction present, pro-inflammatory cytokines are introduced to the tissue, and cells become hypoxic due to decreased access to vascularization; as the adipocyte increase in size, the areas of the cell lacking access to the nutrients delivered by the vasculature, especially oxygen, become dysfunctional and potentially become necrotic exacerbated by macrophage infiltration ([4]).

Because of lack of access to vasculature and increased inflammation, hypertrophy often contributes to a localized hypoxia versus hyperplasia, which is often coined “metabolically healthy” adipose expansion ([4]). Wernstedt Asterholm *et al* define this metabolically healthy adipose tissue by association with appropriate

angiogenesis, vascular remodeling, and extracellular matrix remodeling ([6]). There is dysfunction present in hypertrophy because it often includes an increase in pro-inflammatory adipokines, especially matrix metallopeptidase 9 (MMP9), Interleukin 6 (IL-6), and leptin ([14]).

1. Inflammation and ECM remodeling

Chronic low-grade inflammation present in hypertrophic obesity has the ability to cause remodeling of the adipose tissue due to the increased presence of macrophages and released cytokines. Dysfunction of the extracellular matrix is a result of the macrophage infiltration, adipocyte hypertrophy, and increased ECM remodeling ([4]).

To some extent, ECM remodeling is necessary to maintain adipose tissue health and the average fat cell turns over every 8-12 years, but fibrosis can occur as a result of remodeling in the adipose tissue. Therefore, remodeling of the adipose tissue is a continuous process, but the consequences of excess remodeling that occurs during obesity are of note ([14]). In the obese state, remodeling occurs more often and rapidly, excelling the natural progressive progress and decreasing the efficiency of adipocyte function and disrupting metabolic homeostasis of the adipose tissue ([6,14]).

2. Macrophage infiltration and inflammation

In studies conducted by Toubal *et al*, the pathway controlling obesity-related inflammation in human adipocytes through transcriptional reprogramming was examined. They found that the SMRT-GPS2 copressor complex suppresses the pathway that controls the transcription of monocyte chemoattractant protein-1 (MCP-1) and IL-6 that contributes to macrophage infiltration and inflammatory response ([15]). Other studies have confirmed that nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway works in opposition of the SMRT-GPS2 compressor complex by upregulating inflammatory cytokine, chemokine, and adipokine expression ([16]).

Macrophages are significant contributors to the inflammatory response present in obesity ([17,18]). They are promoted by free fatty acids and chemokines, such as MCP-1, which are upregulated in inflammation. The mobilization of macrophages from the bone marrow initiates the remodeling process of the adipocyte ([2,14,19]). Research shows that Infiltration of macrophages is more evident in visceral WAT in an obese adult, but the subcutaneous tissue showed higher production of collagens and remodeling-related secretory protein. MCP-1, which is highly expressed in the stromal-vascular (SV) fraction of the adipose tissue, and IL-6 are both 2.4 fold and 4.1 fold, respectively, higher in the visceral adipose tissue than in subcutaneous adipose tissue when a pro-inflammatory state is present ([19]). Other

research involving hypertrophic cells by Dalmas *et al* indicates that when adipocytes become hypertrophic, they are more likely to produce cytokines IL-6, Interleukin-8 (IL-8), and markers of inflammation, such as MCP-1. Their lean model equivalent secreted anti-inflammatory cytokines, such as Interleukin-13 (IL-13) and Interleukin-10 (IL-10), adiponectin, and promoted the immunosuppressive, alternative macrophage ([20]).

Pro-inflammatory cytokines promote macrophage infiltration and activation ([19]), causing macrophages to aggregate and form crown-like structures that surround the adipocyte. This is a hallmark of chronic inflammation and can cause remodeling in adipose tissue. The macrophage will attach to the adipocyte, and through phagocytosis, remove the lipid droplet causing adipocyte death and further macrophage infiltration ([14]).

Previous research indicates macrophages can present an inflammatory-controlled response in the pro-inflammatory classic form, as M1 marker, or in an activated, immunosuppressive form, M2 marker. Obesity is highly correlated with the presence of the M1 macrophage, where lean subjects often present with increased M2 macrophages. This increase of M1 macrophages occurs due to the recruitment of resident macrophages that are responsive to the environment and contribute to the polarization of the M2 marker to M1 marker ([17]).

III. Transforming Growth Factor β

a. The TGF- β signaling pathway and metabolic functions

Transforming Growth Factor-β (TGF- β) is a cytokine released from macrophages that is increased in obesity. The regulatory action of TGF- β is robust, as it includes tissue growth and development, immunity, cell differentiation, production of the ECM, and cell cycle control ([21,22]); its actions are highly dependent on cell type and the context of stimulation. TGF- β activates both protein kinases mammalian target of rapamycin complex 1 (mTORC1) and mTOR complex 2 (mTORC2) rapidly, both working in conjunction to support the mechanistic action of TGF- β ([22]).

The mTOR signaling pathway activation by TGF- β is controlled by the activation of cyclooxygenase-2 (COX-2), a bifunctional, rate-limiting enzyme that converts arachidonic acid to prostanoids. Extensive numbers of studies have shown that multiple cell-dependent signaling pathways regulate COX-2, which is induced by cytokines, tumor promoters, and growth factors, and TGF- β has been shown to induce the expression of this enzyme ([23,24]).

The pathway that is activated when TGF- β initiates the phosphorylation of mothers against decapentaplegic homolog 2 (Smad2) and Smad3, which bind and complex with Smad4. This complex is then recognized by the Smad-binding elements in the

nucleus and regulates gene expression in conjunction with other transcription factors ([25]). This increase in gene expression of Smad activates the phosphatidylinositol 3-kinase (PI3K), Protein Kinase B (AKT), and mTOR (PI3K/AKT/mTOR) signaling pathway by increasing COX-2 and Prostaglandin E_2 (PGE_2) activation ([22,24,25]). PGE_2 then activates a G-coupled protein increasing phosphorylation of AKT, mTOR, and its downstream targets such as S6 Kinase (S6K) ([25]). Figure 1 shows the proposed pathway of TGF-β activation in adipocytes adapted from Vo *et al*.

Increased mTORC1 signaling is associated with "pro-growth" signals including: extracellular matrix remodeling, a decrease in anti-inflammatory cytokines, and increase in pro-inflammatory cytokines. mTORC1 activation, however, is mediated by multiple factors, thus challenging the direct effect of TGF- β on this pathway ([25]). Research conducted by Martin *et al* found that phosphorylation of 4E-BP1/2, which influences the translation of proteins for cytokines. Activation of 4E-BP1/2 resulted in increases in anti-inflammatory IL-10 and decreases in pro-inflammatory Interleukin-12 (IL-12) ([26]). mTORC2 is associated with an increased phosphorylation of serine 473 of AKT ([22,26]), which is upstream of mTORC1, and has displayed characteristics of actin re-organization ([26]).

b. TGF- β function in adipocyte differentiation

A study conducted by Bortell *et al* determined the effect on adipocyte differentiation when treated with 100 pM TGF- β1 and showed the effects of cell proliferation and development on the 3T3-L1 pre-adipocytes. After treatment for 36-40 hours, the differentiation of the pre-adipocytes was blocked. The messenger Ribonucleic Acid (mRNA) expression and protein levels of collagen I and fibronectin were decreased, but not causing any physiological changes until later in differentiation, causing a switch in adipogenesis ([27]).

TGF- β KO mice, as discovered by Kulkarni *et al*, are born the same as their wild type (WT) littermates phenotypically, but begin to rapidly waste, losing half of their body weight by 2 weeks of age. They also have early onset of death between three and five weeks of age due to cardiopulmonary complications, triggered by inflammation induced by overwhelming macrophage infiltration ([28]). This suggests that TGF- β does indeed have a robust function that is cell-dependent.

c. TGF- β and cancer

Studies have linked TGF- β to tumorigenesis and the pro-growth response due to high levels of COX-2 expressed in the

cancer cell. In prostate cancer, TGF- β's effect on cell proliferation, migration, and invasion were studied, which demonstrated and confirmed the effects of TGF- β on AKT phosphorylation and COX-2 expression. TGF- β acts as a tumor suppressor in early stages of the cancer by inducing apoptosis, but in later stages, TGF- β demonstrates its pro-growth activity and becomes a tumor promoter ([24]).

Other research has found that suppression of TGF- β signaling and the use of therapeutic rapamycin, an inhibitor of cell proliferation, restored cancer cell apoptosis versus simply using rapamycin to block mTOR signaling. It is pivotal to suppress the apoptotic signals in early stages of cancer progression as a first line of defense ([29]). B. Law *et al* found similar results in both human and rodent cells showing rapamycin potentiates growth arrest that is induced by TGF- β in G_1 non-transformed epithelial cells and restores growth arrest in oncogene-transformed epithelial cells. They also found that TGF- β works in response to rapamycin, but the effects of rapamycin, especially on phosphorylation of S6K, are not changed by the presence of TGF- β ([30]).

d. TGF- β in obesity and other metabolic diseases

In a positively correlative manner with BMI, studies show that expressed levels of TGF- β are higher in obese subjects than

in their lean counterparts. TGF- β is a major inducer of plasminogen activator inhibitor 1 (PAI-1), which is a marker for subjects that have undergone a myocardial infarction or angina ([31]), signifying its relationship to heart disease.

Pfeiffer *et al* researched the relationship between circulating TGF- β levels and the incidence of type II diabetes mellitus, finding that plasma levels of TGF- β were positively correlated with glycosylated hemoglobin, which is a marker for DMII. They also found that the incidence of retinopathy and neuropathy was more prevalent in the subjects with higher plasma TGF- β ([32]). This indicated that there is an association between TGF- β and the growing complications presenting with obesity.

e. Role of TGF- β in fibrosis

TGF- β is strongly associated with synthesis of ECM protein in most fibrogenic cells and is an important regulator of the fibrotic response ([21,33]). It aids in controlling ECM proteins by stimulating production of the fibrogenic agents, such as collagen and fibronectin, and inhibiting degradation of ECM proteins ([14]).

Studies have shown that TGF- β mediates the fibrotic process, but under specific conditions, TGF- β can lead to dysfunction and cause alterations in fibrosis ([25]). Lamouille *et al* found that mTORC2 is related to cell invasion, which accompanies

degradation of the extracellular matrix, due to its association with upregulating MMP9 ([22]).

Fibrosis can occur over time when there is dysregulation in the homeostasis of the profibrotic and antifobrotic cytokines and proteins. Excessive scarring of tissues due to the production, deposition and contraction of an abundance of extracellular matrix proteins define fibrosis ([21]). The importance of the ECM is that it plays a primary role in mechanic support for tissues and when dysregulated, can cause necrosis, leaving the inert lipid droplet, stimulating lipodystrophy ([14]). As mentioned previously with regards to inflammation, the excess of these inert lipid droplets exacerbate insulin resistance and inflammation within the cell.

