Insights on adipose tissue extracellular matrix remodeling: Models of diet-induced obesity and weight loss

by

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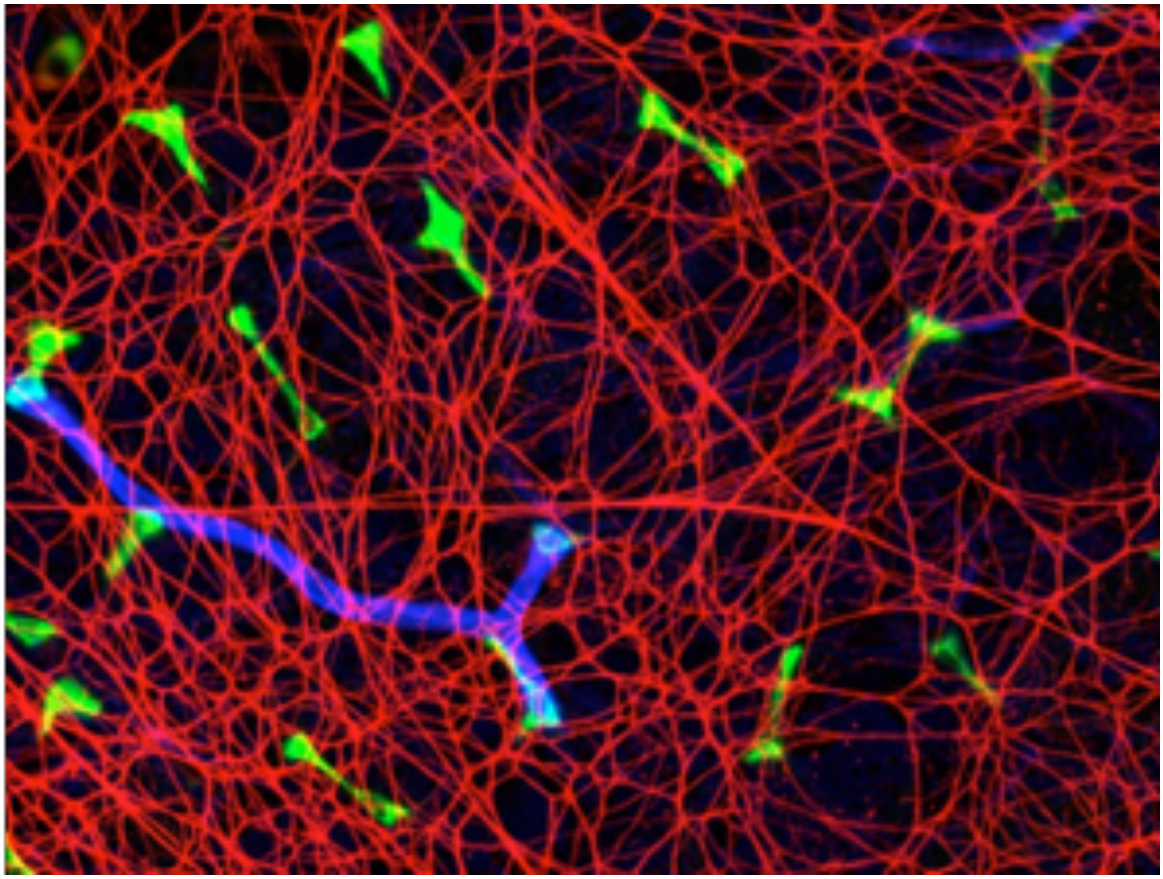

DEDICATION

To my family, who encouraged me to pursue opportunities they never had.

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ADAM, a disintegrin and metalloproteinase
ANOVA, analysis of variance
ATMs, adipose tissue macrophages
BMI, body mass index
C/EBPa, CCAAT/enhancer binding protein alpha
CD, cluster of differentiation
CLS, crown-like structure
CM, conditioned media
COL, collagen
COX, cyclooxygenase
DGAT1
DIO, diet-induced obesity
ECM, extracellular matrix
eWAT, epididymal white adipose tissue
FGF, fibroblast growth factor
GTT, glucose tolerance test
HFD, high fat diet
HGF, human growth factor
HIF1a, hypoxia inducible factor
IGF, insulin growth factor
IHC, immunohistochemistry
IL, interleukin
IP, intraperitoneal
IRS, insulin receptor substrate
ITT, insulin tolerance test
iWAT, inguinal white adipose tissue
LOX, lysyl oxidase
M0, unpolarized macrophage
M1, classically-activated macrophage
M2, resident macrophage
MCP, monocyte chemotactic protein
MGL, macrophage galactose lectin
MLI, mean linear intercept
MMe, metabolically-activated macrophage
MMP, matrix metalloproteinase
mWAT, mesenteric white adipose tissue
ND, normal diet
PAI, plasminogen activator inhibitor

PBS, phosphate buffered saline
PCR, polymerase chain reaction
PDGFR, platelet-derived growth factor
PPAR, peroxisome proliferator-activated receptor
PreAds, preadipocytes
RNA, ribonucleic acid
ROCK, rho-associated coil-coil containing protein kinase
rWAT, peri-renal white adipose tissue
SCA, stem cell antigen
SEM, standard error of the mean
SPARC, secreted protein acidic and rich in cysteine
SVF, stromal vascular fraction
TGF, transforming growth factor
TIMP, tissue inhibitor of metalloproteinase
TNF, tumor necrosis factor
u-PA, plasminogen activator
VEGF, vascular endothelial growth factor
WL, weight loss

ABSTRACT

Obesity and chronic low-grade inflammation are linked to the development of insulin resistance and type 2-diabetes. A component to the response to obesity is the accumulation of extracellular matrix (ECM) in adipose tissue in what is known as adipose tissue fibrosis, which limits the mechanical flexibility of the ECM and promotes metabolic disease by inhibiting metabolic adaptation by altering adipocyte function. Currently, the dynamics of ECM reorganization in adipose tissue with obesity and the sources of ECM components are not well understood. To address this, we investigated the changes in ECM architecture that occur with obesity and weight loss. We also explored the role of adipose tissue macrophages (ATMs) in influencing ECM remodeling in adipose tissues. Experiments addressed the organization of elastin, a relatively unexplored adipose tissue ECM component, and its response to diet induced obesity in mice. Our observations revealed ATM interactions with a dynamic mesh-like elastin network that becomes denser in adipose tissue of obese animals. The elastin matrix was found to be remodeled by obesity independently of MMP-12 and that an MMP-12 deficiency does not influence whole body metabolism. As another major component of adipose tissue ECM, we investigated the source of the excessive collagen production in adipose tissue in the context of weight gain and weight loss, with the hypothesis that ATM-preadipocyte communication is central to ECM production. Preadipocytes ($CD31^{-}CD45^{-}Sca1^{+}PDGFR\,\alpha^{+}$) were found to be a primary source of fibrillar type I collagen, and that obesity expanded the number of collagen-expressing cells, but also induced its expression at a per cell level. Investigations into mechanisms that activate pro-fibrotic preadipocytes revealed that tissue resident macrophages are

a major source of TGF β re and that TGF β an signaling is required for induction of collagen I production in preadipocytes. We also reveal that unexpectedly, weight loss significantly increases fibrotic ECM deposition, and this was associated with persistent adipose tissue metabolic dysfunction despite normalization of body weight and fat pad mass. Data suggests collagen I and elastin- producing preadipocytes increase in density after weight loss. We conclude that preadipocytes are a major source of collagen type I and elasin with both weight gain and weight loss. Our results suggest that ATMs are a source of TGF β and that it is required for activation of a collagen I production in preadipocytes. This data provides insights into how physiologic and pathophysiologic remodeling differs in adipose tissue and identifies potential nodes for intervention to break the link between obesity and disease.

Chapter 1

Introduction: The Regulation of Adipose Tissue Remodeling

Abstract

The ability of adipose tissue to adapt to a changing nutrient environment is critical to the maintenance of metabolic control. Nutrient excess and deficiency alter the shape of adipose tissue drastically and trigger many events that are collectively known as adipose tissue remodeling. The interest in adipose tissue remodeling has been accelerated by the current epidemic of obesity and its associated morbidities including diabetes and metabolic syndrome. Excess adipose tissue ECM deposition has recently been recognized as a hallmark of obesity associated with metabolic disease. This chapter summarizes and highlights the recent work in this area and provides a framework in which to consider how the remodeling events in lean and obese states, with information about the potential role adipose tissue macrophages (ATMs) play in this process. Advancing our understanding of the involvement of macrophages in adipose tissue remodeling will enhance our understanding of one aspect of the new field of "immunometabolism" which connects control systems developed for regulation of immunity with those that control

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metabolism. It also provides insights into how physiologic and pathophysiologic remodeling differs in adipose tissue and identifies potential nodes for intervention to break the link between obesity and disease.

Introduction

As the body's main site of nutrient storage, adipose tissue must be able to quickly respond to both nutrient deprivation and excess. These responses place demands on adipose tissue to be able to dynamically react to the changing nutrient environment. This dynamic alteration of adipose tissue structure, size, and shape is generally referred to as adipose tissue remodeling. While the regulation of energy uptake and fatty acid release by adipocytes is well understood at the molecular and cellular level, a supporting network of non-adipocyte cells and extracellular components provides an additional layer of control over adipocyte function. These stromal components include immune cells such as macrophages, and non-immune cells like vascular endothelial cells and preadipocytes, all of which participate in the regulation of adipocyte function. Understanding the properties of this complex supporting network in adipose tissue has become increasingly important due to the current obesity epidemic. Being overweight or obese can result in adipose tissue dysfunction, which contributes to metabolic disease by ultimately shifting lipid accumulation to non-adipose tissues (e.g. liver and muscle) (217). This introduction focuses primarily on current understandings of the changes in the ECM that occur with obesity that contribute to adipocyte function and dysfunction.

Defining adipose tissue remodeling

The concept of adipose tissue remodeling has been applied broadly to encompass the range of events that occur to adipose tissue depots in response to nutrient excess or deficiency (112, 201, 202) **(Figure 1-1).** Remodeling has been used to describe the changes in the cellular composition of the adipose tissue stromal cell network in response to both adipose tissue expansion and contraction (202, 203). Others use this term to describe the deposition and breakdown of the non-cellular components of adipose tissue that contribute to its structure (e.g. the ECM). To add to this complexity, adipose tissue remodeling has been used to describe the changes in adipose tissue structure in both physiological/adaptive settings (e.g. embryonic development, puberty, pregnancy, and weight loss (67, 106, 156) as well as changes that occur in pathologic settings (e.g. obesity or lipodystrophy (201).

We broadly define adipose tissue remodeling as the process by which adipose tissue changes shape, size, structure, and composition. This definition includes the stromal and ECM changes referenced above, but also encompasses changes in adipocyte size (hypertrophy) and the generation of new adipocytes (hyperplasia/adipogenesis). It also includes alterations in adipose tissue angiogenesis that are important regulators of nutrient delivery to fat. Like most complex systems, these events are all highly interdependent. For example, adipogenesis requires ECM degradation (41), angiogenesis (56), and the coordination with signals from ATMs (114).

Adipocyte maintenance

Two primary mechanisms contribute to the volumetric expansion of adipose tissue. With an acute excess of nutrient intake, enlargement in the size of individual adipocytes (hypertrophy) can occur rapidly due to an increase in lipid (triglyceride) stores. Alternatively, adipose tissue can expand by generating new adipocytes (hyperplasia) from a preadipocyte precursor population. The relative balance between these processes appears to be highly dependent on the location of the fat pad in an organism. For example, in rats, visceral fat depots appear to expand primarily by hypertrophy, while subcutaneous fat depots expanded primarily via hyperplasia (50). Recent lineage tracking studies in mice show that visceral fat develops primarily post-natally which contrasts with subcutaneous adipose tissue (227). With diet-induced obesity, adipogenesis is initiated after four weeks of high calorie diet while subcutaneous adipose tissue expands by hypertrophy primarily for up to 12 weeks.

The mechanisms utilized in human adipose tissue expansion in obesity are not fully delineated, and patterns of fat deposition and expansion vary substantially between individuals. This variation in adipose tissue distribution likely drives the differences in metabolic disease risk seen between individuals with similar degrees of adiposity. A consistent observation in obese individuals is that metabolic dysfunction is associated with large adipocytes and the impaired ability to generate new adipocytes (66, 85). This may be due to altered gene expression in large adipocytes that overexpress inflammatory genes (89). Others have suggested that it is an over abundance of small adipocytes that can no longer hypertrophy that is associated with a more deleterious metabolic phenotype

(136, 137). In both settings, the net effect is the same; the ability of adipose tissue to function as an efficient storage vehicle is impaired, forcing other peripheral tissues to compensate by inappropriately accumulating lipid.

Animal studies to support the benefits of efficient adipose tissue expansion came from a remarkable study by the Scherer lab (99). Mice were generated that lacked leptin and overexpressed adiponectin. In this setting, the mice were severely obese but surprisingly had normal metabolic profiles with normal glucose tolerance and protection from fatty liver disease. The robust capacity of the adipose tissue to act as a nutrient sink in these animals was shown to occur even with high calorie diets as the mice weighed as much as 135g without evidence of abnormal metabolism. The protective phenotype in this setting was associated with preferential partitioning of storage to subcutaneous fat depots, expansion of small adipocytes, and the absence of inflammatory cells (ATMs in particular). Thus, if adipose tissue is able to retain properties that promote efficient fat expansion in the face of tremendous nutrient load, metabolic health may be maintained in with severe obesity. Such mechanisms may be at play in individuals who display a metabolically healthy obese phenotype (13, 23)

Understanding the interaction between adipocyte hypertrophy and hyperplasia requires an understanding of the dynamics and kinetics of adipocyte turnover – an issue that has remained poorly understood until recently in humans. By taking advantage of worldwide exposure to ^{14}Carbon with aboveground nuclear testing in the 1950's, Spalding and colleagues estimated the turnover rate of adipocyte to be approximately 10% per year regardless of BMI status (196). The rate of lipid turnover within adipose tissue appears to be an important variable as well. Metabolic disease is associated with

decreased rates of lipid storage and release from adipocytes (10). In addition, there exists a wide variation in the balance between hyperplasia and hypertrophy in subcutaneous fat depots between individuals independent of adipocyte turnover rates (9). The contribution of these factors to metabolic disease is supported by the observation that women with a predominant adipocyte hypertrophy profile had worse insulin resistance than those with a hypertrophic profile (196). There remain many important unanswered questions in this field regarding whether the number of adipocytes in the adult reach a finite number during obesity, or if there is continuous turnover of adipocyte precursor populations.

Much of what we know about the molecular regulation of adipogenesis is largely derived from *in vitro* cells lines that exhibit adipogenic capacity (184). Over the past several years, there has been significant progress made in identifying specific populations of preadipocytes *in vivo* and understanding their differentiation capacity. This is a fast developing field that overlaps with the efforts to identify multi-potent adipose tissue stem cells with differentiation capacity beyond adipocytes (e.g. chondrocytes and osteocytes) (31). Previous studies have relied on combinations of mouse genetic lineage tracing, surface marker analysis by flow cytometry, and adoptive transfer to validate adipogenic capacity (17, 114).

Using these techniques, a number of putative committed preadipocyte populations have been identified. Using platelet derived growth factor receptor alpha (PDGFR α) reporter mice, Lee *et al* identified a Sca-1^{+} $CD34^{+}$ PDGFR α^{+} population in adipose tissue with the potential to differentiate into either white or brown adipocytes in the setting of lipolysis-stimulated adipose tissue remodeling in mice (115). These observations overlap

with Berry *et al* who report a population of PDGFR α^+ preadipocytes that also co-express CD24 (17, 183). Commitment to the adipocyte lineage is associated with down regulation of CD24 expression. Lineage tracing experiments with adipogenic transcription factors PPARγ and Zfp493 identifies committed preadipocytes tightly associated and possibly coinciding with endothelial cells in adipose tissue (64, 210, 211, 213). How these endothelial derived precursors map onto the PDGFR α^+ precursors has yet to be fully detailed.

Angiogenesis and hypoxia

The expansion of fat is directly related to access to nutrients and gas exchange from the blood supply. All adipose tissue depots are highly vascularized. An extensive capillary network is formed in association with adipocytes (35, 152). In adipose tissue expansion, blood vessel development (angiogenesis) precedes adipogenesis and establishes the architecture of visceral fat pads in mice (67, 70). In humans, angiogenic potential is not equal in all white adipose tissue depots. Explant experiments show a higher angiogenic capacity of subcutaneous adipose tissue compared to visceral adipose tissue samples (61). Adipose tissue from obese patients showed a lower angiogenic capacity that was associated with insulin resistance (197). This is consistent with the observation that obesity decreases the density of capillaries in adipose tissue while increasing the size of larger vessels.

Angiogenesis in adipose tissue is regulated by a wide range of pro- and anti-angiogenic factors. Pro-angiogenic factors are secreted by adipocytes, preadipocytes,

endothelial cells, and inflammatory cells (179). Growing adipocytes produce pro-angiogenic factors that include VEGF, FGF-2, HGF, IGF, TNFα and TGFβ (116). Adipokines can also positively and negatively regulate angiogenesis. Leptin has the potential to activate a pro-angiogenic program (225). Adiponectin has been suggested to have both pro- and anti- angiogenic effects (181, 224). On balance, evidence primarily suggests that Adiponectin is sufficient to promote angiogenesis in overexpression models (99, 109).

Adipogenesis is dependent on appropriate angiogenesis, as it has been shown that the modulation of vascular supply can regulate adipose tissue mass (185). VEGF is referred to as a master regulator of angiogenesis and is essential for the activation of the growth of new blood vessels (168). Blockade of VEGF receptor signaling along with high calorie diets can block adipose tissue angiogenesis and inhibit fat expansion (207). Conversely, overexpression of VEGF in adipose tissue increases blood vessel density and protects against diet-induced obesity, potentially by blunting hypoxia signals and increasing thermogenesis (56).

A potent drive for new blood vessel formation is tissue hypoxia, and there is evidence from several studies that obesity generates a hypoxic environment in adipose tissue (77, 239). This hypoxia may be a means by which adipocyte hypertrophy generates a pro-inflammatory response (171, 172, 178). In vitro, adipocytes and SVF cells respond to a hypoxic environment with an induction of inflammatory cytokine production (58, 158). While this hypoxic environment may relate to decreased diffusion of oxygen in hypertrophic adipocytes, there is evidence that obesity also impairs blood flow to adipose tissue. Total blood flow into white adipose tissue is not increased in

obese patients despite tissue enlargement (222). In addition, obese patients have been shown to have a blunted post-prandial increase in blood flow to adipose tissue (94).

An important outstanding issue in the discussion of adipose tissue hypoxia and obesity is the measurement methods employed. The reported partial pressures of oxygen (44-55 mmHg (171, 172)) are actually quite high compared to many other tissues (e.g. muscle and brain pO_2 are around 30mmHg (30)). Therefore, it is likely more accurate that there is a relative degree of tissue hypoxia occurring in adipose tissue with obesity. More intriguingly, this suggests the possibility that many non-hypoxic mechanisms have yet to be discovered that may be driving many of the angiogenic signals in expanding fat if this is the case.

While many molecular pathways are activated in response to hypoxia, activation of hypoxia-inducible factor-1 α (HIF-1 α) is a key event that is triggered in obese adipose tissue (72). The role of this activation may have less to do with angiogenesis and more to do with regulation of the ECM. Transgenic overexpression of a constitutively active form HIF-1α in adipose tissue did not increase angiogenesis (65). Instead, overexpression of HIF-1α induced the expression of multiple ECM components and ECM remodeling genes including *Col1a1*, *Col3a1*, *Eln*, *Lox*, *Timp1*, and increased tissue inflammation. Overall, while hypoxia may be a drive for new vessel formation in some conditions, in obesity it may primarily serve as a pro-fibrotic and pro-inflammatory signal.

Extracellular matrix composition in adipose tissue

The ECM is a crucial component of adipose tissue that is necessary for maintaining cellular and structural integrity. The ECM is also a source for mechanical signals for proper growth and differentiation, and provides a medium for cell-ECM and cell-cell crosstalk. This communication permits cells to sense and adapt to their changing microenvironment. The generation, maintenance and degradation of adipose tissue ECM is regulated by numerous cellular components in adipose tissue, including inflammatory cells and adipocytes themselves **(Figure 1-2).**

The ECM surrounding adipocytes is composed of numerous protein and carbohydrate components that include collagens, laminins, fibronectin, proteoglycans, and many more (62, 190). Adipocytes develop in the context of a dense ECM matrix that significantly changes throughout development and during adipogenesis. Preadipocyte differentiation triggers a dynamic deposition of all collagen types followed by later degradation of the ECM during the later stages of differentiation (141, 147). ECM scaffolding in fat is also composed of the basement membrane, which contains a large complex of Collagen IV, Collagen VIII, as well as heparan sulfate proteoglycans laminins (176, 190). To bind with the ECM, adipocytes will crosslink their own Collagen VI to the basement membrane. This may be achieved through protein cross-linking via the enzymes Lysyl Oxidase and Transglutaminase, which may modulate adipogenesis (52, 81).

Adipose tissue ECM is composed of both fibrillar (Col I, III) and non-fibrillar (Col IV, VI, VIII) collagens. A unique aspect of adipose tissue is that it contains the

highest concentration of Collagen Type VI compared to other tissues (97, 170). Collagen VI has been shown to correlate with body mass index and be up regulated in adipose tissue with obesity and short term overfeeding (170). ATMs are tightly associated with Collagen VI-rich areas in fat where they demonstrate an alternative-activated phenotype (198). The importance of Collagen VI in controlling adipocyte shape with over nutrition was shown in studies of mice deficient for Collagen VI, who demonstrate an exaggerated capacity for adipocyte hypertrophy (97). Collagen VI cleavage products can also generate signaling molecules which can promote fibrosis via increasing the recruitment of macrophages and activating TGFβ signaling (169). It is also clear that other collagens besides Collagen VI are important in adipose tissue remodeling. In obese children, increased collagen content in adipose tissue correlated with decreased fat cells size and body mass index that was not related to Collagen VI (206).

Beyond the components that make up the ECM, a variety of other control mechanisms tie ECM signals to cellular function. One example of this in adipose tissue is SPARC (secreted protein acidic and rich in cysteine; osteonectin) and its associated complex. The SPARC complex has been implicated in regulating cell-matrix interactions, angiogenesis, activation of matrix metalloproteinases (MMPs), and modification of cytokines and growth factors (27). SPARC binds to numerous proteins including thrombospondins 1 and 2, osteopontin, fibrillar collagens, and tenascins C and X, and may therefore contribute to the organization of the ECM in fat and its interaction with adipocytes (187).

SPARC is induced with obesity and with adipogenesis (33). It is expressed higher in subcutaneous adipose tissue compared to visceral adipose tissue and correlates with

BMI and waist circumference (113). *Sparc*-null mice have larger body weights and larger white adipose depots on normal and high fat diet challenge (26, 150). These observations correlated with decreased adipose tissue collagen content in the *Sparc*-null mice. The mechanism behind these effects may relate to the close relationship between ECM signals and adipogenesis, as even bone marrow derived cells from SPARC-null mice had increased adipogenic potential. This may also relate to the capacity for SPARC to directly inhibit adipogenesis via an enhancement of β-catenin signaling (151).

Extracellular matrix maintenance and turnover in obesity

Both adipose tissue expansion and reduction require an extensive reorganization of the ECM to permit the changes in shape. Gene expression analysis supports the idea that ECM maintenance is dynamically regulated over the lifetime of an adipocyte (127, 157). During the early stages of adipocyte hypertrophy and the development of obesity, there is an increase in ECM deposition (147). As tissue hypertrophy continues, the deposition and maturation of ECM components accumulates and is dynamically modified. Extracellular proteolytic enzymes primarily control ECM degradation. There are many families of proteins that enact this activity, some of which include the MMPs, ADAMs/ADAMTs (a-desintegrin and metalloproteinase with thrombospondin motifs), urokinase type (u-PAs), tissue type plasminogen activators (t-PA) and plasminogen activator inhibitors (PAIs).

A better understood family of ECM degrading systems in adipose tissue is the 20+ member, zinc-dependent family of matrix metalloproteinases (MMPs) (146). MMPs

are loosely classified according to their substrate specificity, although there is substantial redundancy in their activities (223). Still, it is generally believed that the complete MMP collection is able to degrade most, if not all, of the ECM components in tissues. MMPs activity can be regulated by a family of endogenous inhibitors, called the Tissue Inhibitors of Metalloproteinases (TIMPs) which have their own range of specificity of action towards MMPs (220). Beyond their ability to cleave ECM components, MMPs can also target various signaling molecules, growth factors, and cell adhesion molecules with likely effects on adipose tissue function (155). For example, MMP-12 can truncate CXC-type chemokines that are important in macrophage recruitment and mobility (47). MMP can activate pro-IL1β, TNF α , and plasminogen as well as activate other MMPs (69, 188). MMPs can also release and activate TGFβ within the ECM (129, 240). Almost all of these pathways have been implicated in adipocyte function and adipogenesis, which implies the range of activity MMPs play in adipose tissue remodeling.

Transcriptional profiling has shown significant changes in the expression of MMPs and TIMPs in adipose tissue from diet-induced obesity models and genetic models of obesity (34, 132). The predominant source of MMPs and TIMPs are unclear, however adipocytes can stimulate MMP activation in response to macrophage derived inflammatory signals (157). While many MMPs and TIMPs are induced with obesity, some are down regulated or unchanged.

Several studies have shown that MMPs are required for proper adipose tissue expansion. Pharmacological inhibition of MMP activity has been shown to suppress adipocyte differentiation in vitro (169). *In vivo* administration of a broad-spectrum MMP inhibitor was able to significantly restrict the growth of both subcutaneous and visceral

adipose tissue of animals fed high fat diets (48, 119). In this setting, MMP inhibition led to the accumulation of collagen-dense matrix surrounding adipocytes and smaller fat depots, demonstrating the importance of MMPs in adipose tissue ECM degradation and control of adipose tissue shape.

Specific inhibition of MMP-2, an enzyme that can degrade Collagen IV and the basement membrane, can promote differentiation of pre-adipocyte cell lines in vitro (218). However, *in vivo* inhibition of MMP-2 in mice fed a high fat diet decreased body weight gain without an impact on fat pad weight. MMP-2 inhibition reduced adipocyte size and blood vessel density suggesting that MMP-2 may more important in the regulation of adipocyte hypertrophy and angiogenesis. Consistent with this, treatment of obese mice with the MMP-2 inhibitor had a primary influence on blood vessel size, suggesting a role for MMP-2 in maintaining angiogenesis. Similar results were seen with MMP-2 deficient mice which have attenuated weight gain and smaller fat pads when fed a high fat diet (219). The differences between in vitro and *in vivo* results in this study further advocate the need to understand adipose ECM in the context of the entire stromal network.

Another striking example of the limitations of in vitro analysis of adipose tissue ECM come from studies examining the role of the membrane type MMP-14 (MT1-MMP) (41, 42). *Mmp14* null mice exhibit decreased adipose tissue mass. When preadipocytes were cultured on standard tissue culture plates, adipogenesis was observed to proceed undisturbed. However, when placed in a dense three dimensional collagen matrix, preadipocytes from *Mmp14* null mice were unable to grow and differentiate. This ability was restored when the matrix was of a lower density and demonstrated the

requirement of adipocyte derived MMP-14 to cleave and loosen their pericellular ECM and permit adipocyte expansion.

In terms of TIMPs in adipose tissue, the role of TIMP-1 and its role in regulating MMP activity has been the most studied to date. Transgenic over-expression of TIMP-1 increases adipocyte differentiation and lipid storage in mammary gland adipose tissue (4). However, overexpression of human TIMP-1 in 3T3-F442A adipocytes and in transgenic mice had little effects on adipocyte differentiation, adipose tissue expansion, or body weights (49). Attenuating TIMP-1 activity with a neutralizing antibody enhanced 3T3-L1 adipocyte differentiation in vitro and also lead to larger adipocytes *in vivo* (138). Moreover, administering recombinant mouse TIMP-1 to mice resulted in early development of insulin resistance and higher levels of serum non-esterified fatty acid levels along with an increase in hepatic lipid storage. *Timp1* deficient mice have also been studied and show lower fat pad weight, smaller adipocytes, and protection from diet-induced obesity (118). Overall, these studies suggest that TIMP-1 contributes to the remodeling of adipose tissue by inhibiting certain necessary ECM remodeling activities, however the mechanisms of how these events are regulated are unclear.