IV. Lipocalin 2

a. Lipocalin 2 background

Lipocalin 2 (Lcn2), also known as neutrophil gelatinase-associated lipocalin (NAGL), is a 25 kDa adipose-derived cytokine expressed in adipose tissue, belonging to a lipocalin subfamily that binds retinoids, fatty acids, and steroids ([1,2,5,34,3536-38],[3,39,40]). The structure of Lcn2 is similar to that for retinol binding proteins and fatty acid binding proteins, and contains a promoter region with binding sites for nuclear factor- kB (NFkB), glucocorticoid promoter region, and CCAAT/enhancer-binding protein (C/EBP). This

suggests that the adipokine may serve in adipose tissue remodeling and is influenced by inflammation and metabolic conditions which upregulate inflammation or dysfunction ([2,5]).

In research conducted by Jun *et al*, the relationship between wild type and Lcn2 KO male and female mice was examined. When fed a standard chow diet or high-fat diet, the wild type and Lcn2 KO mice showed no changes in phenotype ([5]). Contradicting their findings, Guo *et al* found that when fed a high-fat diet, Lcn2 KO mice are more obese than the wild type mice raised under the same conditions ([35]). Interestingly, studies conducted by I. Law *et al* showed that Lcn2 KO mice were significantly leaner than the WT mice under high fat diet fed conditions ([40]).

b. Lcn2 and inflammation

It was discovered that Lcn2 is secreted from adipocytes in a depot-selective and age-dependent manner. The presence of Lcn2 is upregulated in obesity, hyperlipidemia, infection, and hypoxia. The metabolic changes related to decreased Lcn2 and obesity is associated with increased insulin resistance and inflammation ([2,3,5]). The use of thiazolidinedione in obese mice significantly reduces circulating Lcn2 levels ([39]).

When researching the relationship between Lcn2 and macrophage polarization, evidence shows that Lcn2 deficiency

supports and increase in pro-inflammatory M1 macrophage markers and a decreased expression in the anti-inflammatory M2 macrophage markers. This suggests that Lcn2 plays a protective role against inflammation and macrophage polarization ([1]).

Guo, *et al* researched the effect of Lcn2 on adipogenesis and adipose tissue remodeling using Lcn2 knockout (KO) mice. The Lcn2 KO mice fed a high-fat diet display characteristics of increased adiposity, dyslipidemia, fatty liver disease, and IR ([2]). Lcn2 is significantly upregulated by pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and Lipopolysaccharide (LPS) in 3T3-L1 cells ([2,39]).

Other studies show that Lcn2 expression and secretion is not only regulated by cytokines, but also glucose, norepinephrine (NE), fatty acids, and insulin, although versus insulin, LPS is a more effective stimulant for Lcn2 expression and secretion ([3]). Lcn2 is upregulated by these stimulants, but the mechanism as to how Lcn2 regulates inflammation is still unclear.

c. Lcn2 and obesity

With support showing that adipose tissue is a major contributor to the synthesis and secretion of adipokines, such as Lcn2, that control the inflammatory response, glucose metabolism, and action of insulin, there is evidence that obesity alters the

normal functions of this organ ([39]). Research conducted by Zhang *et al* demonstrated that Zucker obese rats that had significantly increased mRNA levels of Lcn2 in both the liver and epididymal adipose tissue.

Due to its relationship with obesity and inflammation, Lcn2 has a suspected role in the remodeling of adipose tissue with changes in expression of ECM genes. Previous research in our lab found inguinal depots are more responsive to high-fat diet than epididymal in KO mice and feeding a high-fat diet led to an increase in fat cell size and decrease in number of fat cells present, impairing adipogenesis. Wild type mice fed a high-fat diet for 30 weeks showed an increase in cell count, indicating hyperplasia, versus the KO mice, which displayed an increase in the diameter of the cells, which would indicate hypertrophy ([2]).

Decreased adipogenesis, tissue fibrosis, and hypertrophy are all related to insulin resistance caused by obesity-related inflammation, due to the alterations in the glucose transporter type 4 (GLUT4) transporters for carbohydrate (CHO) metabolism and increased expression of pro-inflammatory cytokines ([2]). Human studies support these findings in that overweight subjects with type II diabetes had higher circulating levels of markers regulating inflammation such as TNFα and IL-8, as well as increased mRNA expression of diabetes predictors ([41]).

Research shows that there is a positive correlation between circulating Lcn2 levels and percentage of body fat percentage, blood pressure levels, insulin resistance, and altered lipid panels. Song *et al* researched this by measuring Lcn2 levels in obese mice and found positive correlations between these parameters; Wang *et al* found similar results in which circulating Lcn2 levels were reflective of increased adiposity, insulin resistance, and triglyceride levels ([38]).

Other studies have shown that circulating levels of Lcn2 are related to obesity in women, but do not serve as a marker for insulin resistance ([42]). In contrast, studies have also found that plasma Lcn2 levels do not correlate with markers of obesity or metabolic disease. Catalan *et al* reported that there were no differences between the plasma levels of Lcn2 in obese versus lean subjects, although there was a reported association between obesity and a Lcn2/MMP9 complex ([43]).

d. Lcn2 and cancer

Because of the role Lcn2 plays in inflammation and has been extensively studied as a tumor-associated protein, its connection as a complex with MMP9 is of relevance. Associations have been made between increased Lcn2 levels, especially the Lcn2/MMP9 complex, and the prevalence of cancer. Koh *et al*

found that in gastric cancer, Lcn2 and MMP9 expression levels were upregulated by hepatocyte growth factor (HGF), which increased the signaling of the PI3K/AKT/mTOR pathway. Gastric tumors were found to have increased Lcn2/MMP9 complex, suggesting increased invasion and progression of the tumor cells ([16]). Ruiz-Morales et al also found cancer stage-dependent increase in the Lcn2/MMP9 complex in lung adenocarcinoma ([34]). Other studies have confirmed that increased expression of Lcn2 is linked to increased incidence of liver ([44]), esophageal ([36]), prostate ([45]), colorectal ([46]), and oral cancers ([47]).

e. TGF- β and Lcn2 in activation of mTOR signaling

The role of TGF- β in the activation of mTOR signaling is related to its relationship with PGE_2. It is known from preliminary studies in our lab that the production of PGE_2 is increased in Lcn2 KO adipocytes, suggesting that the presence of Lcn2 may reduce the levels of PGE_2, thus decreasing the mTOR signaling. TGF- β also works to upregulate the pathway involving PGE_2, thus signaling in the Lcn2 KO mice, in conjunction with TGF- β would increase the mTOR signaling versus the wild type mice.

Other research from our lab shows that with a decreased expression of TGF- β in the inguinal adipose depots, there was a higher rate of hypertrophy present in the mice overall. The rationale

is that the decrease in hypertrophy is compensating for the ability of the depots to increase lipid storage capacity, undergoing adipogenesis ([2]).

In the wild type versus the Lcn2 KO mice, there were significant differences in mRNA expression and protein expression for extracellular matrix proteins, collagen I and III, fibronectin 1 and TGF- β in the epididymal adipose tissue KO mice when fed a high fat diet. In the SV fraction of epididymal adipose tissue, the opposite effect was found in that the Lcn2 KO mice, both collagen III and fibronectin were down-regulated versus their wild type equivalents; in inguinal adipose tissue, there were no significant alterations in the extracellular matrix genes when comparing the wild type and KO models ([2]).

V. Summary and Objectives

In summary, obesity causes metabolic distress and dysregulates cytokines and adipokines such as TGF- β and Lcn2. TGF- β is a pro-inflammatory cytokine upregulated in obesity, but its relationship with Lcn2, an adipokine that is also positively correlated with obesity is still unclear. Due to the qualities that both adipokines possess in extracellular matrix remodeling and inflammation, looking into the TGF- β effect in Lcn2 deficient mice may provide insight on these processes. Since this relationship has not been studied in adipose tissue, it is necessary to acquire more information about the function of

Lcn2 under inflammatory stimuli and the association with metabolic diseases and its metabolic consequences is needed.

Our hypothesis is that Lcn2 KO mice will have dysregulated extracellular matrix remodeling and altered pro-inflammatory and anti-inflammatory cytokine responses versus their wild type counterparts. Therefore, our objectives are to determine how Lcn2 is involved in the effect of TGF- β on inflammation and extracellular matrix remodeling in inguinal adipocytes and how Lcn2 KO will influence pro-inflammatory and anti-inflammatory cytokines and dysregulate ECM remodeling.

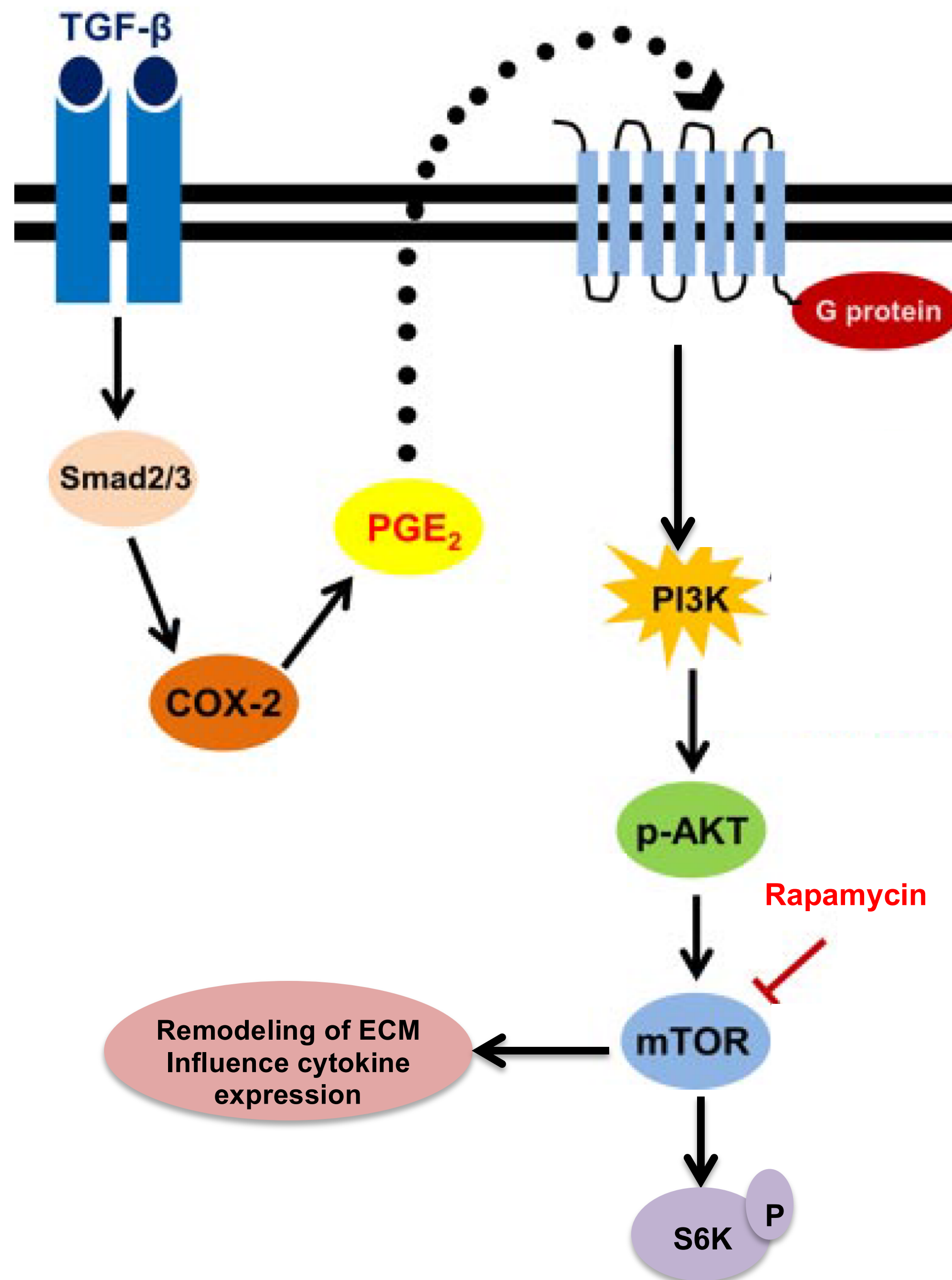


Figure 1. A proposed pathway suggesting TGF- β increases COX-2 and PGE_2 expression to activate the PI3K/AKT/mTOR signaling pathway in adipocytes, thus influencing remodeling of the ECM and dysregulation of cytokine expression.