The role of TIMP-3 in adipose tissue and adipocyte differentiation has also been evaluated. Macrophage-specific TIMP-3 over-expression suggested that TIMP-3 was protective towards obesity-induced inflammation and metabolic dysfunction (139). Conversely, *Timp3* deficient mice showed an increase in both hepatic steatosis and adipose tissue inflammation (140). In vitro, TIMP-3 expression is down regulated during adipogenesis suggesting that low TIMP-3 expression is required to permit adipocyte

hypertrophy (15). Overall, these results suggest that TIMP-3 may exert another layer of control over the extent and timing of ECM degradation in adipose tissue.

Collectively, these studies suggest that a tightly controlled balance of ECM remodeling enzymes is necessary for proper adipocyte expansion and can influence the inflammatory set point of adipose tissue. However, MMPs are often difficult to study because they can be regulated at multiple post-transcriptional levels. Because of the multi-step regulatory program of MMPs, future studies focused on how MMP activity is regulated in adipose tissue are required.

Adipose tissue fibrosis in obesity and weight loss

The growing interest in adipose tissue remodeling and the ECM relates to the strong correlation between adipose tissue fibrosis and obesity-associated metabolic dysfunction (202, 203). Obesity is associated with an increase in adipose tissue fibrosis, a prominent signature in visceral adipose tissue depots that is not regularly observed in subcutaneous depots in animals, but may be more prominent in human adipose tissues (54, 97). Obesity induces a strong induction of ECM component gene expression, growth factors, and enzymes involved in post-translational modifications (20, 74). With obesity, it is believed that the imbalance of ECM deposition, ECM degradation, and ECM component crosslinking results in accumulation of insoluble fibrotic regions. Beyond obesity, fibrosis and expression of certain collagens (e.g. Collagen VI) negatively correlate with insulin sensitivity, further implicating fibrosis as a signature of adipose tissue dysfunction and poor metabolic health (198). Still, while the majority of findings

are consistent in seeing a denser ECM with obesity, the mechanisms by which pathogenic fibrosis develops is not understood, and discrete criteria describing at which point the fibrotic accumulations result in detrimental influence on adipocyte maintenance and in metabolism are still unclear (53).

Currently, it is not well understood how modifications of the ECM can affect adipose tissue's ability to store, release, and maintain proper energy balance. Current data suggests that adipose fibrosis limits the mechanical flexibility of the ECM, which may limit the ability of adipocytes to expand and contract in response to metabolic demand (9, 10). *In vitro* studies suggest that mechanical stress due to an increasingly dense ECM inhibits preadipocyte differentiation and hypertrophy (40-42). This limits new adipocyte formation and may limit the normal capacity of adipose tissue to store and sequester lipid and contributes to the increase in lipid deposition in peripheral non-storage tissues such as the liver and muscle. *In vitro* experiments culturing adipocytes in dense ECM limits their sensitivity to insulin, and decreases adiponectin expression and secretion (117). Additionally, clinical studies reveal that obese patients have increased ECM density and stiffness in subcutaneous adipose tissues (1). Some reports suggest a possible protective role for adipose tissue fibrosis might be to limit adipocyte hypertrophy in visceral adipose depots, as smaller adipocyte sizes are associated with a lower inflammatory profile (9, 10). Overall, the majority of pre-clinical data supports the model that fibrosis promotes metabolic dysfunction, however potential benefits to fibrosis have been observed in clinical settings. The differences between the pre-clinical and clinical settings may relate to the range of BMI being evaluated, depot being sampled, methods of quantitation of fibrosis, and the heterogeneity of human obesity and diabetes status.

The negative effects of ECM on metabolism are highlighted in an animal model with impaired ECM deposition. Mice deficient for Collagen VI, an ECM component enriched in adipose tissue, were crossed onto a leptin-deficient ob/ob background which displays genetic obesity. The lack of *Col6* permitted unrestricted expansion of adipocyte size (97). This resulted in an improvement of metabolic parameters and glucose tolerance compared to the leptin-deficient controls. This was also associated with fewer crown-like structures (CLSs) that represent regions of adipocyte death and inflammation. These findings indicate that increasing the flexibility of the ECM may in fact improve the ability of adipocytes to adapt to metabolic demand. This may also indicate that adipocyte hypertrophy per se is not sufficient to induce an inflammatory response and that the ECM may be a key mediator in translating adipocyte stress.

To date, whether or not weight loss resolves adipose tissue fibrosis in humans and if it is pathogenic or protective is still under investigation. With weight loss, the ECM undergoes significant reorganization that allows for a decrease in adipocyte size (11, 29, 106). Weight loss can improve a variety of metabolic and inflammatory factors related to obesity, however in many cases the risk of cardiovascular disease remains high (237). For example, normal weight adults that were obese as children have a 2-3 fold higher risk of developing diabetes (177). A striking observation is that the accumulation of ECM components appears to persist and even increase in obese patients that have undergone weight loss (53, 54). This suggests that resolution of adipose tissue fibrosis may not be possible and suggests a potential mechanism for ongoing risk of metabolic disease in formerly obese individuals.

Fibrosis and mechanical stress

One consequence of fibrotic adipose tissue is an increase in the physical stiffness of the microenvironment. Increased rigidity of the microenvironment can promote self renewal of adipocyte progenitors while decreasing their adipogenic capacity (32). This is consistent with the observations that *Mmp14* deficient mice have impaired adipocyte formation due to the loss of local collagen breakdown (41).

A few studies have evaluated the mechanisms by which the mechanical properties of the microenvironment modify adipocyte function. Mechanical stretching of 3T3-L1 adipocytes inhibits differentiation via ERK-mediated down regulation of PPAR γ 2 (209). Applying a mechanical stress to human preadipocytes by compressive force inhibited adipocyte differentiation via COX-2 mediated down regulation of PPARγ2 and C/EBPα (78). These effects were not observed when the compression was done after differentiation, suggesting that preadipocytes may be primarily responsive to mechanical stress from fibrosis.

The Rho/Rho-Kinase (ROCK) signaling pathway plays an important role in the mechanotransduction of stress, which converts physical stimuli from the microenvironment into biochemical signals. ROCK is activated in adipose tissue of obese Zucker rats, and ROCK activation correlates with the development of glucose intolerance via phosphorylation of insulin receptor substrate-1 (IRS-1) (92). ROCK also induces the expression of inflammatory cytokines MCP-1 and TNFα (73, 148). Inhibition of ROCK activity by overexpression of an adipocyte-specific dominant-negative RhoA attenuated adipocyte hypertrophy, weight gain, and macrophage recruitment to adipose

tissues (68). ROCK inhibition also improved glucose metabolism in mice after high fat diet challenge. Hypertrophy of 3T3-L1 adipocytes was found to correlate with increased Rho-Kinase activity and expression of MCP-1 and TNF α. Together, these data may demonstrate the importance of adipocyte mechanical sensing in metabolic and inflammatory control.

Changes in macrophage populations with obesity

ATMs are the dominant leukocyte population in adipose tissue in both lean and obese states. Both human and mouse ATMs can be subdivided into two general groups based on functional observations, however additional studies suggest this model is overly simplistic (122, 228). More recent studies however, have described an additional distinct ATM subtype associated with obesity, termed the "metabolically active" or MMe subtype (107). The resident ATM population has properties that overlap with alternatively activated macrophages (M2) and is associated with suppression of the immune response and with ECM remodeling. Resident ATMs are found in all adipose tissue depots and are the dominant population in lean states. A subtype of $CD11c^{+}$ ATMs are induced with obesity and are associated with tissue damage, pro-inflammatory signaling, and the generation of T_{H1} cytokines such as TNF α which is a common hallmark of classically activated macrophages (M1). The functions and roles of MMe macrophages in ECM remodeling, while being associated with metabolic dysfunction, have not yet been fully explored and remains a promising research avenue.

Which ATM population contributes more to adipose tissue ECM deposition and to pathogenic fibrosis is unclear. Some studies have attributed remodeling capacity more to the $CD11c^+$ M1 ATM subset than the resident ATM subset (25, 192). This largely due to the association between $CD11c^+$ ATM accumulation and adipose tissue fibrosis in obese mice. These observations are not surprising given that the M1/M2 paradigm is an in vitro oversimplification and *in vivo* tissue macrophage likely assume activity along a broad spectrum. The position of ATMs in direct contact with adipocytes, vascular endothelial cells, preadipocytes, and the ECM leave them poised to integrate signals from a myriad of inputs and alter the behavior of the remodeling environment **(Figure 1-3).** It is becoming clear that ATM plasticity is a result of the diverse environment of adipose tissue.

Developmental remodeling of adipose tissue appears to be crucially controlled by resident ATMs (67). Depleting macrophages during development with clodronate liposomes inhibited adipogenesis by perturbing angiogenesis in the developing fat pad. In post-natal development, ATMs are concentrated in the tips of growing visceral fat pads where they may be contributing to ongoing angiogenesis and adipocyte formation by regulating VEGF signaling (35, 204).

In adult animals, resident M2 ATMs provide critical signals that support adipogenesis in many types of adipose tissue remodeling. Lee *et al* recently identified an adipogenic niche that was formed in during prolonged lipolysis (114). This model of studying reorganizations via lipolysis generated CLS due to the death of white adipocytes that were enriched in resident M2 ATMs. This new structure also appears to be critical to the generation of new adipocytes, as the ATMs provide signals that trigger preadipocyte

proliferation and new adipogenic foci. Osteopontin was identified as a signal emanating from the M2 ATMs and sensed by the preadipocytes as a migratory and stimulatory signal. Osteopontin deficient mice failed to form these regenerative adipogenic foci.

An important contribution of this paper by Lee *et al* is the comparison between macrophage activation paradigms between several models of induced adipose tissue "remodeling." These included physical disruption, diet induced obesity, and neogenesis of adipose tissue in a matrigel plug. All of these conditions led to the formation of similar adipogenic foci, however the macrophage activation profiles differed substantially between these conditions. This suggests a plasticity of ATM function depending on the triggers for adipose tissue remodeling and the importance of the local tissue microenvironment in generating a macrophage response.

A hallmark of obese adipose tissue is the presence of CLS that develop around necrotic adipocytes (43, 62). CLS density is associated with insulin resistance and a pro-inflammatory environment in adipose tissue (18, 145, 198). The formation of CLS is presumably due to excessive adipocyte hypertrophy and subsequent stress leading to a pattern of cell death that resembles necrosis or pyroptosis – a caspase-dependent type of programmed cell death.

The primary component of CLS are lipid-laden macrophages, however multiple leukocytes have been observed to be concentrated there as well, including lymphocytes, eosinophils, and mast cells (6, 234). CLS are enriched for $CD11c^{+}$ M1-like ATMs which take up lipids and cellular debris and generate pro-inflammatory cytokines. The function of ATMs in this context may be to process and prevent lipid release out of adipose tissue. Increasing macrophage lipid storage by overexpression of diacylglycerol acyltransferase

1 (DGAT1) results in reduced adipose tissue inflammation and an improved metabolic profile in obese mice (104). In addition, this accumulation of M1 ATMs in CLS is primarily dependent upon the influx of monocytes from the circulation in response to chemotactic signals in adipose tissue (123, 153, 161).

Macrophage regulation of ECM deposition and degradation

CLS are also associated with dense ECM deposition (43). The localization of M1 ATMs to these areas suggests that M1 ATMs promote a local pro-fibrotic environment. The cells in adipose tissue involved in the deposition of ECM have not been clearly delineated. In many other pro-fibrotic settings, fibroblasts are a major contributor to the promotion of ECM accumulation. In response to pro-inflammatory damage signals and growth factors such as TGF β, fibroblasts can be converted into pro-fibrotic myofibroblasts that express Collagen I and smooth muscle actin (α-SMA) (102). In adipose tissue, fibroblasts are abundant and are similar in morphology with adipocyte precursors.

Evidence suggests a coupling between macrophage pro-inflammatory signals and the generation of myofibroblasts in obese adipose tissue. Pro-inflammatory signals can activate gene expression of ECM components in preadipocytes (96). Conditioned media from ATMs can induce the gene expression of α-SMA in adipocyte precursors (24). In this system, TGFβ signaling was required to promote a "myofibroblast" phenotype and TGF β was sufficient to induce promote induce the gene expression TGF β can also induce the migration and contractility of adipose tissue fibroblasts suggesting direct links

between myofibroblasts and mechanical stress (90). Secreted factors from pro-inflammatory M1 macrophages can also block the adipogenic capacity of preadipocytes (19, 123). How this may be coupled to a shift toward a myofibroblast phenotype has yet to be fully delineated. Additionally, the evidence suggesting that these pro-fibrotic cells actually produce ECM proteins or promote their crosslinking and assembly has not been elucidated.

It is not clearly understood how the pro-fibrotic capacity overlaps with the ability of macrophages to degrade the ECM (131). In other fibrotic settings, macrophages are important in degrading the collagen network, and in adipose tissue, ATMs have been shown to have significant ECM remodeling potential in obesity (198). Still, it is unclear if the promotion of ECM degradation pathways (e.g. MMP production) seen in obesity can be resolved even with excessive accumulation of ECM.

Significance of adipose tissue ECM research

Pre-clinical mouse models suggest an integrated model of macrophage control of adipose tissue fibrosis. In lean states, resident ATMs participate in the maintenance of adipose tissue by promoting regenerative adipogenesis and ECM remodeling to permit adipocyte expansion and contraction upon demand. Obesity challenges the balance of this system by inducing adipocyte hypertrophy and stress, which promotes M1 ATM accumulation and reprogramming of the M2 ATM population. Pro-fibrotic signals from ATMs (either $CD11c^+$ or resident) promote the conversion of preadipocytes to a pro-fibrotic phenotype that promotes ECM deposition. Fibrosis increases the mechanical

stress upon the adipocytes and preadipocytes that further potentiates adipocyte dysfunction and stress. In response to the pro-fibrotic environment, ATMs attempt to degrade and remodel the ECM and restore the system to homeostasis. However, in the face of ongoing nutritional excess, this may not be sufficient to restore normal adipose tissue function and thus metabolic dysfunction is promoted.

The understanding of ECM remodeling will have to take place in the context of dynamic nutrient states such as weight gain and weight loss in order to have a more complete understanding of the pathogenesis of metabolic disease. In addition, how remodeling differs between visceral and subcutaneous fat depots may unlock the links between visceral adiposity and metabolic disease. Increasing our understanding of how adipose tissue fibrosis is generated and if it can be resolved may provide novel therapeutic strategies that are needed in the face of the obesity epidemic.

In this dissertation, we take a step-wise approach to adipose tissue fibrosis to address three major gaps in knowledge relating how the ECM is modified in the context of weight gain and weight loss. In the following chapter, we examine the architecture and organization of a previously unexplored adipose tissue ECM component, the elastin matrix, and investigate it mechanisms of its regulation with the development of obesity. In the third chapter, we investigate the organization of the collagen matrix with the goal of identfying the cellular sources of the excess collagen production in visceral and subcutaneous adipose tissues, and identify a potential mechanism of stromal cell pro-fibrotic activation. In Chapter 4, we investigate the question of whether or not fibrosis is reversible after weight loss by examining adipose tissue ECM architecture and components both before and after weight loss. Finally, we conclude with a discussion of

our findings in the context of the field of obesity and diabetes research.

Summary Points:

- The ECM is a crucial component of adipose tissue that is necessary for maintaining adipocyte function and structural integrity
- Insulin resistance is associated with adipose tissue inflammation, excess ECM deposition, and adipose tissue fibrosis.
- Excess adipose tissue ECM may limit the ability of adipocytes to expand and contract in size in response to metabolic demand.

Future Directions

While many groups have provided a functional base of knowledge of the events components of adipose tissue remodeling, there are still many important unresolved questions in the field:

- What are mechanisms that regulate the deposition of ECM components in across physiological and pathological spectrums and how does this differ between adipose tissue depots?
- What causes the shift in balance resulting in favoring ECM deposition over ECM degradation in adipose tissue?
- How do interactions between ATMs and preadipocytes or ATMs and mature adipocytes influence how the ECM is produced and modified?
- Does weight loss reverse the fibrotic status of the adipose tissue ECM architecture?

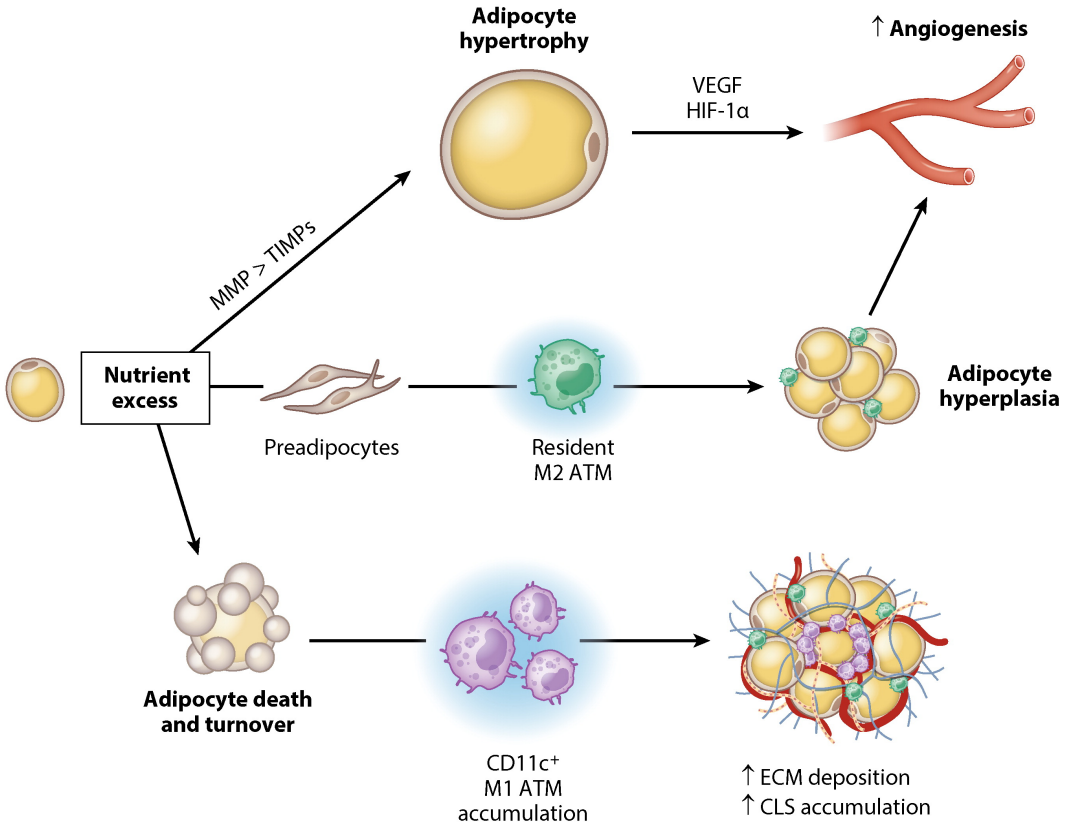

Figure 1-1. Adipose tissue remodeling during obesity. With nutrient excess (chronic or acute), numerous events are triggered that influence adipose tissue shape. A balance between adipocyte hypertrophy, hyperplasia, and death contributes to the nutrient storage capacity of a fat depot. Hypertrophy and hyperplasia are linked to angiogenic cues that maintain nutrient availability to adipocytes. Adipocyte death is a trigger for the accumulation of inflammatory adipose tissue macrophages (ATMs) that form crown-like structures (CLSs), which is a hallmark of adipose tissue stress. Extracellular matrix (ECM) degradation and deposition in the microenvironment are regulated differently in these conditions. Abbreviations: CD11c+, cluster of differentiation 11c or integrin alpha X; HIF, hypoxia-inducible factor; MMP, matrix metalloproteinase; TIMPs, tissue inhibitors of metalloproteinases; VEGF, vascular endothelial growth factor.

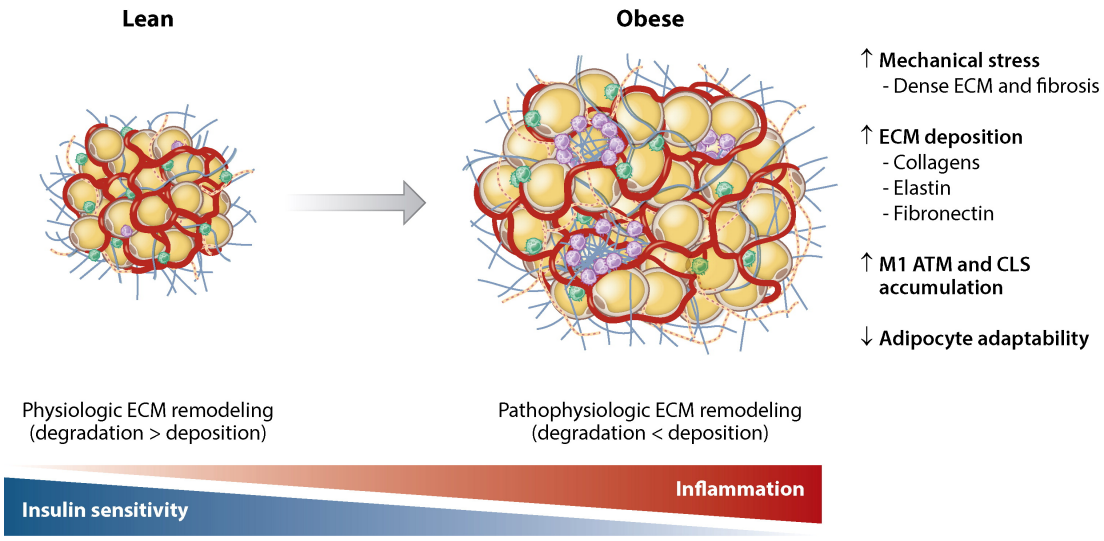

Figure 1-2. Dynamic regulation of adipose tissue extracellular matrix (ECM) with obesity. In lean states, the adipose tissue ECM is remodeled in response to acute physiologic changes in nutrient availability. This remodeling permits adipocyte expansion and contraction in response to nutrient demands. Both conditions require a relative dominance of processes that degrade the ECM over those that deposit ECM. With chronic obesity, this system is altered. In concert with a decrease in insulin sensitivity and inflammation, ECM accumulates around adipocytes. This increases mechanical stress by tipping the balance away from ECM degradation and toward ECM deposition. In concert with this, adipose tissue macrophages (ATMs) are activated and accumulate in ECM-dense regions. The net effect is a loss of adipocyte flexibility as a nutrient storage depot, which contributes to the development of metabolic disease and insulin resistance. Abbreviations: CLS, crown-like structure; M1 ATM, classically activated macrophage.

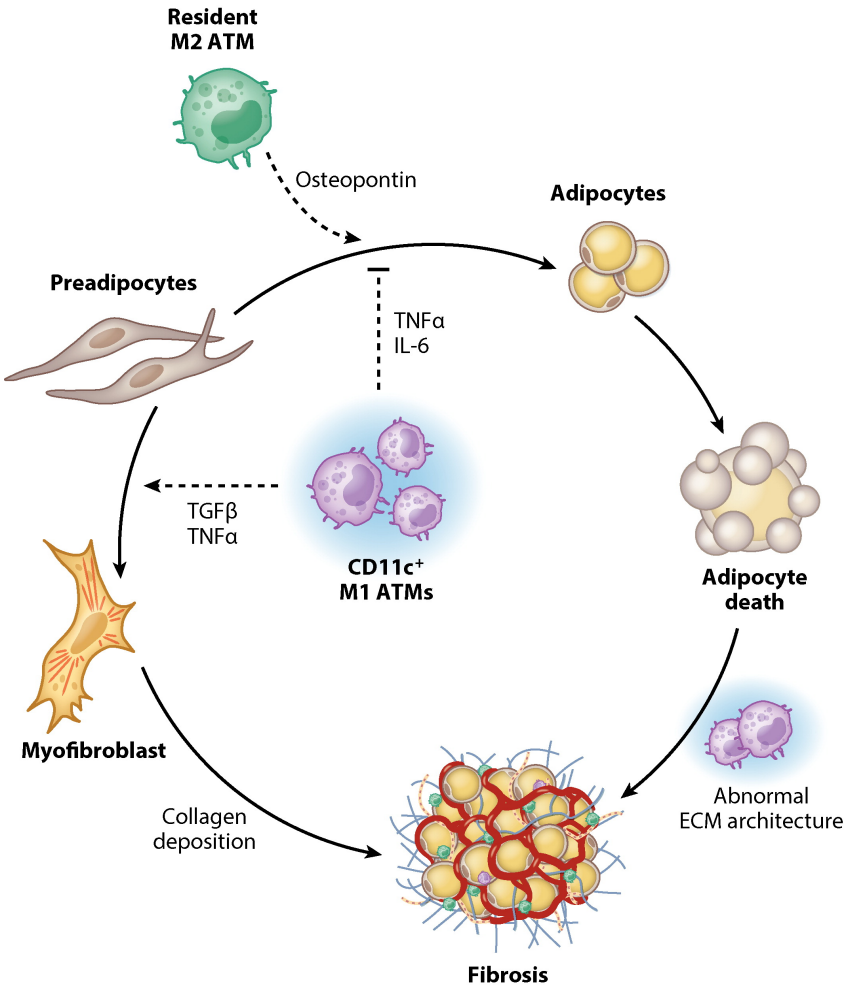

Figure 1-3. Adipose tissue macrophages (ATMs) and the regulation of adipose tissue remodeling. ATMs sit at the interface of many of the pathways that regulate adipocyte health. Adipogenesis is promoted by resident alternatively activated macrophage (M2) ATM signals (e.g., osteopontin) and is suppressed by pro-inflammatory cytokines from classically activated macrophage (M1) ATMs. Blockade of adipogenic potential can trigger a myofibroblasts or "pro-fibrotic" phenotype in preadipocytes by M1 ATM-secreted factors. Adipocyte death is a potent adjuvant for M1 ATM activation, which promotes abnormal extracellular matrix (ECM) organization and fibrosis in conjunction with formation of myofibroblasts. Abbreviations: IL-6, interleukin-6; TGF β, transforming growth factor beta; TNF α, tumor necrosis factor alpha.

Chapter 2.

Obesity-Induced Remodeling of the Adipose Tissue Elastin Network is Independent of the Metalloelastase MMP-12

Abstract

The ECM plays important roles in maintaining adequate adipose tissue function and in metabolic regulation. Here we have examined the organization of a relatively unexplored adipose tissue ECM component, elastin and its response to diet induced obesity in mice. Additionally, we have explored the regulation and requirement of macrophage metalloelastase, MMP-12, in adipose tissue ECM remodeling in obesity. In visceral fat depots, elastin fibers form a mesh-like net that becomes denser with diet-induced obesity. In contrast, the elastin fibers in subcutaneous adipose depots are more linear in organization, and are tightly associated with ATMs. We found that *Mmp12* is produced predominantly by ATMs and can be induced with both short- and long-term high fat diet challenge and rapid remodeling induced by lipolysis. This contrasts with *Mmp14* and *Timp1*, which are further induced only after chronic obesity in non-ATM populations. We examined obese transgenic *Mmp12*$^{-/-}$ mice and found an increase in

Portions of this chapter are in press: **Martinez-Santibanez G**, Singer K, Cho KW, Delproposto J, Mergian T., Lumeng CN. Obesity-induced remodeling of the Adipose Tissue Elastin Network is Independent of the metalloelastase MMP-12. *Adipocyte* 2014.

gene expression of ECM genes with diet-induced obesity, but showed few significant differences in metabolic parameters, elastin matrix density, or in adipose tissue inflammation. Together, these studies reveal the architecture and diet-induced regulation of the elastin matrix and suggest that MMP-12 is not required for elastin matrix remodeling or for the metabolic dysfunction that occurs with obesity.