CHAPTER 2

LIPOCALIN 2 DEFICIENCY INFLUENCES TGF-β EFFECT ON INFLAMMATION AND EXTRACELLULAR MATRIX REMODELING IN INGUINAL ADIPOCYTES

I. Introduction

As the prevalence of overweight and obesity continue to rise globally, so does the risk of obesity-related diseases such as type II diabetes mellitus (DMII), insulin resistance (IR), cancers, heart disease and death ([4,6]). Obesity is commonly associated with caloric surplus, genetics, metabolic stress, aging, and medications, although obesity is considered a preventable disease ([2,3]); the imbalance created between energy intake and expenditure is the major contributor to the accumulation of excess fat mass, leading to the development of obesity.

White adipose tissue (WAT) serves as the main site for energy storage and works as an endocrine organ to regulate energy homeostasis, inflammatory response, the secretion of adipokines and cytokines, and to control the action of insulin. Adipose tissue also has mechanical function to protect major organs from damage by serving as insulation. There are two forms of energy storage in the functional adipocyte, hypertrophy, which is an increase in cell size, versus hyperplasia, which is an increase in number of adipocytes ([2,4,14].

Although both forms of storage, hyperplasia and hypertrophy, increase the amount of total adipose tissue, adipocyte hypertrophy is more closely associated with increased occurrence of apoptosis, hypoxia, and cytokine secretion as well as dysregulation of fatty acid homeostasis ([14]). Adipocyte dysfunction associated with obesity is present when cells are hypertrophic, hypoxic, and/or have

increased inflammation due to decreased access to vascularization. Chronic low-grade inflammation present in hypertrophic obesity has the ability to cause remodeling of the adipose tissue due to the increased presence of macrophages and pro-inflammatory cytokines. Dysfunction of the extracellular matrix (ECM) is a result of the macrophage infiltration, adipocyte hypertrophy, and ECM remodeling ([4]). In obesity, remodeling occurs more frequently and rapidly, excelling the natural progressive progress of fibrosis and decreasing the efficiency of adipocyte function and metabolic homeostasis of adipose tissue ([6,14]).

Transforming Growth Factor-β (TGF- β) is a cytokine released from macrophages that is increased in obesity and plays a major role in ECM remodeling of tissues. The regulatory action of TGF- β includes tissue growth and development, immunity, cell differentiation, production of the ECM, and cell cycle control ([21,22]). TGF- β activates both protein kinases mammalian target of rapamycin complex 1 (mTORC1) and mTOR complex 2 (mTORC2) rapidly ([22]) by indirectly activating the phosphatidylinositol 3-kinase (PI3K), Protein Kinase B (AKT), and mammalian target of rapamycin (mTOR) (PI3K/AKT/mTOR) signaling pathway ([22,24,25]).

Increased mTOR complex 1 (mTORC1) signaling is associated with "pro-growth" signals including: ECM remodeling, a decrease in anti-inflammatory cytokines, and increase in pro-inflammatory cytokines. TGF- β aids in controlling ECM proteins by stimulating production of the fibrogenic agents, such as collagen and fibronectin, and inhibiting degradation of ECM proteins through

activation of mTOR. The ECM plays a primary role in mechanic support for tissues and when dysregulated, can leave increased inert lipid droplets ([14]). Excess inert lipid droplets in circulation exacerbate IR and inflammation within the cell ([9]).

Lipocalin 2 (Lcn2), also known as neutrophil gelatinase-associated lipocalin (NAGL), is a 25 kDa adipose-derived cytokine expressed in adipose tissue, belonging to a lipocalin subfamily that binds retinoids, fatty acids, and steroids ([1-3,5,34-38,40]). The structure of Lcn2 is similar to that for retinol binding proteins and fatty acid binding proteins, and contains binding sites for nuclear factor- kB (NFkB) and CCAAT/enhancer-binding protein (C/EBP), as well as glucocorticoid responsive element within the promoter region ([2,5]).

Research shows that there is a positive correlation between circulating Lcn2 levels and percentage of body fat percentage, blood pressure levels, insulin resistance, and altered lipid panels ([37,38]). In our previous studies, Lcn2 is shown to play a role in adipose tissue remodeling, inflammation, and adipocyte dysfunction ([1,2,5]). We also found that TGF- β expression in adipose tissue was dysregulated in Lcn2 KO mice ([2]), suggesting a potential link between Lcn2 and TGF- β in terms of their function in adipose tissue remodeling.

Due to the qualities that both adipokines possess the role in extracellular matrix remodeling and inflammation, looking into the TGF- β effect on the regulation of ECM and inflammatory cytokines in Lcn2 deficient adipocytes may provide insight on these processes. Since this relationship has not been studied in adipose tissue, it is necessary to acquire more information about the function

of Lcn2 under inflammatory stimuli and the association with metabolic diseases is needed.

We hypothesize that Lcn2 deficiency leads to dysregulated extracellular matrix remodeling and altered pro-inflammatory and anti-inflammatory cytokine responses via modulating the action of TGF- β. Therefore, our objectives are to determine how Lcn2 regulates the effect of TGF- β on inflammation and extracellular matrix remodeling in inguinal adipocytes. We performed the experiments to address how Lcn2 KO influences the response of adipocytes to TGF- β in the production of pro-inflammatory and anti-inflammatory cytokines and ECM proteins.

Our results demonstrate that TGF- β down-regulates Lcn2 expression at both the mRNA and protein levels in inguinal adipocytes. Lcn2 KO adipocytes have lower levels of TGF- β expression, but normal levels of ribosomal protein S6 kinase (p70S6K) phosphorylation and normal response to TGF- β stimulation in mTORC1 signaling activation. However, the inhibitory effect of rapamycin on TGF- β expression is attenuated in Lcn2 KO adipocytes. Moreover, Lcn2 KO impairs the rapamycin potentiation of TGF- β effect on the expression of ECM proteins, but shows little effect on dysregulation of cytokines.

II. Results

Lcn2 expression in inguinal adipocytes is regulated by TGF- β

It is known that the presence of Lcn2 in white adipose tissue (WAT) is depot- specific, as well as gender-, and age dependent ([5]). Previous research indicates that there is an ample amount of Lcn2 in inguinal WAT in mice fed either a high-fat diet or regular chow; in these studies, protein expression and messenger Ribonucleic Acid (mRNA) expression of Lcn2 was more abundant in the inguinal WAT versus the epididymal WAT ([1,3]). Due to the depot-specific response, we examined the effect of TGF- β on Lcn2 gene expression and protein expression in inguinal stromal vascular (SV) adipocytes. SV cells of wild type (WT) and Lcn2 KO mice were isolated from inguinal adipose tissue and cultured in vitro. Differentiation was induced and matured adipocytes were treated with TGF- β. In response to 10 ng/mL TGF-β treatment for 24 hours, mRNA expression of Lcn2 decreased significantly ($p<0.0005$), as shown in Figure 1A. Consistent with the mRNA expression, protein expression of Lcn2, when treated with TGF- β, rapamycin, or both, showed a decrease in expression from the basal condition (Figure 1B). Protein levels of Lcn2 in wild type (WT) adipocytes were increased, interestingly, when treated with 10 ng/mL TGF- β for 1 hour versus the control, but when treated for 24 hours,

protein expression decreased in the TGF- β group versus the control, which is consistent with the mRNA expression (Figure 1C). Figure 1D indicates that TGF- β also has an impact on Lcn2 secretion in WT adipocytes. Lcn2 secretion was decreased in the 1 hour, 4 hour, and 24 hour treatment groups versus the WT control. These results indicate that Lcn2 expression in inguinal adipocytes is regulated by TGF- β in a time-dependent manner.

TGF- β expression is decreased, but TGF- β action on mTOR signaling activation is unaffected in Lcn2 KO inguinal adipocytes.

In our previous research, we have shown that mRNA levels of TGF- β were decreased more in the Lcn2 knock out (KO) mice fed a high-fat diet than in the WT mice ([2]). Figure 2A shows our results of mRNA expression in inguinal SV differentiated adipocytes support these findings showing decreased levels in the Lcn2 KO versus the WT adipocytes ($p<0.01$). However, when treated with 10 ng/mL TGF- β for 15, 30, and 60 minutes, WT and Lcn2 KO adipocytes similarly display a time-dependent increase in the phosphorylation of p70S6K, indicating that Lcn2 adipocytes have a normal response to TGF- β in mTOR signaling activation (Figure 2B). This suggests that the TGF- β effect on mTOR signaling activation is not impaired, although TGF- β expression is decreased in Lcn2 KO inguinal adipocytes.

Previous research indicated that cyclooxygenase-2 (COX-2) expression is upregulated by cytokines, such as TGF- β ([23]), and is the rate-limiting enzyme that increases prostaglandin (PG) synthesis, such as prostaglandin E_2 (PGE_2). PGE_2 activates a G-coupled protein that activates the PI3K/AKT/mTOR signaling pathway ([24]). Therefore, we also looked at protein expression of COX-2 to determine the TGF- β effect on the activation of the PI3K/AKT/mTOR signaling pathway. Interestingly, we found that COX-2 protein levels were higher in Lcn2 KO adipocytes than in WT adipocytes under the basal condition (Figure 3A). When treated with TGF- β for 1, 4 and 24 hours in WT and Lcn2 KO adipocytes, Figure 3A showed that that TGF- β treatment exacerbates the time-dependent upregulation of COX-2 seen in both WT and Lcn2 KO adipocytes, as the most significant upregulation in protein expression was seen after 24 hours with TGF- β treatment. Moreover, TGF- β had a more significant effect on upregulation of COX-2 in Lcn2 KO adipocytes than the WT adipocytes at the 24-hour time point (Figure 3A). These results indicate the TGF- β upregulation of the COX-2 protein expression after 24 hours correlated with the activation of the mTOR signaling pathway. These results also suggest that Lcn2 deficiency does not affect TGF- β action on COX-2 expression and mTOR signaling activation.