Introduction

The proper function of adipocytes is dependent on stromal cells in adipose tissue, as well as the organization and composition of the ECM. Adipose tissue ECM is composed of a diverse network of proteins that include collagens (Type I, III, V, and VI), fibronectin, and elastin (53). The healthy physiologic expansion of adipose tissue requires a tightly regulated balance of ECM deposition, degradation, and post-translational modifications (40, 41). Obesity promotes ECM accumulation in adipose tissue, which can contribute to fibrosis and ultimately impair its function as an efficient nutrient storage organ (81). Associations between adipose tissue fibrosis and metabolic disease has led to a considerable interest in understanding the mechanisms that regulate adipose tissue ECM remodeling (203).

Elastin is an ECM protein found in almost all tissues, but there is a paucity of information regarding its distribution and regulation in adipose tissue. Elastin is a heavily cross-linked protein that enables tissues to stretch and return back to its original conformation (76, 98). In human adipose tissue, the density of the elastin network was higher in tissues from lean individuals compared to obese individuals, suggesting

remodeling of the elastin network with increased adiposity (197). An interaction between collagen and elastin networks in adipose tissue has been suggested, as elastin expression is decreased in Collagen VI-deficient mice, which have a less dense collagen matrix that is permissive to adipocyte hypertrophy (97). Inhibition of lysyl oxidase, an enzyme that crosslinks both collagen and elastin fibers, can improve metabolic parameters in obese animals (65, 128). In addition to this structural role, elastin-derived peptides have been shown to contribute to insulin resistance by blocking glucose uptake in muscle and adipose tissue (22).

The degradation of the ECM proteins such as collagen and elastin is controlled by proteinases that include matrix metalloproteinases (MMPs) and their endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) (34, 119). We focus on MMP-12 is a metalloelastase because it has the capacity to degrade elastin, and because it plays important roles in tissue fibrosis and injury in other tissues (71, 83, 230). *Mmp12* expression is highly induced in adipose tissue of mice with diet-induced obesity, and its expression has been reported in both mature adipocytes as well as in ATMs (34, 82, 132, 192). The importance of *Mmp12* in obesity-induced metabolic dysfunction remains unclear as one report failed to identify any metabolic phenotype in *Mmp12*$^{-/-}$ mice despite a decrease in ATMs and another found that *Mmp12* deficiency provided protection from metabolic dysfunction and obesity-induced adipose tissue inflammation (14).

In this chapter, we describe an extensive network of elastin fibers in adipose tissue that are regulated by diet-induced obesity. Elastin deposition is increased in visceral adipose tissue depots with obesity and there are significant qualitative differences in the cross linking of elastin fibers between depots. Since we observe a

strong physical association between ATMs and elastin we have examined the response to dietary obesity in *Mmp12*$^{-/-}$ mice and observe changes in ECM gene expression but no significant change in metabolic measures with *Mmp12* deficiency.

Materials and Methods

Animal Studies.

C57BL/6J and B6.129X-*Mmp12*^{tm1Sds}/J (*Mmp12*$^{-/-}$) mice were obtained from The Jackson Laboratories. Male C57 mice, (6 weeks of age) were fed a normal diet (ND, 4.5% fat Research Diets, New Brunswick, NJ), or High fat diet (HFD, 60% fat from lard; Research Diets). For *Mmp12*$^{-/-}$ experiments, littermate males were fed a HFD *ad libitum* starting at 8 weeks of age. For lipolysis experiments, mice fed a ND or HFD for 16 weeks were injected with 1mg/kg of CL316243 or PBS-vehicle intraperitonealy (IP) for three consecutive days. All procedures were approved by the University of Michigan University Committee on Use of Care of Animals and were in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals.

Quantitative real-time PCR.

Tissues were frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from adipose tissues, SVCs, or FACS-sorted cells using the RNeasy Lipid Tissue Kit or QIAzol lysis reagent (Qiagen, Valencia, CA) followed by DNAse digestion according to manufacturer's instructions. cDNA was generated from 0.5-1ug of RNA

using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, CA). Real-time PCR analysis was performed using SYBR Green PCR Master Mix (Applied Biosystems) and StepOnePlus thermocycler (Applied Biosystems). Relative expression was performed in duplicate samples and determined using the $2^{-\Delta}$ [elati]method after normalizing to *Arbp*. *Mmp12* to *Timp1* ratios calculated by dividing *Mmp12* relative expression levels by *Timp1* relative expression levels.

Microscopy and Histology.

Tissues used for sectioning were fixed and stored in 10% Buffered Formalin. Anti-Elastin antibodies for immunofluorescence staining were kindly provided by Dr. Robert Mecham (Washington University in St. Louis). Macrophages were stained with anti-MGL-1 (AbCam) or anti-Mac-2 (eBiosciences) antibodies. Whole mount immunofluorescence microscopy was performed as described (123). Images were captured using an Olympus inverted microscope fitted with an Olympus DP72 camera. ImageJ software (NIH) was used to measure adipocyte size and to assemble composite images. Mean Linear Intercept (MLI) was used to measure the mean free distance between elastin matrix fibers, and was performed by point counting method as described (103). Elastin positive area (%Eln+ area) was calculated with ImageJ software.

Analysis of adipose tissue stromal vascular fraction (SVF).

SVF isolation and flow cytometry were performed as described (144). Antibodies used were: CD45-eFluor405, CD64-PE, and CD11c-APCCy7, F480-APC, CD11b-APCCy7, in addition to propidium iodide viability stain. Flow Cytometry was performed

using a FACS CANTO II Flow Cytometer (BD Biosciences) and sorting on a FACSAria III. Analysis with FlowJo software (Tree Star, Ashland, OR).

Metabolic studies.

Glucose and Insulin tolerance tests were performed as described (143). Plasma insulin levels were measured using a mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL). Body composition analysis (body fat, lean mass, free fluid) was performed by the Animal Phenotyping Core at the University of Michigan's Nutrition and Obesity Research Center using the NMR-based analyzer (Minispec LF90II, Bruker Optics).

Statistical analysis.

Data are expressed as the mean +/- the standard error of the mean (SEM). GraphPad Prism software (GraphPad Software, La Jolla, CA) was used for statistical analyses. Differences between groups were determined using unpaired two-tailed Student's *t*-test, where a $p \leq 0.05$ was considered significant.

Results

Adipose tissue depot specific differences in the organization of the elastin matrix

While the collagen network is relatively well characterized in adipose tissue, little is known about the distribution of the adipose tissue elastin network and how this changes with obesity. We assessed elastin composition in adipose tissue using several

complementary methods. First, Verhoeff's stain, previously used to identify elastin in other tissues, was performed on section from visceral (epididymal; eWAT) and subcutaneous (inguinal; iWAT) white adipose tissue depots from lean (16wks ND) and obese (16wks HFD) male C57BL/6 mice. In lean mice, Verhoeff^{+} regions were more prominent in iWAT compared to eWAT **(Figure 2-1A).** With obesity, Verhoeff^{+} areas increased in eWAT and were concentrated around crown-like structures (CLSs) - regions of adipocyte death and ATM accumulation. In contrast, iWAT from obese mice had reduced Verhoeff^{+} regions relative to lean tissues. To verify the specificity of Verhoeff's stain for elastin, we performed immunohistochemistry (IHC) for elastin on eWAT and iWAT from lean and obese **(Figure 2-1B).** In eWAT, elastin^{+} regions were increased with HFD and recapitulated the observations with Verhoeff stain. Less elastin^{+} staining was observed in iWAT compared to eWAT and there were no significant changes in elastin positivity in iWAT with HFD. The iWAT staining did not match the Verhoeff's reagent and suggests non-specific staining in iWAT may limit the use of the Verhoeff's reagent.

As a third approach, elastin was assessed in fixed whole mount adipose tissue samples by immunofluorescence microscopy. Elastin fibers in eWAT of ND mice formed a highly cross-linked mesh-like network around adipocytes **(Figure 2-2A).** In stark contrast, elastin fibers in iWAT were organized in individual fiber bundles with less cross-linking compared to eWAT **(Figure 2-2B).** After 16 weeks of HFD, the elastin network in eWAT showed an increase in the density of elastin fibers and with more intersecting fibers **(Figure 2-2C).** The iWAT elastin network in obese mice also demonstrated a qualitative increase in intersecting fibers **(Figure 2-2D).** Quantification

of elastin$^+$ regions confirmed the increase in elastin fibers in eWAT, but not iWAT with HFD **(Figure 2-2E).** The mean linear intercept (MLI) method was utilized to quantify reticulation (increased branching is associated with a decrease in MLI) (8, 126). This demonstrated a significant increase in elastin branching in eWAT compared to iWAT and an increase in branching with HFD feeding (decrease in MLI) **(Figure 2-2F).** No significant changes in elastin (*Eln*) gene expression were seen in eWAT or iWAT suggesting that post-transcriptional regulation of elastin degradation/formation may be a critically regulated step **(Figure2-2G).**

Mmp12 expression is induced in ATMs with obesity.

MGL1$^+$ resident ATMs are found in all adipose tissue depots and are the dominant macrophage population in lean mice (124). In iWAT, MGL1$^+$ ATMs were observed to be tightly associated with elastin fibers **(Figure 2-2H).** The close physical association between ATMs and elastin fibers led us to explore the hypothesis that macrophages may participate in the remodeling of the elastin matrix via the macrophage metalloelastase MMP-12.

We examined *Mmp12* expression in four adipose tissue depots (eWAT, iWAT, peri-renal adipose tissue (rWAT) and mesenteric adipose tissue (mWAT)) after a short, 4-week HFD challenge to induce rapid fat expansion. This short term HFD treatment induced significant adipose tissue expansion without accumulation of CD11c$^+$ ATMs (data not shown). Gene expression analysis revealed that *Mmp12* was induced at least 80 fold in all four depots after HFD challenge when compared to lean age-matched ND-fed controls **(Figure 2-3A).** In contrast, the expression of *Timp1,* an endogenous inhibitor of

MMP-12, was not altered by obesity in eWAT or iWAT, but was induced in mWAT and rWAT depots **(Figure 2-3B).** Expression of *Mmp14,* a pericellular collagenase, decreased in eWAT with short term HFD, but did not change in other depots **(Figure 2-3C).** Long-term HFD (16wks) induced both *Mmp12* and *Timp1* expression in eWAT **(Figure 2-3D,E).** *Mmp14* increased in eWAT after HFD **(Figure 2-3F).**

Based on the range of reports in the literature, we next set out to clarify the source of *Mmp12* expression in adipose tissue after diet-induced obesity. We analyzed *Mmp12* gene expression from total adipose tissue or from adipose tissues that had been fractionated to separate mature adipocytes and the stromal vascular fraction (SVF). Consistent with previous reports, the SVF was identified as the major site of *Mmp12* induction **(Figure 2-4A).** FACS cell sorting was then used to separate ATMs ($F4/80^{+}CD11b^{+}$) and Non-ATMs from the SVF. *Mmp12* expression was enriched in the ATM sub-fraction from both lean and obese groups and was induced by HFD only in the ATM sub-fraction. **(Figure 2-4B)** In contrast, the expression of *Timp1* and *Mmp14* were more prominently expressed in the non-ATM sub-fraction, where they were both induced with HFD **(Figure 2-4,-D).** Overall, our data demonstrate a rapid induction of *Mmp12* in ATMs with fat expansion that precedes other genes involved in ECM remodeling.

Lipolytic remodeling activates Mmp12 expression.

To observe rapid adipose tissue remodeling in a different context, we employed the use of an acute lipolysis model (106). To examine the regulation of *Mmp12* gene expression in this setting, we assessed age-matched lean (ND) and obese (16-week HFD) mice after three days of treatment with a d adrenergic receptor agonist, CL-316243 (CL).

Compared to lean vehicle-treated controls, *Mmp12* was induced by CL compound **(Figure 2-5A).** In obese mice, *Mmp12* was highly expressed compared to lean controls, however CL did not induce further *Mmp12* expression in obese mice. CL had no significant effects on *Timp1* expression or *Mmp14* expression in lean or obese mice **(Figure 2-5B,C).**

Mmp12 deficiency does not alter weight or metabolism with high fat diet feeding.

From our observations, we hypothesized that MMP-12 would play a critical role in modifying the elastin matrix to permit adipocyte hypertrophy in response to high calorie diets and obesity. Therefore, we tested the impact of *Mmp12* deficiency on adiposity and measures of metabolic dysfunction and inflammation in adipose tissue. To assess this, $Mmp12^{-/-}$, $Mmp12^{+/-}$, and $Mmp12^{+/+}$ littermates were fed ND or HFD for 10 weeks. In adult lean mice, there were no significant differences in body weight or glucose metabolism as seen by fasting glucose measurements (data not shown). In the HFD fed group, $Mmp12^{-/-}$ and $Mmp12^{+/-}$ mice had higher body weights compared to $Mmp12^{+/+}$ during the later stages of HFD feeding **(Figure 2-6A).** However, body composition analysis showed no significant differences in percent lean or fat mass. To evaluate glucose regulation, we measured fasting glucose and insulin levels, and performed glucose and insulin tolerance tests and observed no significant differences between genotypes (**Figure 2-6B).** Tissue weights of the liver, eWAT, iWAT, and rWAT fat depots did not differ between genotypes, indicating that adipose tissue expansion was not dependent upon *Mmp12* **(Figure 2-6C).** Adipocyte size **(Figure 2-6D)** and density (# of

adipocytes per gram, not shown) did not differ between genotypes, nor did expression of the markers of adiposity *Lep* or *AdipoQ* **(Figure 2-6E).**

To evaluate if *Mmp12* plays a role in chronic obesity and metabolism, we fed *Mmp12*$^{-/-}$ and *Mmp12*$^{+/+}$ HFD for 25-27 weeks. In contrast to short term feeding, no significant differences in body weight, body composition, fat pad weights, or glucose tolerance were observed between genotypes (data not shown). Together, these data suggest that an *Mmp12* deficiency does not play a significant role in adipose tissue expansion, metabolism, or chronic obesity.

Mmp12 deficiency does not influence ECM deposition or ATM accumulation

We assessed long term HFD fed *Mmp12*$^{-/-}$ and *Mmp12*$^{+/+}$ mice for adipose tissue ECM and did not observe significant differences in the deposition of total collagen as measured by Picrosirius collagen stain (data not shown). Similarly, we did not see differences in the organization of the elastin matrix in eWAT as measured by Verhoeff's stain **(Figure 2-6F).** Because *Mmp12* may impact ATM function, we examined CLS formation and observed a mild qualitative decrease in the number of Mac-2^{+} CLS in eWAT of *Mmp12*$^{-/-}$ animals similar to other reports **(Figure 2-6G)** (14). However, flow cytometry analysis failed to detect any differences in the quantity of CD11c^{+} ATMs and CD11c^{-} resident ATMs in long term HFD fed *Mmp12*$^{+/+}$ and *Mmp12*$^{-/-}$ mice (data not shown).