As indicated by other research in human carcinoma cells, rapamycin potentiates the effect of TGF- β in a dose-dependent manner ([30]). Rapamycin is a known mTOR inhibitor, especially with regards to

mTORC1 signaling. Activation of mTOR is evidenced by phosphorylation of downstream targets such as p70S6K ([25]). To determine how Lcn2 deficiency affects rapamycin regulation of TGF- β expression in SV differentiated inguinal adipocytes, we assessed the mRNA expression levels of TGF- β in both WT and Lcn2 KO adipocytes when treated with 25 nM rapamycin for 24 hours. When evaluating the mRNA expression in WT adipocytes, rapamycin significantly inhibits TGF- β expression ($p<0.05$), however in Lcn2 KO adipocytes the inhibitory effect of rapamycin is disrupted, as evidenced by a significant increase in TGF- β when treated with rapamycin ($p<0.05$) (Figure 2A).

To determine the impact of Lcn2 deficiency on the effect of rapamycin, we detected the protein levels of p70S6K phosphorylation. Mature adipocytes were collected after 1 hour and 24 hours of treatment with TGF- β and rapamycin. Figure 3B shows that rapamycin blocks the phosphorylation of p70S6K in both WT and Lcn2 KO adipocytes in the presence or absence of TGF- β, with a more significant decrease in the Lcn2 KO adipocytes, especially after 1 hour of treatment with TGF- β compared to 24 hour treatment, whereas total S6K remained consistent (Figure 3B). Treatment with TGF- β alone led to the upregulation of p70S6K in both WT and Lcn2 KO adipocytes, although phosphorylation of S6K is greater in the Lcn2 KO adipocytes than the WT after 1 hour. The data indicates that Lcn2 deficiency does not influence the inhibitory effect

of rapamycin on mTOR signaling by evidence of reduced phosphorylation of p70S6K in both WT and Lcn2 KO adipocytes at a similar level.

The effect of TGF- β and rapamycin on inflammation in Lcn2 KO adipocytes

The above results indicate that rapamycin has an inhibitory effect on TGF- β evidenced by decreased expression of TGF- β when adipocytes were treated with rapamycin versus the control. Research indicated that TGF- β and mTOR signaling interact, which is inhibited by rapamycin, and rapamycin enhances the action of TGF- β ([24,25,29,40]). Since increased mTORC signaling, which is associated with "pro-growth" signals causes dysregulation of the ECM remodeling and inflammatory cytokines ([25]), it is imperative to determine if Lcn2 deficiency alters the effect of TGF- β and rapamycin on pro- and anti-inflammatory cytokines ([1]). Therefore, we determined the regulation of pro-inflammatory cytokines such as Interleukin-6 (IL-6), tumor necrosis factor α (TNFα), and Monocyte Chemoattractant Protein-1 (MCP1) in WT and Lcn2 KO adipocytes treated with TGF- β for 20 hours. With regards to the effect of TGF- β on the WT and Lcn2 control cells, Figure 4A shows there is a significant decrease in MCP1 in both groups ($p<0.05$, $p<0.0001$, respectively), but not in TNFα or IL-6. mTOR signaling was then blocked using rapamycin with TGF- β,

which resulted in similar results in that there was no significant effect of TGF- β in WT or Lcn2 KO cells for TNFα or IL-6 (Figure 4B and 4C, respectively), but there was a significant decrease in MCP1 ($p<0.0001$). There is not a significant difference between the WT and Lcn2 KO adipocytes in any of the pro-inflammatory cytokines, suggesting that Lcn2 deficiency does not have a significant affect on the TGF- β effect on MCP-1, TNFα or IL-6. There was, however, a significant increase ($p<0.001$) in IL-6 and MCP-1 expression as a result of rapamycin treatment independent of TGF- β in both WT and Lcn2 KO adipocytes (Figure 4A and 4C, respectively). Interestingly, we saw that IL-6 expression also increased more in the Lcn2 KO adipocytes compared to the WT adipocytes ($p<0.05$) in response to rapamycin. This implies that Lcn2 impairs the rapamycin effect on gene expression of IL-6 (Figure 4C).

We also determined the mRNA expression of M2 macrophage markers in adipocytes treated after differentiation for 20 hours with TGF- β alone or in combination with rapamycin. When looking at M2 macrophage markers, Macrophage mannose receptor 1 (MRC-1) and Arginase 1 (Arg1), there is a significant decrease in mRNA expression in MRC-1 gene expression in Lcn2 KO and WT adipocytes when treated with TGF- β ($p<0.005$, $p<0.05$, respectively)(Figure 4D). Rapamycin either alone or in combination with TGF- β does not affect MRC-1 mRNA expression (Figure 4D). There is also no difference in MRC-1 expression between WT and Lcn2 KO adipocytes in response to TGF- β and/or rapamycin. The mRNA

expression of Arginase 1 showed differential regulation of TGF- β as significant increases in WT adipocytes, but not in the Lcn2 KO adipocytes. The Lcn2 adipocytes, however, did show an increase in mRNA expression of Arg1 in response to the TGF- β, but it was not significant (Figure 4E). Furthermore, WT and Lcn2 KO adipocytes show a significantly different response to the treatment of TGF- β and rapamycin in combination. When treated with TGF- β and rapamycin together, the mRNA expression of Arg1 was significantly decreased in WT adipocytes ($p<0.05$), but increased in Lcn2 KO adipocytes ($p<0.05$) (Figure 4E). This implies that Lcn2 does impair the effect of TGF- β and rapamycin on gene expression of Arg1.

The effect of TGF- β and rapamycin on extracellular matrix in Lcn2 KO adipocytes

Because TGF- β is strongly associated with synthesis of ECM protein in most fibrogenic cells and is an important regulator of the fibrotic response ([21,33]), its relationship with Lcn2, which also has a know relationship with adipose tissue remodeling due to its ability to regulate high fat diet-induced adipogenic defect and inflammation ([2,5]) was of interest. Hence, we investigated the effect of TGF- β and rapamycin on ECM proteins in Lcn2 KO adipocytes. The mRNA expression of proteins supporting the ECM, Collagen 1A1 (Col1A1), Collagen 3A1 (Col3A1), and matrix metallopeptidase 9 (MMP9), were evaluated when treated with

TGF- β and rapamycin for 20 hours. With regards to the effect of TGF – β in both WT and Lcn2 KO adipocytes, there were significant decreases in Col3A1 and significant increase in MMP9 that were independent of Lcn2 deficiency ($p<0.0001$); there was no significant change in Col1A1 gene expression compared to the controls (Figure 5A, 5B and 5C, respectively).

When mTOR signaling was blocked with rapamycin, a significant difference in the effect of TGF- β between WT and Lcn2 KO adipocytes were seen for both MMP9 (Figure 5B) and Col1A1 (Figure 5C) ($p<0.005$). While rapamycin significantly augmented the stimulatory effect of TGF- β on MMP9 and Col1A1 expression in WT adipocytes, rapamycin has much less of an effect in Lcn2 KO adipocytes. This suggests that Lcn2 deficiency impairs TGF- β and mTOR signaling interaction. However, there were no significant changes in mRNA expression of Col3A1 (Figure 5A). There was a decreased response in the Lnc2 KO adipocytes versus the WT adipocytes when treated with both TGF- β and rapamycin, as well as a significant increase in mRNA expression of Col3A1as a result of rapamycin treatment alone in the Lcn2 KO adipocytes compared to the WT adipocytes ($p<0.05$) as shown in Figure 5A.

When treated with 10 ng/mL TGF- β for 1, 4 and 24 hours in WT adipocytes and 24 hours in Lcn2 KO adipocytes, MMP9 protein expression displayed abnormal results as seen in Figure 5D. TGF- β significantly upregulated protein expression of MMP9 in WT adipocytes treated for 1 and 4 hours, as well as Lcn2 KO adipocytes treated for 24

hours. However, WT adipocytes treated with TGF- β for 24 hours showed a significant decrease in protein expression of MMP9 versus the control. These results contradict the MMP9 mRNA expression seen in Figure 5B which displays a 4.7-fold increase from the basal conditions in both WT and Lcn2 KO adipocytes when treated with TGF- β alone, and when treated with TGF- β and rapamycin, an 8.8-fold increase in Lcn2 KO adipocytes and 10.8-fold increase in WT adipocytes. We hypothesize that decreased MMP9 protein levels could be due to a feedback mechanism that decreases mRNA expression once MMP9 transcription has been significantly upregulated, thus giving opposite results in protein expression and mRNA expression. However, MMP9 protein levels were markedly upregulated by TGF- β treatment for 20 hours in Lcn2 KO adipocytes (Figure 5D).

From these experiments, we concluded that Lcn2 deficiency does not significantly affect the TGF- β effect on gene expression of the ECM. Lcn2 deficiency, however, does impair the rapamycin effect on ECM proteins, particularly in the presence of TGF- β. Combined, these results suggest that mTOR signaling is involved in Lcn2 regulation of the TGF- β effect on ECM remodeling and cytokine production, but the exact mechanism is still unknown.

III. Discussion

TGF- β's growing role in ECM remodeling in tissues and known relationship to activation of the mTOR signaling pathway has made this an inflammatory cytokine of interest ([21,22,24,25]). In our previous studies, dysregulation of TGF- β has been suggested to potentially contribute to impaired ECM remodeling in adipose tissue of Lcn2 KO mice. In this study, we further explored the role of TGF- β on Lcn2 expression in inguinal adipocytes to obtain a better understanding of the function of Lcn2. In WT adipocytes, where the presence of Lcn2 is abundant ([2,3]), we observed the effect of TGF- β treatment on mRNA levels of Lcn2; WT adipocytes treated with TGF- β for 24 hours showed a significant decrease in mRNA expression and protein expression compared to the control. This suggests that TGF- β regulates Lcn2 expression in inguinal adipocytes, although the exact mechanism is still unknown.

TGF- β is known to increase the expression of rate-limiting enzyme COX-2 in various different cell types, which in turn activates the PI3K/AKT/mTOR signaling pathway ([23,24]), thus increasing mTOR signaling and phosphorylation of its downstream targets such as S6K ([22,25]). Our previous results show

significantly decreased mRNA levels of TGF- β in adipose tissue of Lcn2 KO mice fed a high-fat diet compared to the WT mice, indicating that Lcn2 has a role in expression of TGF- β ([2]). Our results in WT and Lcn2 KO adipocytes concur with previous findings showing a significant decrease in mRNA expression. However, when the WT and Lcn2 KO adipocytes were treated with TGF- β for a short time period (15-60 minutes), as well as a longer time-period (1hr-24hrs), normal phosphorylation of p70S6K was displayed, suggesting that although TGF-β expression is decreased, deficiency of Lcn2 in adipocytes does not have an effect on mTOR signaling activation. However, when WT and Lcn2 KO adipocytes were treated with TGF- β for 1, 4, and 24 hours, COX-2 protein expression did show a significant increase in the 24-hour treatment group, with a greater upregulation in the Lcn2 KO adipocytes than in the WT adipocytes. The COX-2 protein expression levels were higher in the Lcn2 KO adipocytes than in the WT adipocytes, even in the basal state. This upregulation in COX-2 expression was also seen in prostate cancer cells ([24]) and human mesangial cells ([23]), suggesting increased activation of mTOR. However, in this study, upregulation of COX-2 expression does not correlate with an increase in phosphorylation of p70S6K, implying that COX-2 may not be the only pathway that mediates TGF- β activation of mTORC1. It is also possible that COX-2 is involved in the activation of mTORC2, which phosphorylates AKT on site Ser473 ([22]). Increased upregulation of COX-2 in Lcn2 KO adipocytes versus the WT adipocytes indicates that Lcn2 may negatively regulate COX-2 expression.

In fibroblasts, human target of rapamycin was found to inhibit the activity of mTORC1, and not mTORC2, when stimulated with TGF- β treatment ([25]). Another study found that the action of TGF- β alone or rapamycin alone had no significant effect on Mv1Lu transformed cells, but when treating the cells with both TGF- β and rapamycin together, there was a significant, dose-dependent effect on cell proliferation ([30]), suggesting an interaction between TGF- β and mTOR signaling pathways. We then investigated if this pathway interaction is affected in Lcn2 adipocytes. First, we studied the impact of Lcn2 deficiency on the rapamycin effect of TGF- β. We found that rapamycin significantly decreases the mRNA expression of TGF- β in the WT adipocytes, but significantly increases mRNA expression of TGF- β in the Lcn2 KO adipocytes. Moreover, treatments with rapamycin, or both TGF- β and rapamycin for 1 hour led to a significant decrease in phosphorylation of p70S6K in both WT and Lcn2 KO adipocytes when treated with rapamycin or both TGF- β and rapamycin, with a slightly more significant decrease seen in Lcn2 adipocytes than the WT. After 24 hours of treatment with TGF- β and rapamycin, p70S6K phosphorylation was significantly decreased in Lcn2 KO adipocytes compared to WT adipocytes in the basal and TGF- β treated states. Rapamycin or rapamycin plus TGF- β completely blocks p70S6K phosphorylation in both WT and lcn2 KO adipocytes. There was no significant difference in the effect of rapamycin and TGF- β treatment on mTOR signaling activation between the WT and Lcn2 KO adipocytes after 24 hours. Therefore, these results indicate that Lcn2 does not affect the inhibitory effect of rapamycin on mTOR signaling.

Although there was no difference between WT and Lcn2 KO adipocytes with regards to the effect of rapamycin inhibiting mTOR signaling when treated with TGF- β, rapamycin did have a significant inhibitory effect on mTOR signaling when treated with TGF- β in adipocytes. Other research has demonstrated the inhibitory effect of rapamycin in cancer cells ([24,29,30]) and fibroblasts ([25]), and has found a relationship between increased mTOR signaling and dysregulation of inflammatory cytokines ([25,26]) and ECM remodeling ([25]). Thus, identifying the relationship between TGF- β treatment and inflammation in WT and Lcn2 KO adipocytes is of interest. When treated with TGF- β, the chemokine responsible for macrophage infiltration, MCP1, showed a significant decrease in mRNA expression. The effect of TGF- β was not exacerbated when treated together with rapamycin, although there was a significant increase when treated with only rapamycin. There was however, no difference in the response between WT and Lcn2 KO adipocytes. In pro-inflammatory cytokines, TGF- β treatment alone or with rapamycin had no significant effect on mRNA expression of TNFα and IL-6 and no difference between the WT and Lcn2 KO adipocytes. Because there was no significant difference between the WT and Lcn2 KO adipocytes, we conclude that Lcn2 does not have an effect on the TGF- β- mediated inflammatory response in adipocytes. Rapamycin, however, independent of TGF- β may have a significant effect on inflammatory cytokines with regards to its significant increase in both MCP-1 and IL-6 gene expression, especially in IL-6, where a significant difference was seen between the increase in WT and Lcn2 KO

adipocytes. This suggests that Lcn2 deficiency impairs the rapamycin effect on gene expression with regards to cytokine IL-6.

Next we examined the effect of TGF- β on M2 macrophage markers, Arg1 and MRC-1 in Lcn2 KO adipocytes. The mRNA expression in Arg1 and MRC-1 were significantly increased and significantly decreased, respectively, with a more profound increase in the WT adipocytes than the Lcn2 KO adipocytes in Arg1 expression. When mTOR signaling was blocked using rapamycin with the TGF- β treatment, there was a significantly different response in Arg1 expression between WT and Lcn2 KO adipocytes. For instance, blocking mTOR signaling activation leads to a significant decrease in TGF- β induction of Arg1 expression in WT adipocytes, but an increase in Lcn2 KO adipocytes. This notably significant difference in response between the WT and Lcn2 KO adipocytes when treated with TGF- β and rapamycin implying that Lcn2 deficiency impairs the effect of rapamycin and TGF- β interaction on gene expression of macrophage marker, Arg1.

Lcn2 KO adipocytes were found to have increased mRNA expression levels of collagens and fibronectin compared to WT type adipocytes ([2]) with a suspected role of TGF- β in controlling local concentrations of ECM proteins ([21]). Suggesting that Lcn2 has a role in ECM remodeling due to decreased TGF- β levels in inguinal adipocytes displaying increased hypertrophy and depot expansion ([2]), the effect of TGF- β and rapamycin on ECM in WT and Lcn2 adipocytes is of interest. Therefore, we researched the mRNA expression in ECM proteins Col1A1, Col3A1, and MMP9 after treatment with TGF- β,

rapamycin, and TGF- β and rapamycin. Collagen proteins Col1A1 and Col3A1 showed opposite results of each other with regards to gene expression when treated with TGF- β for 24 hours. Significant decreases in Col3A1 and no significant change in Col1A1 with TGF- β treatment led us to investigate the effect of rapamycin. When treated with TGF- β and rapamycin, Col1A1 showed a significant increase in gene expression, in WT, but not Lcn2 adipocytes, whereas Col3A1 showed a significant decrease in gene expression when treated with TGF- β and rapamycin with a significant difference between the WT and Lcn2 KO adipocytes; in both collagen proteins, the WT adipocytes displayed significantly higher gene expression than the Lcn2 KO adipocytes. This suggests that Lcn2 plays a role in regulating the interaction between mTOR and TGF- β signaling pathways in terms of their effect on ECM protein expression.

Of note, MMP9 showed a significant increase in mRNA expression with TGF- β treatment and further increase as a result of TGF- β treatment blocked with rapamycin. Moreover, there was a significant difference between the WT and Lcn2 KO adipocytes, with a lesser increase in Lcn2 KO adipocytes. This suggests that Lcn2 deficiency impairs TGF- β and mTOR signaling interaction with regards to MMP9 mRNA expression. However, the effect of TGF β and rapamycin on MMP9 protein expression contradicts the gene expression. When treated with TGF- β, there was a time-dependent increase in MMP9 protein expression for WT adipocytes at 1 and 4 hours, but a decrease in expression at 24 hours. For the Lcn2KO adipocytes treated with TGF- β for 24 hours, there was, however, an increase in protein expression of MMP9 expression. This

suggests that there may be a feedback mechanism regulating the protein expression of MMP9, controlled by gene expression. Therefore, we concluded that Lcn2 deficiency does not significantly affect the TGF- β effect on gene expression of the ECM protein such as Col1A1, Col3A1, and MMP9, but does interfere with rapamycin potentiation of the TGF- β effect. These results, however, do indicate that mTOR signaling is involved in Lcn2 regulation of the TGF- β effect on ECM remodeling.

In conclusion, this research has demonstrated that TGF- β down-regulates Lcn2 expression at both the mRNA and protein expression levels in inguinal adipocytes. Furthermore, Lcn2 KO adipocytes have lower levels of TGF- β expression, but normal levels of S6K phosphorylation and normal response to TGF- β stimulation in mTORC1 signaling activation. Research has also shown the exacerbated effect of TGF- β when mTOR signaling is blocked by rapamycin; however, we have shown that the inhibitory effect of rapamycin on TGF- β expression is attenuated in Lcn2 KO adipocytes, specifically. Moreover, Lcn2 KO impairs the rapamycin potentiation of TGF- β effect on the expression of ECM proteins, but shows little effect on dysregulation of cytokines. Other studies have shown that Lcn2 deficiency increases ECM remodeling and fibrosis in adipose tissue, with a more profound response in inguinal adipose tissue with regards to adipocyte hypertrophy when mice were fed a high-fat diet. Combined, our results indicate that Lcn2 plays a role in the inhibitory effect of rapamycin on TGF- β expression and mTOR signaling activation.

In future research, investigating complex-specific mTOR signaling in response to TGF- β and rapamycin would be of interest to see if there is a relationship to specific signaling pathways with regards to ECM remodeling and inflammatory cytokine dysregulation. It would also be beneficial to conduct similar research, using TGF- β in whole tissue or whole-body relations to systemic, low-grade inflammation to see if there is a different response when other organs are involved; on a larger spectrum, research investigating the application to human obesity and chronic inflammation, and how the TGF- β and mTOR signaling pathway could be manipulated to reduce systemic low-grade inflammation and ECM remodeling. Lastly, further research would be needed to investigate the detailed mechanism of how Lcn2 affects the mTOR signaling pathway to influence the ECM and inflammation.

IV. Materials and Methods

a. Animal studies

C57BL/6 WT and Lcn2 KO mice provided by Dr. Alan Aderem (Institute for Systems Biology, Seattle, Washington), which were originally generated by Dr. Shizuo Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, were used in this study. Through gene targeting the Lcn2 KO mice were generated in embryonic stem cells from the mouse strain 129, and the targeted embryonic stem cells were injected into C57BL/6 blastocysts ([35]) as described in research done by Flo, *et al.* C57BL/6 mice were purchased form the Jackson Laboratory (Bar Harbor, ME). Before the mice were used for experiments, the Lcn2-null mice were backcrossed onto the B6 background for 10 generations. A heterozygous mating scheme was used to generate WT and Lcn2 KO mice for the experiments.