Mmp12 deficient animals express higher levels of ECM genes

Finally, we assessed if *Mmp12* deficiency influences ECM gene expression in adipose tissue. From the eWAT from HFD- fed *Mmp12*$^{-/-}$ and *Mmp12*$^{+/+}$ mice, small but statistically significant increases in the expression of *Col1a, Col5a,* C*ol6a3,* and *Tgfb1* in eWAT of *Mmp12*$^{-/-}$ mice were observed **(Figure 2-7A).** We did not observe differences in expression of genes involved in crosslinking ECM components and ECM processing (Figure 7B). Evaluation of genes related to macrophage migration and inflammation in adipose tissue showed an increase in *Csf1*, but a decrease in *Mrc1* gene expression (a marker of alternatively activated macrophages) **(Figure 2-7C).** No significant differences in expression of chemokine genes were observed **(Figure 2-7D).**

Discussion

The makeup of the adipose tissue ECM has the potential to influence whole-body metabolism by modifying the ability of adipocytes to store nutrients. Of the ECM components, adipose tissue elastin is one of the least understood. Elastin has unique mechanical properties that provide tissue flexibility and resilience to deformation (5). Our findings demonstrate 1) a distinct depot-specific architecture of the elastin network in adipose tissue with more a dense network of elastin fibers in visceral compared to subcutaneous adipose tissues, 2) speculation of the use of elastin fibers perhaps as a scaffold by ATMs, 3) ATMs are the main source of the metalloelastase *Mmp12* that is induced with lipolysis, short term, and chronic HFD challenge, and 4) obese *Mmp12*$^{-/-}$

mice have increase expression of ECM genes, but do not demonstrate significant differences in glucose metabolism or adipose tissue inflammation compared to WT mice.

In several tissue fibrosis settings, the density of elastin has been shown to increase after injury (21, 226). Our results match a study reporting the pericellular localization of heterogenic elastin fibers around adipocytes (5). Our results add further detail about its organization and changes with obesity and suggest the importance of post-translational regulation of elastin stability and fiber organization in adipose tissue. The stark differences in elastin organization and mesh density between the visceral and subcutaneous depots may shed some light into why the expansion and inflammatory potential of these two fat pads are different. We posit that the organization of elastin fibers may be responsible for the metabolic and inflammatory differences between visceral the subcutaneous adipose depots.

Our immunostaining revealed the surprising observation that many resident ATMs are bound to elastin fibers, and was more clearly observed in subcutaneous depots. This may fall in line with observations that elastin and its fragments can stimulate macrophage chemotaxis (84, 91). ATMs may be using elastin fibers as an organizing scaffold or alternatively located there poised to degrade or remodel the elastin network.

To further study the role of MMP-12 in adipose tissue, we challenged *Mmp12*$^{-/-}$ mice to short term and long term HFD. We observed a small increase in total body weight in the *Mmp12*$^{-/-}$ mice compared to WT controls with the short-term treatment. This effect was not observed with a longer HFD challenge. Our short term diet challenge results are comparable to the study by Lee *et al* , however our studies differ because they demonstrated a profound increase in adipose tissue mass in *Mmp12*$^{-/-}$ mice which we did

not observe (111). It is unclear why the body weight differences did not persist with chronic feeding in our studies. In both cohorts however, we did not observe significant metabolic differences related to *Mmp12* deficiency. This observation concurs with Bauters *et al* which show no metabolic phenotype despite an decrease in ATMs, and conflicts with Lee *et al* who suggest that *Mmp12* plays a role in aggravating insulin resistance (14, 111). Differences in experimental design and husbandry conditions (e.g. microbiome) may explain our differing results. Bauters *et al* used a mouse strain on a mixed genetic background. Lee *et al* used *Mmp12*$^{-/-}$ animals on a C57BL/6 background, yet it is unclear if littermate controls were used. We feel that the use of littermate and heterozygous controls is required to control for husbandry conditions.

The observation that *Mmp12* is robustly and rapidly induced with lipolytic and obesogenic stimuli in iWAT may be consistent with a requirement for elastin to be remodeled to permit adipocyte morphological changes such as with hypertrophy. However, from our mouse studies, it appears that overall *Mmp12* is dispensable in the overall regulation of the elastin network. Our findings further emphasize the importance of overlapping and redundant function of MMPs and other ECM remodeling enzymes. It suggests that other elastases such as cathepsins and neutrophil elastases which have been implicated in obesity and metabolic disease may play a more prominent role in ECM reorganization (205, 238). Overall, while we have added further detail in one of the components of the adipose tissue ECM, our data suggests that MMP-12 may not play a significant role in regulating the elastin matrix, metabolism, or in modifying adipose tissue macrophage recruitment or phenotypes in mice.

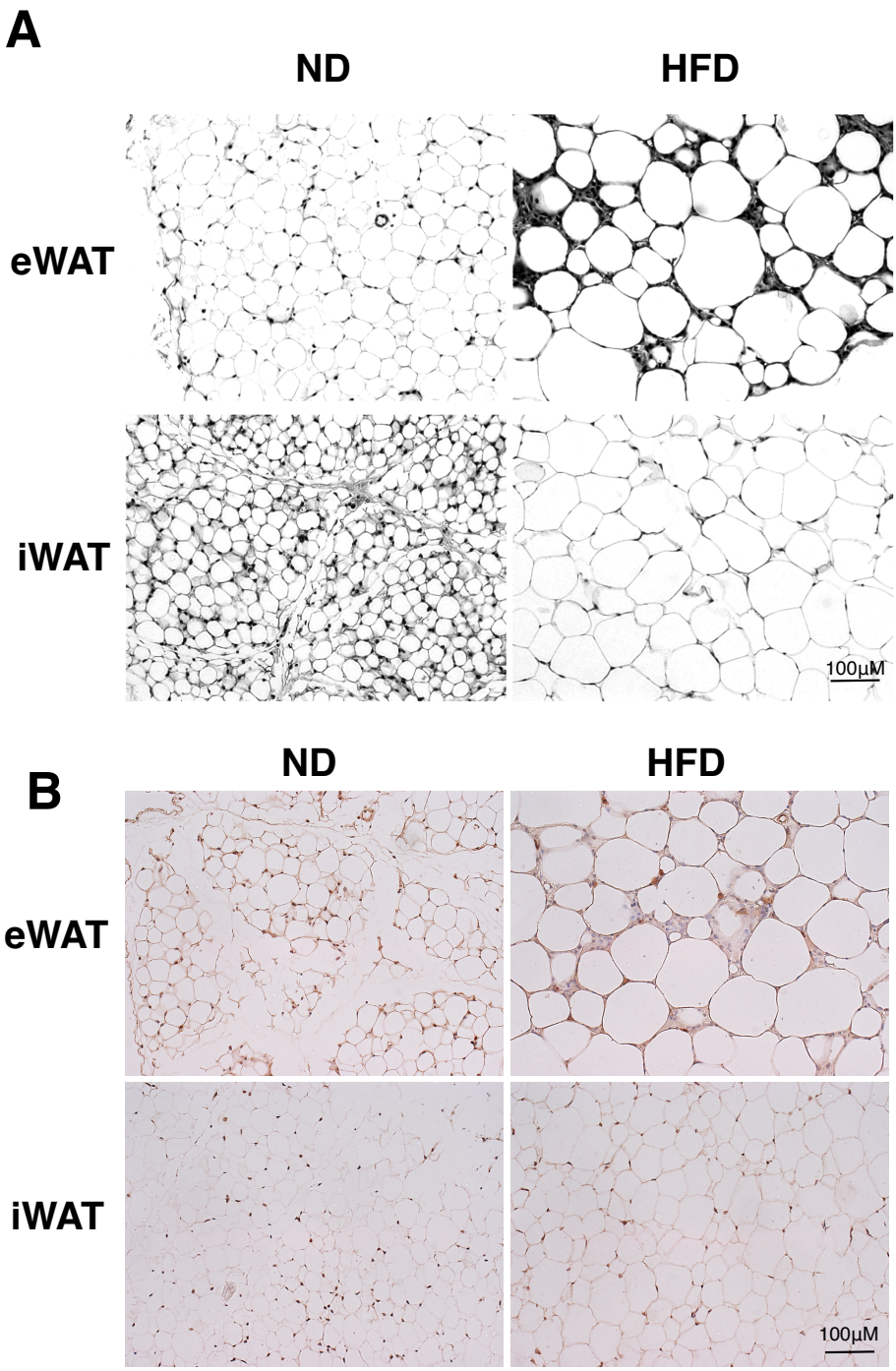

Figure 2-1. High fat diet-induced obesity modifies the adipose tissue elastin matrix. Paraffin embedded sections were stained with (A) Verhoeff Van Giesonlism, or in modifying adipose tissue macrophage recruitment or phenotypes in mice. of MMPs and other ECM remodeling enzymes. It sugges) mouse epididymal (eWAT) and subcutaneous inguinal (iWAT) tissues. Representative images shown from one of 5 replicate samples with similar results. (scale bar = 100 μ M).

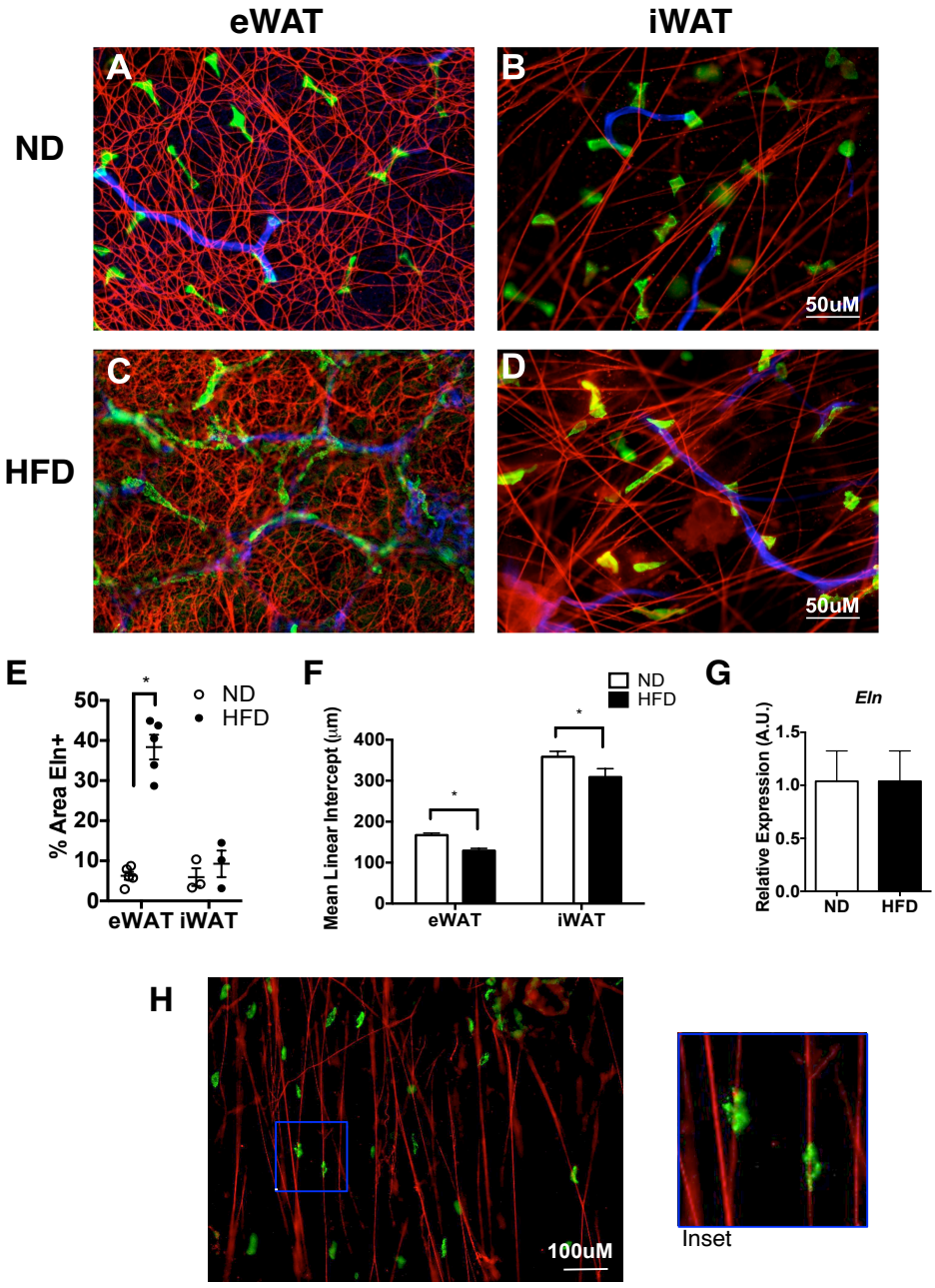

Figure 2-2. Elastin matrix architecture differences between visceral and subcutaneous tissues. Immunofluorescence imaging of elastin (red), resident ATMs (MGL1; green), and blood vessels (Isolectin; Blue). (A,B) Representation of whole mount tissues from lean (ND) eWAT and iWAT and (C,D) obese (HFD) eWAT and iWAT. (scale bar = 50 μ M). (E) Graphical representation of percent elastin positive (%Eln^{+} area) and (F) reticulation by Mean Linear Intercept (MLI). (G) Gene expression analysis of *Elastin* from eWAT from ND and HFD animals. (H) Representative image of ATMs bound to Elastin matrix (scale bar = 100 μ M). (mean ± SEM; n= 2-4 per group*p<0.05)

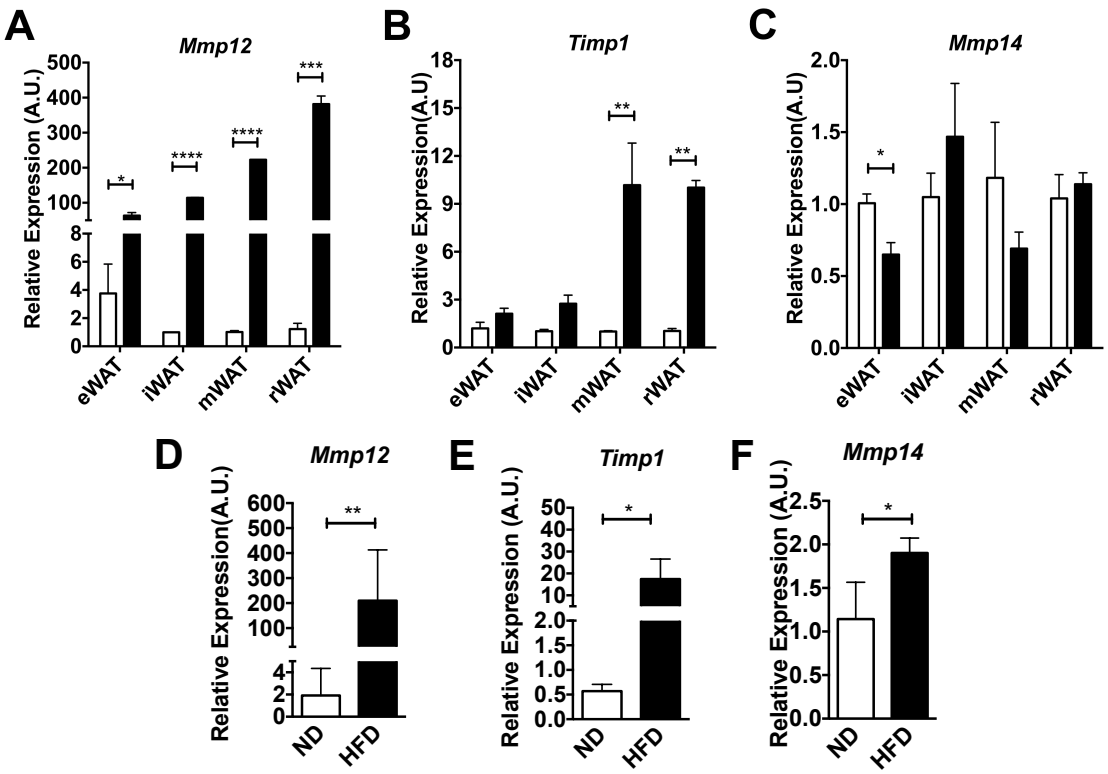

Figure 2-3. *Mmp12* is dynamically regulated in adipose tissue with short term and long-term diet induced obesity. Analysis of (A) *Mmp12,* B) *Timp1*, and (C) *Mmp14* gene expression in major fat pad depots (mWAT = mesenteric, rWAT = perirenal) from lean (ND) and obese (HFD) mice after 4 weeks (n=4). (D-E, G) eWAT gene expression analysis for (D) *Mmp12*, (E) *Timp1*, and (F) *Mmp14* from animals on ND or HFD for 16 weeks. (mean ± SEM; n=4-6 per group, *p<0.05, **<0.01***0.001, ****0.0001)

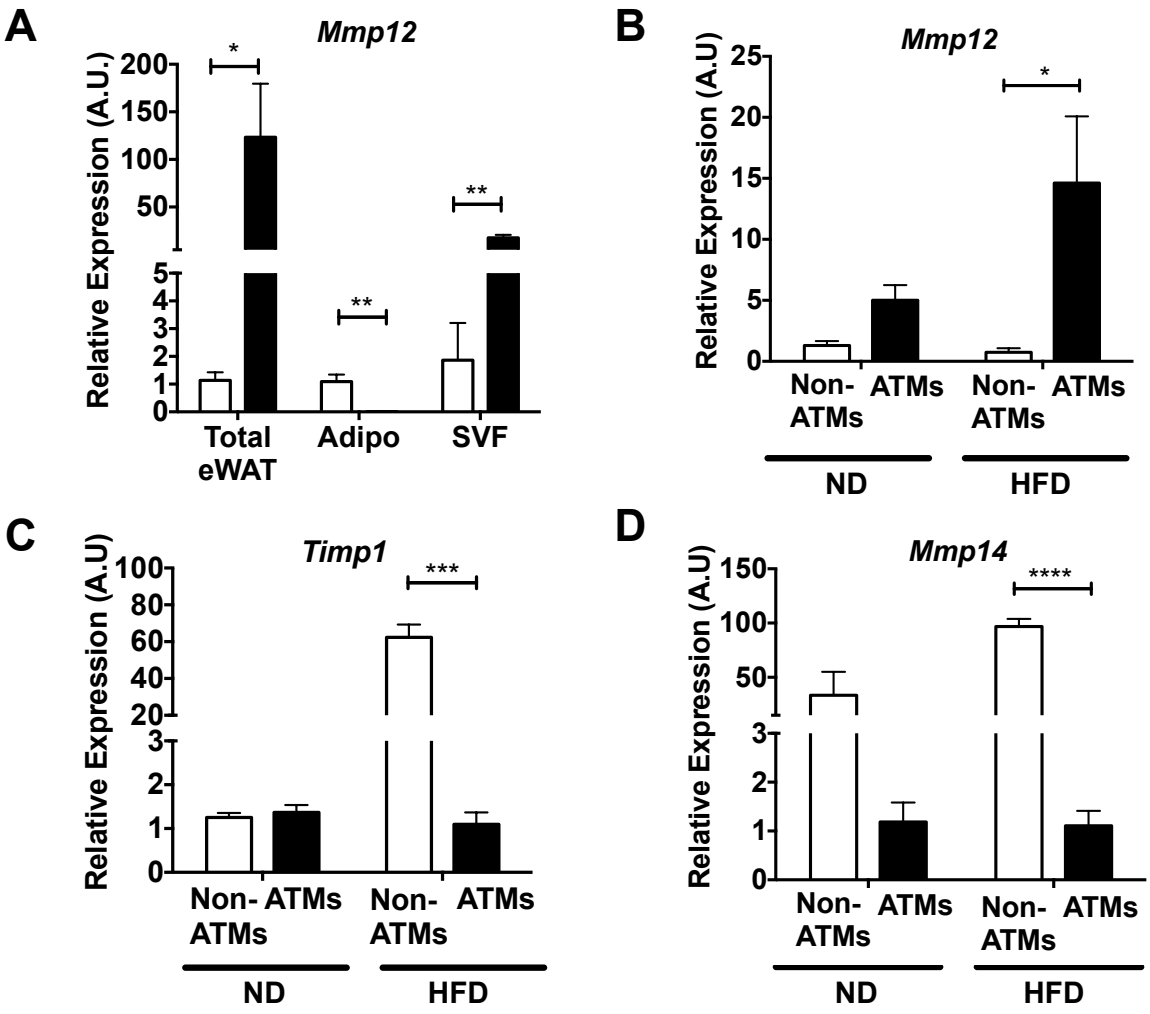

Figure 2-4. *Mmp12* is primarily induced in adipose tissue macrophages with HFD. (A) Expression of *Mmp12* from total eWAT, dissociated adipocyte fraction (Adipo), and the stromal vascular fraction (SVF) normalized to total ND eWAT levels. Expression analysis of (B) *Mmp12*, (C) *Timp1*, and (D) *Mmp14* from FACS sorted eWAT SVF. (ATMs= Black bars, F4/80^{+} CD11b^{+}, Non-ATMs = White bars, F4/80^{-}CD11b^{-}). (mean ± SEM; n=4 per group, *p<0.05, **<0.01***0.001, ****0.0001)

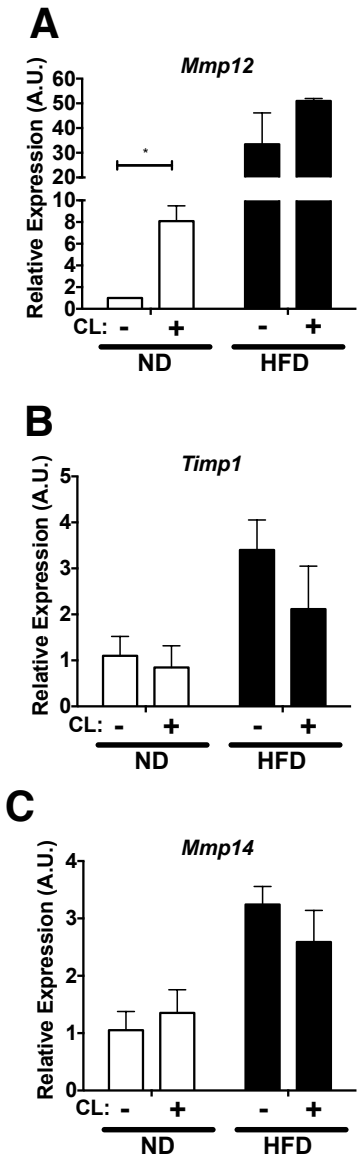

Figure 2-5. Model of acute lipolysis induces *Mmp12* in visceral adipose tissues. Gene expression analysis of (A) *Mmp12*, (B) *Timp1*, and (C) *Mmp14* in eWAT from ND (white bars) and HFD (black bars) fed mice after I.P. injection of CL-316243 (CL) for 3 days. (mean ± SEM; n=3 per group, *p<0.05)

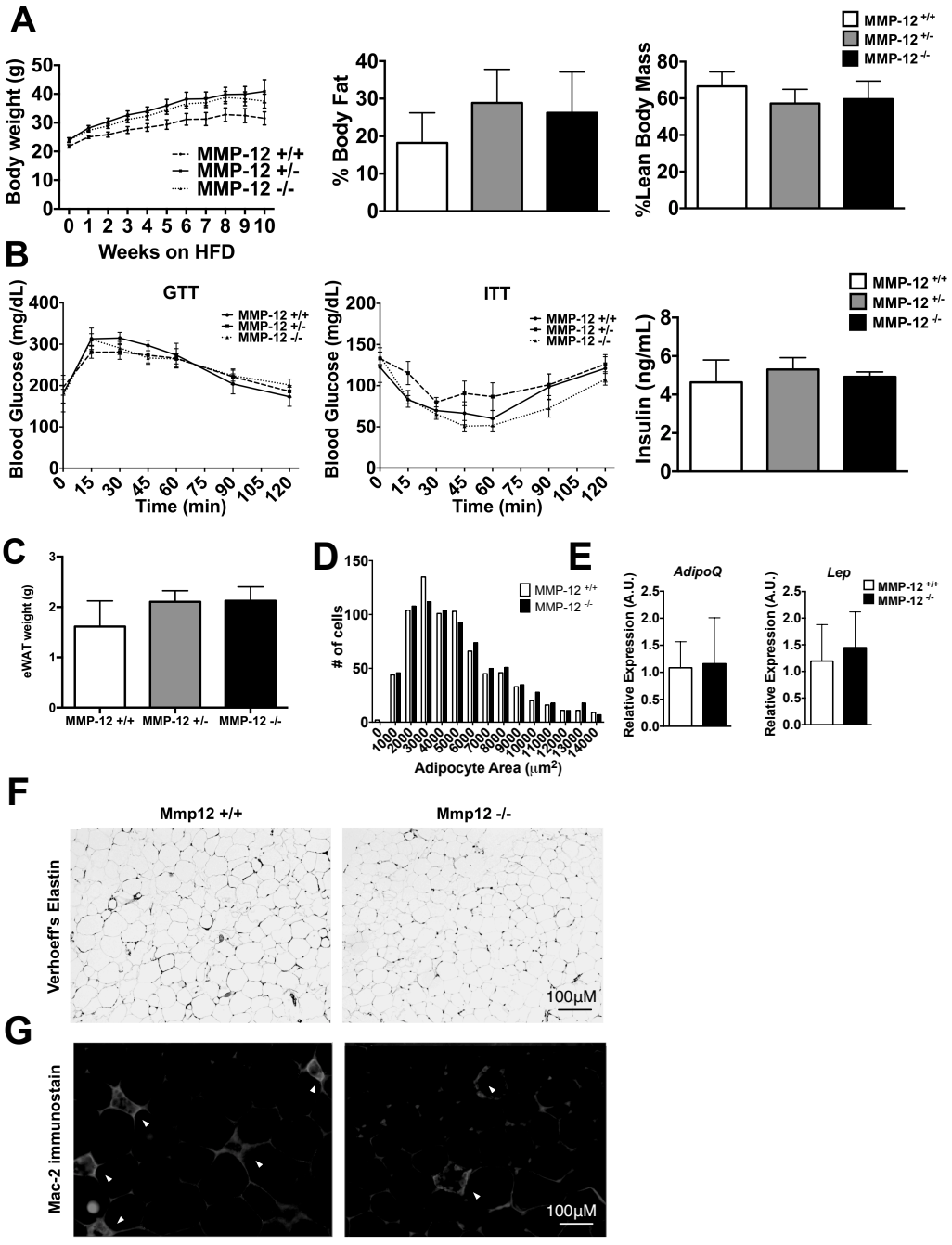

Figure 2-6. *Mmp12* deficiency does not influence body composition or metabolism. (A) Body weights were measured in male *Mmp12*$^{-/-}$, *Mmp12*$^{+/-}$, and *Mmp12*$^{+/+}$ mice fed HFD for 10 weeks, body composition was measured at 8 weeks of HFD. (B) Glucose Tolerance Tests (GTT), Insulin tolerance tests (ITT), and fasting serum insulin concentrations were examined. (C) eWAT weights were recorded after sacrifice, and (D) a graphical representation of adipocyte number by size was recorded. (D) *AdipoQ* and *Lep* gene expression was examined. (F) Verhoeff's stain and (G) immunofluorescence stain for Mac-2^{+} ATMs in HFD-fed *Mmp12*$^{-/-}$ mice and wild type controls shown in gray scale. (scale bar = 100 μ m) (mean ± SEM; n=4-10).