Animals were housed in a specific pathogen-free facility at the University of Minnesota. Animal handling followed the National Institutes of Health guidelines, and experimental procedures were approved by the University of Minnesota Animal Care and Use Committee. The mice were allocated into groups (3-4 mice per cage) and fed a regular chow diet, with free access to water in all studies. At 14 weeks of age, the mice were killed and adipose tissues were collected for the isolation of stromal-vascular (SV) cells.

b. Isolation of primary adipocytes and SV cells

Primary adipocytes and SV cells were isolated from WT and Lcn2 KO mice. After mincing, inguinal fat pads were digested with collagenase (2 mg/mL solution) in digestion vials containing Krebs-Ringer bicarbonate HEPES buffer (pH 7.4), 200 nM adenosine, and 3.5% BSA. After a 2-hour digestion, adipocytes and SV cells were separated by centrifugation at 1200 rpm for 10 minutes and washed twice with Krebs-Ringer bicarbonate HEPES buffer. After the final wash, SV cells were collected and cultured.

c. Cell Culture

SV cells isolated from inguinal adipose tissue of WT and Lcn2 KO mice were cultured in DMEM with 10% bovine calf serum (Sigma Aldrich, Saint Louis, MO) and 100 IU/mL

penicillin/streptomycin (Invitrogen, Carlsbad, CA) until confluent. Two days after confluence, the cells were induced for differentiation with using a differentiation cocktail containing 10% fetal bovine serum (Sigma Aldrich, Saint Louis, MO), and 390 ng/mL dexamethasone (Sigma Aldrich, Saint Louis, MO). The differentiation cocktail was replaced by 10% fetal bovine serum, 100 IU/mL penicillin/streptomycin and 1 ug/mL insulin after two days. The cells were cultured for six days following. On day 7 of differentiation, the adipocytes were starved in DMEM with 1 mg/mL glucose and 0.5% bovine calf serum for 4 hours, then treated with 10 μg/mL insulin and 10 ng/mL TGF-β (R & D System, Minneapolis, MN), 25 nM rapamycin, or both treatments for various amounts of time as noted in the figure legends. Both conditioned media and cells were collected for protein or mRNA detection.

d. RNA Extraction for Quantitative real-time RT-PCR

Total RNA was extracted from adipocytes using TRIzol reagent (Intvitrogen, Carlsbad, CA) according to manufacturer's instructions. After treatment with RQ1 DNase (Promega, Madison, WI), 1 μg RNA was used to synthesize the cDNA in reverse transcription with 5 mM oligo dT primer, 0.5 uM dNTP, 20 units RNasin Ribonuclease Inhibitor (Promega, Madison, WI), 5 mM DTT, and 100 units SuperScript II Reverse Transcriptase

(Intvitrogen, Carlsbad, CA). Teal-time-PCR was performed using SYBR GreenER qPCR SuperMix Universal kit (Intvitrogen, Carlsbad, CA) with an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Results were analyzed using the software supplied with the 7500 system and presented as levels of expression relative to that of controls after normalizing to TBP-2 using the ΔΔCt method ([3]).

e. Protein Preparation for Western Blotting

Adipocytes were solubilized in a lysis buffer containing 25 nM Tris-HCl pH 7.5, 0.5 mM EDTA, and 25 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1% Nonidet P-40 and protease inhibitor cocktails (Diagnostic Roche, Branchburg, NJ). The lysates were centrifuged at 12,000 g for 10 minutes, and supernatants were collected. Protein concentrations of lysates were detected with bicinchoninic acid method (Pierce, Rockford, IL). Equivalent proteins or same volume of conditioned media were loaded and separated on SDS-PAGE and then electro-transferred to nitrocellulose membranes. Membranes were incubated with anti-Lcn2 (R&D System, Minneapolis, MN), anti- MMP9 (R & D System, Minneapolis, MN), anti-p70S6K (Cell Signaling Technology, Danvers, MA), and anti-actin (Cell Signaling Technology, Danvers, MA) antibodies after blocking with TTBS (20 nM Tris-HCl pH 7.5, 0.5 M NaCl, 0.1% Tween-20) containing 5% skim milk (Fisher

Scientific, Pittsburgh, PA). The membranes were then washed in TTBS and incubated with corresponding secondary antibodies conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The signals were detected by ECL plus Western Blotting Detecting Reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) ([3]).

f. Statistical analysis

The results are expressed as mean ± SEM. Differences in parameters between WT and Lcn2 KO adipocytes were evaluated using a two-group *t* test with a 0.05 two-sided significance level. Significance was considered a *P* value <0.05.

BIBLIOGRAPHY

1. Guo H, Jin D, Chen X. Lipocalin 2 is a regulator of macrophage polarization and NF-kappaB/STAT3 pathway activation. *Mol Endocrinol*. 2014;28(10):1616-1628. doi: 10.1210/me.2014-1092 [doi].

2. Guo H, Bazuine M, Jin D, Huang MM, Cushman SW, Chen X. Evidence for the regulatory role of lipocalin 2 in high-fat diet-induced adipose tissue remodeling in male mice. *Endocrinology*. 2013;154(10):3525-3538. doi: 10.1210/en.2013-1289 [doi].

3. Zhang Y, Foncea R, Deis JA, Guo H, Bernlohr DA, Chen X. Lipocalin 2 expression and secretion is highly regulated by metabolic stress, cytokines, and nutrients in adipocytes. *PLoS One*. 2014;9(5):e96997. doi: 10.1371/journal.pone.0096997 [doi].

4. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol*. 2011;11(2):85-97. doi: 10.1038/nri2921 [doi].

5. Jun LS, Siddall CP, Rosen ED. A minor role for lipocalin 2 in high-fat diet-induced glucose intolerance. *Am J Physiol Endocrinol Metab*. 2011;301(5):E825-35. doi: 10.1152/ajpendo.00147.2011 [doi].

6. Wernstedt Asterholm I, Tao C, Morley TS, et al. Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling. *Cell Metab*. 2014;20(1):103-118. doi: 10.1016/j.cmet.2014.05.005 [doi].

7. Patel P, Abate N. Role of subcutaneous adipose tissue in the pathogenesis of insulin resistance. *J Obes*. 2013;2013:489187. doi: 10.1155/2013/489187 [doi].

8. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature*. 2006;444(7121):847-853. doi: nature05483 [pii].

9. Chaves VE, Frasson D, Kawashita NH. Several agents and pathways regulate lipolysis in adipocytes. *Biochimie*. 2011;93(10):1631-1640. doi: 10.1016/j.biochi.2011.05.018 [doi].

10. Tchkonia T, Thomou T, Zhu Y, et al. Mechanisms and metabolic implications of regional differences among fat depots. *Cell Metab*. 2013;17(5):644-656. doi: 10.1016/j.cmet.2013.03.008 [doi].

11. Villena JA, Cousin B, Penicaud L, Casteilla L. Adipose tissues display differential phagocytic and microbicidal activities depending on their localization. *Int J Obes Relat Metab Disord*. 2001;25(9):1275-1280. doi: 10.1038/sj.ijo.0801680 [doi].

12. Matsuzawa Y, Shimomura I, Nakamura T, Keno Y, Tokunaga K. Pathophysiology and pathogenesis of visceral fat obesity. *Ann N Y Acad Sci*. 1995;748:399-406.

13. Lee YH, Mottillo EP, Granneman JG. Adipose tissue plasticity from WAT to BAT and in between. *Biochim Biophys Acta*. 2014;1842(3):358-369. doi: 10.1016/j.bbadis.2013.05.011 [doi].

14. Sun K, Kusminski CM, Scherer PE. Adipose tissue remodeling and obesity. *J Clin Invest*. 2011;121(6):2094-2101. doi: 10.1172/JCI45887 [doi].

15. Toubal A, Clement K, Fan R, et al. SMRT-GPS2 corepressor pathway dysregulation coincides with obesity-linked adipocyte inflammation. *J Clin Invest*. 2013;123(1):362-379. doi: 10.1172/JCI64052 [doi].

16. Koh SA, Lee KH. HGF mediated upregulation of lipocalin 2 regulates MMP9 through nuclear factor-kappaB activation. *Oncol Rep*. 2015. doi: 10.3892/or.2015.4189 [doi].

17. Lumeng CN, DelProposto JB, Westcott DJ, Saltiel AR. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal

differences in macrophage subtypes. *Diabetes*. 2008;57(12):3239-3246. doi: 10.2337/db08-0872 [doi].

18. Xu H, Barnes GT, Yang Q, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest*. 2003;112(12):1821-1830. doi: 10.1172/JCI19451 [doi].

19. Zhu Y, Tchkonia T, Stout MB, et al. Inflammation and the depot-specific secretome of human preadipocytes. *Obesity (Silver Spring)*. 2015;23(5):989-999. doi: 10.1002/oby.21053 [doi].

20. Dalmas E, Clement K, Guerre-Millo M. Defining macrophage phenotype and function in adipose tissue. *Trends Immunol*. 2011;32(7):307-314. doi: 10.1016/j.it.2011.04.008 [doi].

21. Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. *FASEB J*. 2004;18(7):816-827. doi: 10.1096/fj.03-1273rev [doi].

22. Lamouille S, Connolly E, Smyth JW, Akhurst RJ, Derynck R. TGF-beta-induced activation of mTOR complex 2 drives epithelial-mesenchymal transition and cell invasion. *J Cell Sci*. 2012;125(Pt 5):1259-1273. doi: 10.1242/jcs.095299 [doi].

23. Rodriguez-Barbero A, Dorado F, Velasco S, Pandiella A, Banas B, Lopez-Novoa JM. TGF-beta1 induces COX-2 expression and PGE2 synthesis through

MAPK and PI3K pathways in human mesangial cells. *Kidney Int*. 2006;70(5):901-909. doi: 5001626 [pii].

24. Vo BT, Morton D,Jr, Komaragiri S, Millena AC, Leath C, Khan SA. TGF-beta effects on prostate cancer cell migration and invasion are mediated by PGE2 through activation of PI3K/AKT/mTOR pathway. *Endocrinology*. 2013;154(5):1768-1779. doi: 10.1210/en.2012-2074 [doi].

25. Rahimi RA, Andrianifahanana M, Wilkes MC, et al. Distinct roles for mammalian target of rapamycin complexes in the fibroblast response to transforming growth factor-beta. *Cancer Res*. 2009;69(1):84-93. doi: 10.1158/0008-5472.CAN-08-2146 [doi].

26. Martin S, Saha B, Riley JL. The battle over mTOR: An emerging theatre in host-pathogen immunity. *PLoS Pathog*. 2012;8(9):e1002894. doi: 10.1371/journal.ppat.1002894 [doi].

27. Bortell R, Owen TA, Ignotz R, Stein GS, Stein JL. TGF beta 1 prevents the down-regulation of type I procollagen, fibronectin, and TGF beta 1 gene expression associated with 3T3-L1 pre-adipocyte differentiation. *J Cell Biochem*. 1994;54(2):256-263. doi: 10.1002/jcb.240540214 [doi].

28. Kulkarni AB, Karlsson S. Transforming growth factor-beta 1 knockout mice. A mutation in one cytokine gene causes a dramatic inflammatory disease. *Am J Pathol*. 1993;143(1):3-9.

29. Gadir N, Jackson DN, Lee E, Foster DA. Defective TGF-beta signaling sensitizes human cancer cells to rapamycin. *Oncogene*. 2008;27(8):1055-1062. doi: 1210721 [pii].

30. Law BK, Chytil A, Dumont N, et al. Rapamycin potentiates transforming growth factor beta-induced growth arrest in nontransformed, oncogene-transformed, and human cancer cells. *Mol Cell Biol*. 2002;22(23):8184-8198.

31. Alessi MC, Bastelica D, Morange P, et al. Plasminogen activator inhibitor 1, transforming growth factor-beta1, and BMI are closely associated in human adipose tissue during morbid obesity. *Diabetes*. 2000;49(8):1374-1380.

32. Pfeiffer A, Middelberg-Bisping K, Drewes C, Schatz H. Elevated plasma levels of transforming growth factor-beta 1 in NIDDM. *Diabetes Care*. 1996;19(10):1113-1117.

33. Rockey DC, Bell PD, Hill JA. Fibrosis--A common pathway to organ injury and failure. *N Engl J Med*. 2015;373(1):96. doi: 10.1056/NEJMc1504848 [doi].

34. Ruiz-Morales JM, Dorantes-Heredia R, Arrieta O, Chavez-Tapia NC, Motola-Kuba D. Neutrophil gelatinase-associated lipocalin (NGAL) and matrix metalloproteinase-9 (MMP-9) prognostic value in lung adenocarcinoma. *Tumour Biol*. 2015;36(5):3601-3610. doi: 10.1007/s13277-014-2997-3 [doi].

35. Guo H, Jin D, Zhang Y, et al. Lipocalin-2 deficiency impairs thermogenesis and potentiates diet-induced insulin resistance in mice. *Diabetes*. 2010;59(6):1376-1385. doi: 10.2337/db09-1735 [doi].

36. Du ZP, Wu BL, Xie YM, et al. Lipocalin 2 promotes the migration and invasion of esophageal squamous cell carcinoma cells through a novel positive feedback loop. *Biochim Biophys Acta*. 2015;1853(10 Pt A):2240-2250. doi: S0167-4889(15)00243-8 [pii].

37. Song E, Fan P, Huang B, et al. Deamidated lipocalin-2 induces endothelial dysfunction and hypertension in dietary obese mice. *J Am Heart Assoc*. 2014;3(2):e000837. doi: 10.1161/JAHA.114.000837 [doi].

38. Wang Y, Lam KS, Kraegen EW, et al. Lipocalin-2 is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans. *Clin Chem*. 2007;53(1):34-41. doi: clinchem.2006.075614 [pii].

39. Zhang J, Wu Y, Zhang Y, Leroith D, Bernlohr DA, Chen X. The role of lipocalin 2 in the regulation of inflammation in adipocytes and macrophages. *Mol Endocrinol*. 2008;22(6):1416-1426. doi: 10.1210/me.2007-0420 [doi].

40. Law IK, Xu A, Lam KS, et al. Lipocalin-2 deficiency attenuates insulin resistance associated with aging and obesity. *Diabetes*. 2010;59(4):872-882. doi: 10.2337/db09-1541 [doi].

41. Samaras K, Botelho NK, Chisholm DJ, Lord RV. Subcutaneous and visceral adipose tissue gene expression of serum adipokines that predict type 2 diabetes. *Obesity (Silver Spring)*. 2010;18(5):884-889. doi: 10.1038/oby.2009.443 [doi].

42. Auguet T, Quintero Y, Terra X, et al. Upregulation of lipocalin 2 in adipose tissues of severely obese women: Positive relationship with proinflammatory cytokines. *Obesity (Silver Spring)*. 2011;19(12):2295-2300. doi: 10.1038/oby.2011.61 [doi].

43. Catalan V, Gomez-Ambrosi J, Rodriguez A, et al. Increased adipose tissue expression of lipocalin-2 in obesity is related to inflammation and matrix metalloproteinase-2 and metalloproteinase-9 activities in humans. *J Mol Med (Berl)*. 2009;87(8):803-813. doi: 10.1007/s00109-009-0486-8 [doi].

44. Chung IH, Chen CY, Lin YH, et al. Thyroid hormone-mediated regulation of lipocalin 2 through the Met/FAK pathway in liver cancer. *Oncotarget*. 2015;6(17):15050-15064. doi: 3670 [pii].

45. Ding G, Fang J, Tong S, et al. Over-expression of lipocalin 2 promotes cell migration and invasion through activating ERK signaling to increase SLUG expression in prostate cancer. *Prostate*. 2015;75(9):957-968. doi: 10.1002/pros.22978 [doi].

46. Barresi V, Reggiani-Bonetti L, Di Gregorio C, Vitarelli E, Ponz De Leon M, Barresi G. Neutrophil gelatinase-associated lipocalin (NGAL) and matrix

metalloproteinase-9 (MMP-9) prognostic value in stage I colorectal carcinoma. *Pathol Res Pract*. 2011;207(8):479-486. doi: 10.1016/j.prp.2011.05.012 [doi].

47. Peng X, Li W, Johnson WD, Torres KE, McCormick DL. Overexpression of lipocalins and pro-inflammatory chemokines and altered methylation of PTGS2 and APC2 in oral squamous cell carcinomas induced in rats by 4-nitroquinoline-1-oxide. *PLoS One*. 2015;10(1):e0116285. doi: 10.1371/journal.pone.0116285 [doi].

APPENDICES

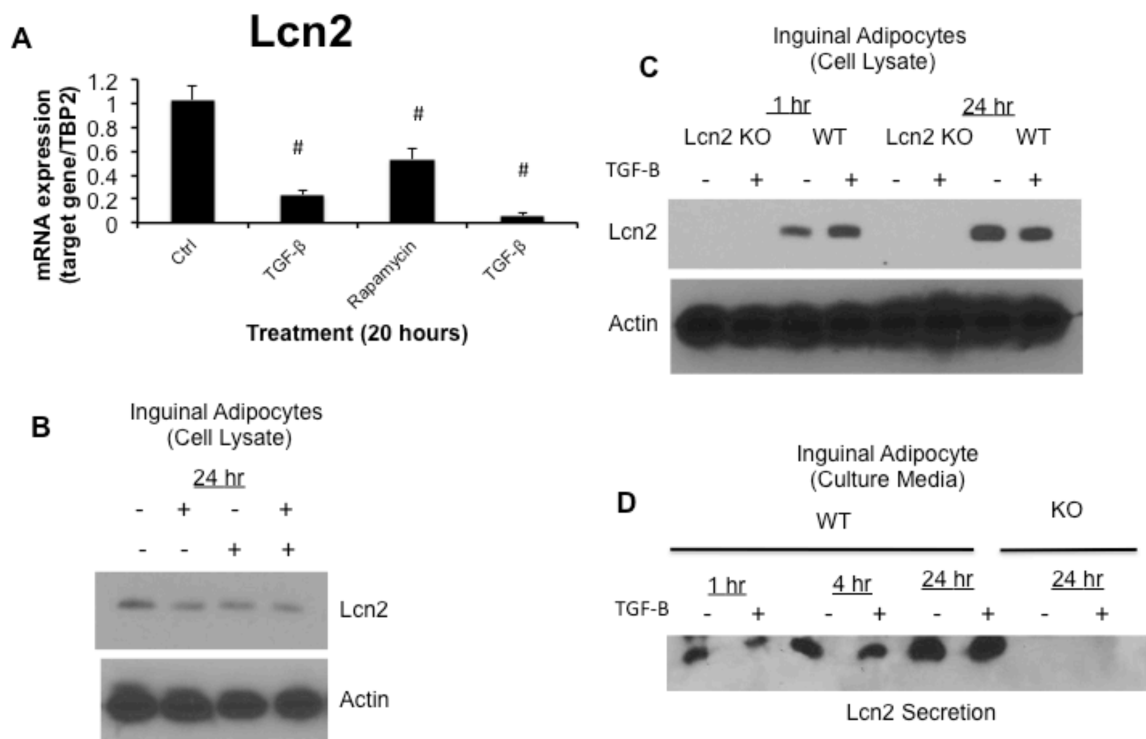
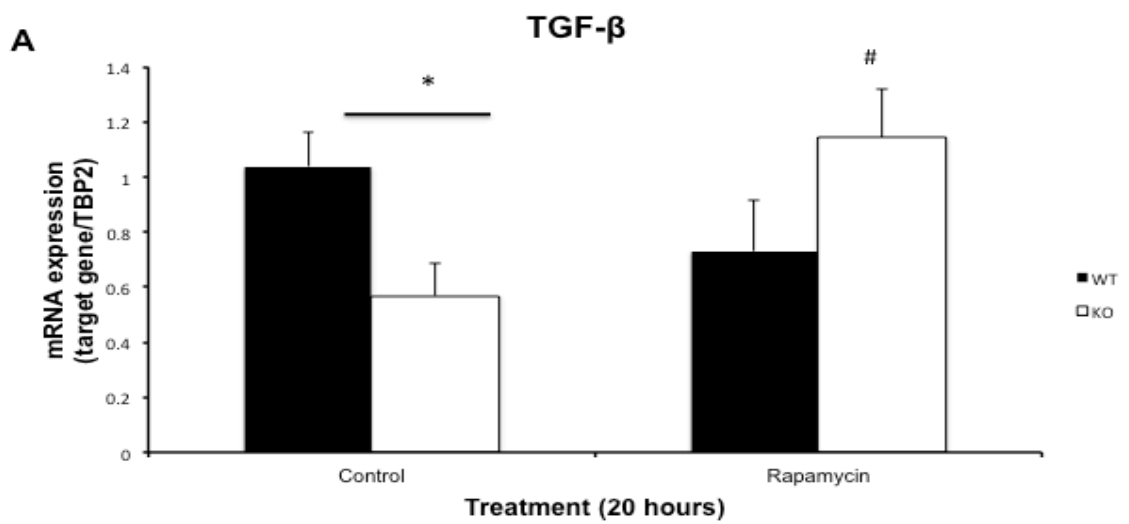


Figure 1. A shows the mRNA expression of Lcn2 in WT adipocytes when treated with TGF- β, rapamycin, and TGF- β + rapamycin for 20 hours. Results indicate mean ± SE. #, $p < 0.05$ from WT control. B and C, Lcn2 protein expression in WT adipocytes treated with TGF- β, rapamycin, or TGF- β and


A
TGF-β
mRNA expression
(target gene/TBP2)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
#
WT
KO
Control
Rapamycin
Treatment (20 hours)

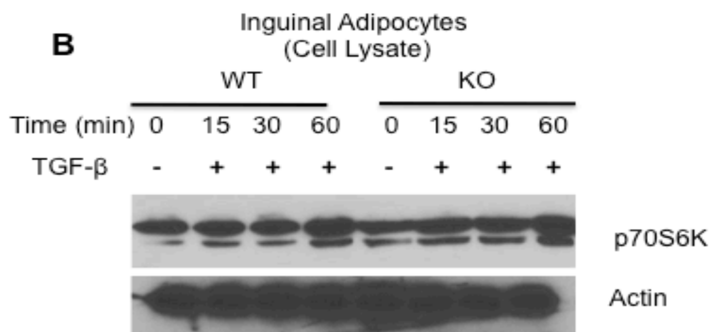

B
Inguinal Adipocytes
(Cell Lysate)
WT
KO
Time (min) 0 15 30 60 0 15 30 60
TGF-β - + + + - + + +
p70S6K
Actin

Figure 2. A, the mRNA expression of TGF- β in WT and Lcn2 KO adipocytes when treated with rapamycin for 20 hours. Results indicate mean ± SE. #, $p < 0.05$ from WT control; *, $p < 0.05$, WT vs. Lcn2 KO. B, p70S6K protein expression in WT and Lcn2 KO adipocytes when treated with TGF- β for 15,30, 60 minutes.

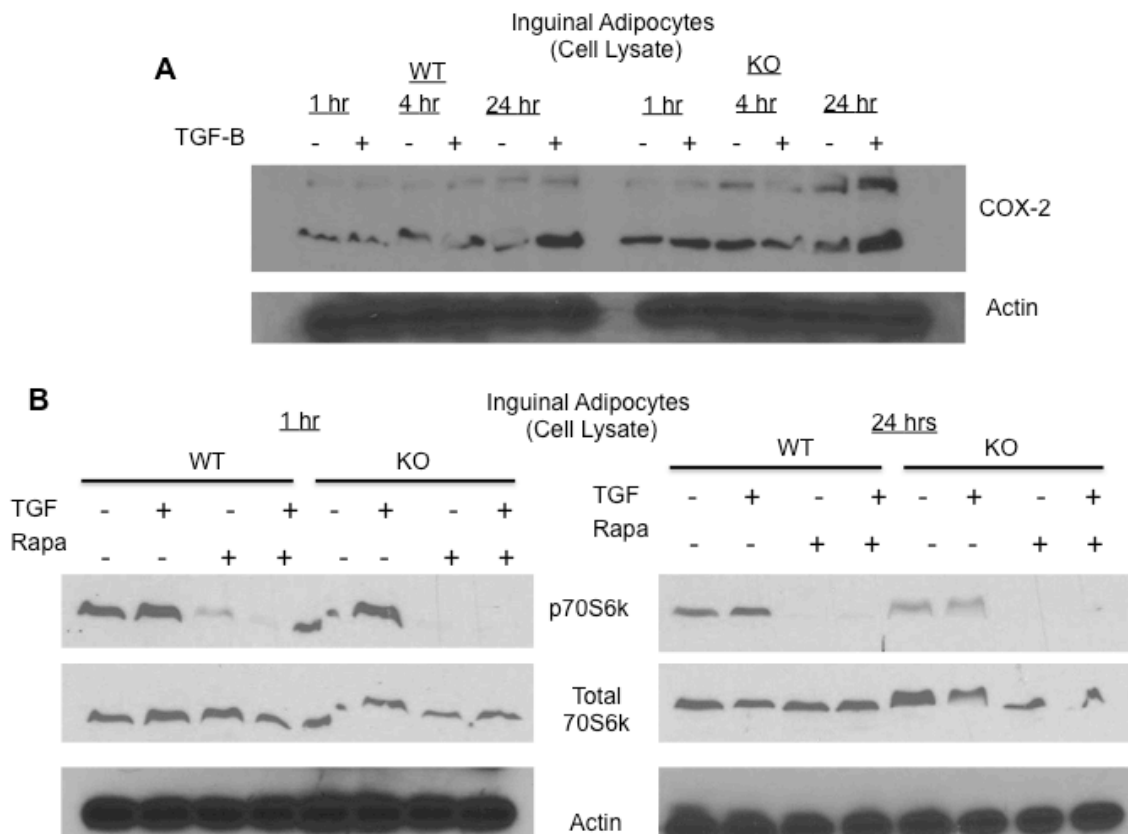


Figure 3. A, protein expression of COX-2 in WT and Lcn2 KO adipocytes when treated with TGF- β for 1, 4, or 24 hours. B, protein expression of p70S6k and total S6K in WT and Lcn2 KO adipocytes treated with TGF- β, rapamycin, or

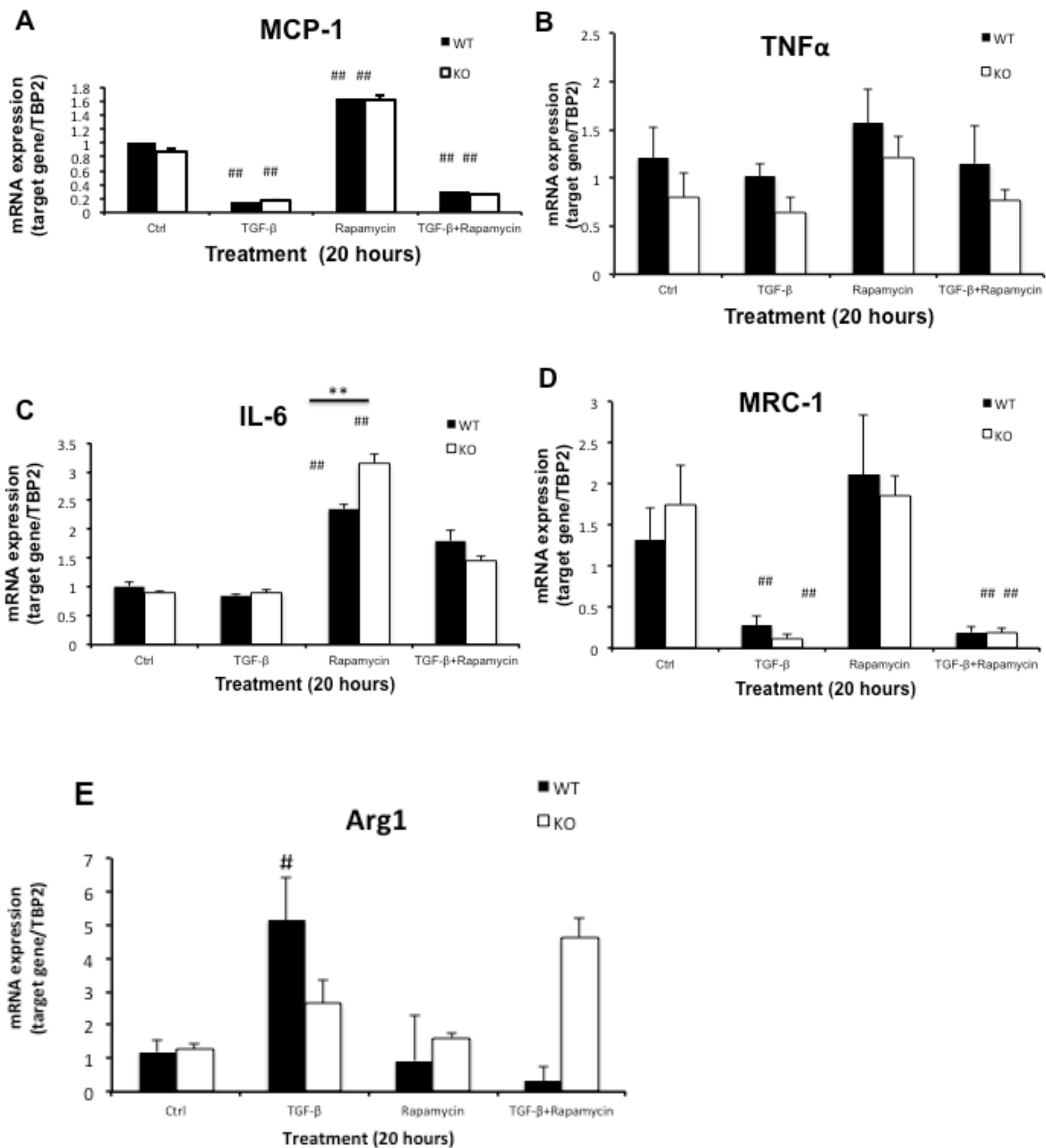


Figure 4. A-E, the mRNA expression of MCP-1 (A), TNFα (B), IL-6 (C), MRC-1 (D), Arg1 (E) in WT and Lcn2 KO adipocytes, when treated with TGF- β, rapamycin, or TGF- β and rapamycin for 20 hours. Results indicate mean ± SE. #, $p < 0.05$; ##, $p < 0.005$ from WT control; *, $p < 0.05$; **, p , 0.005, WT vs. Lcn2 KO.

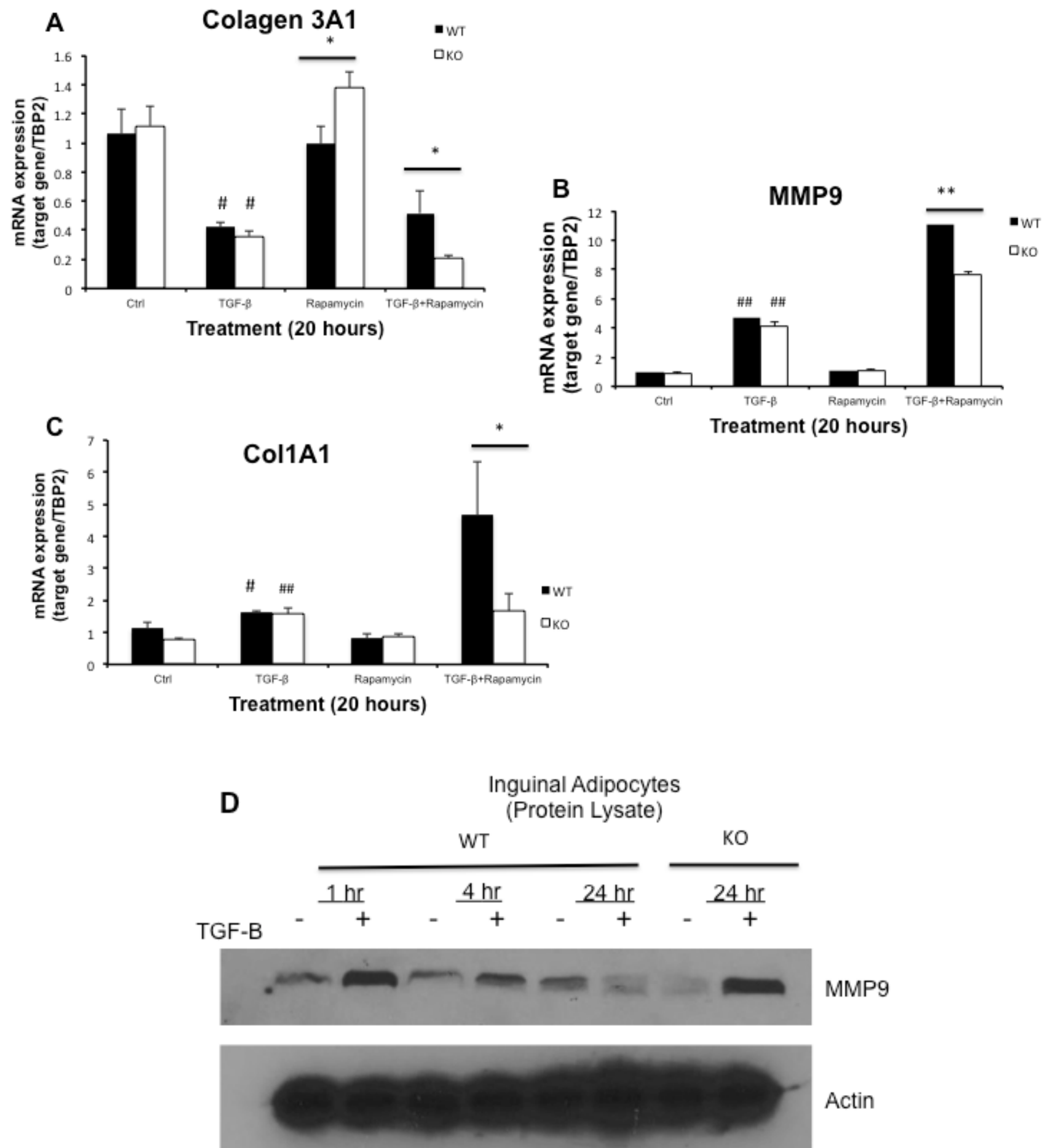


Figure 5. A-C, the mRNA expression of Col3A1 (A), MMP9 (B), Col1A1 (C) in WT and Lcn2 KO adipocytes when treated with TGF- β, rapamycin, or TGF- β and rapamycin for 20 hours. Results indicate mean ± SE. #, $p < 0.05$; ##, $p < 0.005$ from WT control; *, $p < 0.05$; **, p , 0.005, WT vs. Lcn2 KO. D, protein expression of MMP9 expression in WT and Lcn2 KO adipocytes treated with TGF- β for time periods as noted.