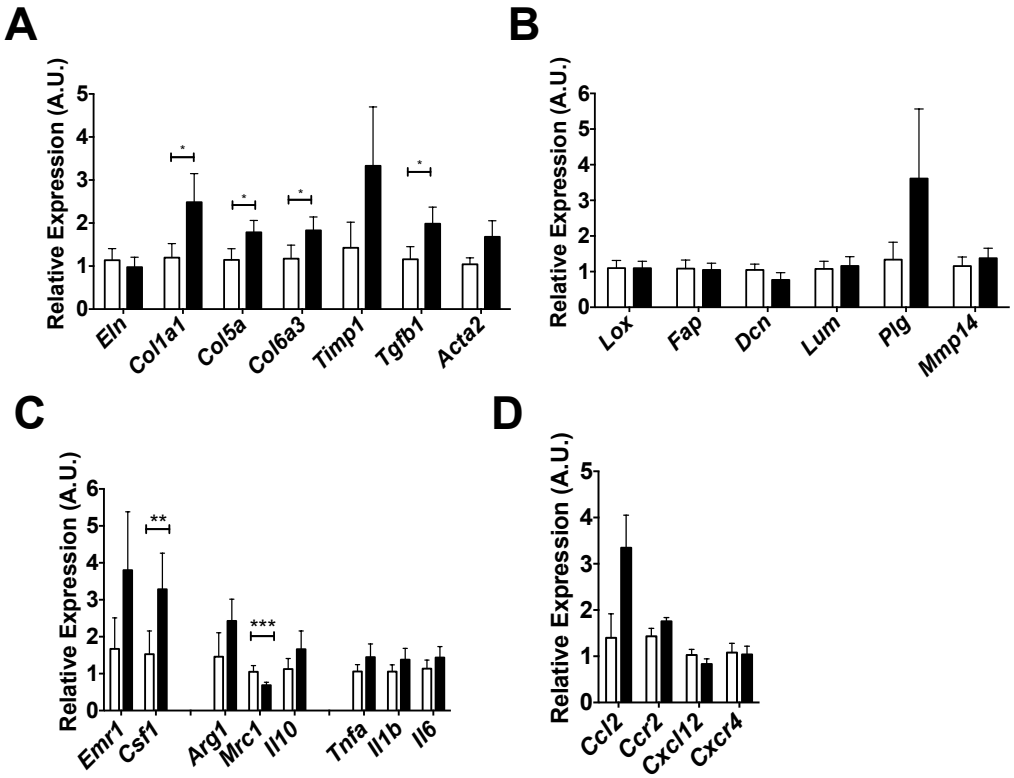

Figure 2-7. Gene expression analysis of markers of extracellular matrix remodeling in *Mmp12*$^{-/-}$ mice. *Mmp12*$^{+/+}$ data shown in white bars, *Mmp12*$^{-/-}$ in black bars. (A,B) Gene expression analyses of ECM remodeling-related genes and markers of ECM maturity and crosslinking, (C,D) inflammatory cytokines and chemokines are shown (mean ± SEM; n=4-8, *p<0.05, **<0.01***0.001).

Chapter 3.

Diet-Induced Obesity Expands Collagen-I^{+} Preadipocyte Populations in Mice

Abstract

The density of the adipose tissue ECM increases with chronic obesity and can lead to adipose tissue fibrosis, which is associated with insulin resistance and metabolic disease. Adipose tissue fibrosis correlates with the accumulation of ATMs and stromal cells in matrix-dense regions, however these interactions are not well defined. Here, we set out to identify stromal factors contributing to ECM production and the mechanisms by which this program is activated in diet-induced obesity. Adipose tissue fibrosis was noted to be prominent in visceral but not subcutaneous depots only after prolonged high fat diet feeding. Microarray analysis and identification of Collagen I+ preadipocytes by flow cytometry demonstrate that preadipocytes are the major source of collagen I production with diet-induced obesity. ColI^{+} preadipocytes accumulated in visceral fat pads to a greater extent than subcutaneous fat pads. Secreted factors from visceral, but not subcutaneous adipose tissue induced collagen I in preadipocytes, and TGF -1 was identified as a likely candidate for this as it is induced in ATMs in obese mice. *In vitro*

Currently under review: **Martinez Santibanez G**, Zamarron BF, Geletka L. Lingaya MA, Lucas H, Maley N, Singer K, Morris D, Muir L, Chun TW, O'Rourke B, Lumeng CN. Diet-induced obesity expants Collagen-I+ preadipocyte populations. *Submitted AJP Endo*

experiments revealed that alternative activated macrophages, but not classical or metabolically activated macrophages induced expression of fibrillar type I collagen in primary preadipocytes via TGFu-1 signaling pathway. We conclude that preadipocytes are the main source of collagen I expression and implicate TGFß-dependent signals between macrophages and preadipocytes in triggering adipose collagen I production.

Introduction

Obesity is a strong risk factor for type 2 diabetes and is associated with metabolic disturbances relating to nutrient sensing and regulation (166). Adipose tissue is the body's main energy repository and one of its most important functions is to store and release nutrients according to metabolic demand (112). The progression from a lean to an obese state involves changes in adipose tissue structure that include adipocyte hypertrophy (increased cell size) and hyperplasia (new adipocyte formation) (202). This transformation is regulated by cells that include preadipocytes, endothelial cells and immune cells such as macrophages. Many of these adipose tissue stromal cells undergo quantitiative and qualitative changes with obesity and contribute to the development of insulin resistance and progression to diabetes (12, 63, 235).

The ECM plays a pivotal role in adipose tissue remodeling events and comprises a substantial portion of the adipose tissue non-cellular bulk (133, 176, 233). ECM remodeling, the active deposition and degradation of the ECM proteins, plays crucial roles in adipogenesis, adipocyte function, and adipose tissue structural integrity (41, 42, 53). Adipose tissue ECM is comprised of many structural protein and carbohydrate

components, predominantly the family of collagens – both fibrillar (e.g. Type I) and non-fibrillar (e.g. Type VI) (53, 133, 170). With obesity, an imbalance of ECM deposition, degradation, and crosslinking of ECM compounds results in higher accumulations of insoluble collagen fibrils, often referred to as adipose tissue fibrosis. Adipose tissue fibrosis is a hallmark of obesity and is a feature of metabolically dysfunctional adipose tissue (53, 198, 203). Obesity induces the expression of a wide range of genes related to ECM production (20, 74). While it is widely accepted that the interstitial deposition of fibrotic material is associated with obesity, mechanisms by which fibrosis develops and at what point the excess deposition becomes a pathological concern are still unresolved (53, 134).

Pre-clinical studies suggest that the dense ECM deposition seen with adipose tissue fibrosis limits the ability of preadipocytes to differentiate and form new adipocytes and of adipocytes to hypertrophy with nutrient excess (41, 42, 198). In this setting, adipocytes fail to efficiently store nutrients as fatty acids leading to ectopic lipid deposition in tissues such as the liver and muscle, with negative effects on whole-body metabolism (75). Decreasing ECM deposition by genetic deletion of non-fibrillar collagen type VI was shown to permit adipocyte hypertrophy and improve whole-body glucose metabolism in mice (97). The relationship between adipose tissue fibrosis, ECM, and structural measures in humans may be more complex, but ECM physiology has been shown to differentiate healthy and unhealthy obese individuals (1, 108).

In other fibrotic diseases, ECM deposition is associated with tissue damage and a local inflammatory reaction driven by leukocytes. The inciting factor in obese adipose tissue may be physical and mechanical stress of enlarged adipocytes. For reasons still

now known, this hypertrophy is associated with the activation of leukocytes such as mast cells and macrophages (75, 198) that may promote a fibrotic response. ATMs are heterogeneous and are best known for their roles in regulating chronic inflammation and their contribution to the development of insulin resistance (12, 122, 228). Resident tissue ATMs are present in lean and obese states and express markers of M2 or alternatively activated macrophages and may play an immunosuppressive role to prevent inflammation and facilitate preadipocyte differentiation (160). Obesity induces the generation of $CD11c^+$ ATMs with some features of M1 or classically activated macrophages that have the capacity to inhibit adipocyte differentiation by generating pro-inflammatory signals (43, 125). While many studies support the concept that the M1-M2 spectrum of macrophage activation contributes to metabolic disease, recent studies suggest this model is overly simplistic and that a distinct "metabolically active" macrophage activation state (MMe) is a feature of ATMs in obesity and diabetes (107, 122, 142, 149, 159).

In many settings of tissue repair and fibrosis, macrophages play important roles as effectors and initiators of ECM remodeling (110, 231). Evidence also supports an important role for ATMs in obesity associated ECM remodeling. There is a reported association of M2c "remodeling" phenotype macrophages in this context (43, 60, 96, 198). ATMs play critical roles in the developmental growth and remodeling of adipose tissue (160, 191, 202). In obese states, ATMs co-localize with preadipocytes in regions undergoing remodeling and new adipocyte formation (114). Macrophage-secreted factors such as inhibin beta A, a TGFß family member, have been identified as factors secreted from classically activated macrophages that induce a pro-fibrotic phenotype in preadipocytes (60, 96). However, the influence of the range of macrophage activation

states on preadipocyte fibrosis and the importance of these cells in the fibrotic response are unresolved.

Here, we investigate the regulation of ECM in adipose tissue in mouse models of diet-induced obesity (DIO) with the goal of identifying the cells responsible for ECM deposition with obesity and to understand their regulation. Using novel flow cytometry techniques to identify collagen-expressing adipose stromal cells, we support a primary role for preadipocytes in the induction of fibrillar type I collagen production with DIO. We have examined how macrophage-derived signals influence preadipocyte ECM production and demonstrate that the M2 alternative activation state, but not M1 or MMe activated macrophages have the strongest effect on induction of preadipocyte collagen I expression via TGFβ signaling. We demonstrate that a pro-fibrotic profile in resident ATMs is induced in obese mice, suggesting that they are a potential source of pro-fibrotic signals. Our observations advance our understanding of ATM activation states and their supporting role in regulating adipose tissue ECM deposition, which may ultimately influences whole body metabolism.

Materials and Methods

Animal Studies

C57BL/6J Male mice were fed ad libitum normal diet (4.5% calories from fat; PMI Nutrition International) or high fat diet (60% calories from fat; Research Diets) starting at 6 weeks of age for durations as described. Animal procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan

and were conducted in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals.

Total Collagen assessment

Total collagen staining was performed on formalin-fixed paraffin-embedded tissue sections using the Picrosirus Red Stain Kit (Polysciences, Inc. Warrington, PA). Images were captured using an Olympus inverted microscope fitted with an Olympus DP27 camera. Image J software (NIH) was used to measure density. Hydroxyproline content was measured in 50mg of tissue input using the Hydroxyproline assay kit from QuickZyme Biosciences (Leiden, Netherlands, dist. Cedarlane Labs Burlington, NC).

Flow Cytometry and Cell sorting

Adipose tissues were dissociated as previously described (36). Fractionation of major adipose tissue populations was performed using a combination of centrifugation, biotinylated anti-CD31 antibodies (Affymetrix eBioscience, San Diego, CA) and lineage depletion kit from Milteny Biotech, Inc. (San Diego, CA). Fluorescence activated cell sorting was performed on cells stained $CD11b^{+}F4/80^{+}$ or $CD11b^{-}F4/80^{-}$ (Affymetrix eBioscience, San Diego, CA) using a FACSaria (BD Biosciences, San Jose, CA). For intracellular collagen staining, cells were incubated in Fc Block then stained with surface antibodies (Affymetrix eBioscience, San Diego, CA) and Live/Dead viability stain (Thermo Fisher Scientific-Life Technologies, Carlsbad, CA) for leukocytes ($Live/Dead^{-}$ $CD64^{+}$, $CD45^{+}$) preadipocytes ($Live/Dead^{-}CD31^{-}CD45^{-}Sca\text{-}1^{+}$, PDGFR α^{+}) and endothelial cells ($CD31^{+}$) for 45 min at 4°C. Briefly, intracellular stains were performed

using FOXP3 Fix/Perm buffer (BioLegend, San Diego, CA) followed by 15 min block with 0.5% goat serum, a 1hr incubation with 0.5 μ g of rabbit Collagen Type I antibody (Rockland, Limerick, PA), three washes, and 30 min incubation with 0.2µg of goat anti-rabbit AlexaFluor 647 antibody (Thermo Fisher Scientific-Life Technologies, Carlsbad, CA). Flow cytometry performed on FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA.) and analyzed with FlowJo data analysis software (Ashland, OR).

Explant tissue culture and conditioned media

Tissue explants were cut to 150mg pieces and cultured in serum-free AIM-V AlbuMAX (BSA) media for 48 hours at 37°C (Thermo Scientific-Life Technologies Carlsbad, CA). For cytokine array analysis, tissues were cultured in serum-free DMEM with antibiotics for 48 hours 37°C. Explant conditioned media was pooled and assessed with the Proteome Profiler Cytokine Array (Panel A) (R&D systems, Minneapolis, MN). Immunoblots were quantified after background subtraction for integrated pixel density with ImageJ. Secreted TGF β -1 protein from tissue explants was collected in serum-free DMEM and measured with ELISA at the University of Michigan Cancer Center Immunology core facility.

Cell culture experiments

3T3-L1 mouse preadipocytes from American Type Culture Collection (Manassas, VA) were cultured and differentiated as described (121). Cells were treated with recombinant IL-1 β at 10pg/mL and IL-1Ra at 40pg/mL for 48hrs (R&D systems, Minneapolis, MN). Primary stromal cells were cultured in 10% heat-inactivated FBS and

high glucose DMEM and Primocin (Invivogen, San Diego, CA). ALK5/TGF β signaling inhibitor SB431542 (Tocris, Bristol, UK) was used at 100nM for 48hrs. ImageStream analysis was performed at the University of Michigan flow cytometry core facilities using ImageStreamX Mark II flow cytometer (EMD-Millipore-Amnis, Seattle, WA). Immunofluorescence staining was performed on 10% buffered formalin-fixed cells with Collagen Type I or Pro-collagen I antibody (Rockland, Limerick, PA) AlexaFluor 488 antibody (Thermo Fisher Scientific-Life Technologies, Carlsbad, CA), aSMA-Cy3 antibodies (Sigma-Aldrich, St. Louis, MO), and DAPI (Thermo Fisher Life Technologies, Carlsbad, CA). For 3T3-L1 macrophage conditioned media exposure experiments, 2mLs of undiluted conditioned media was added to 50K cells/well in 6 well dishes.

Bone marrow-derived macrophages and activation

Bone marrow cells were isolated from mice by flushing tibias and fibulas. Cells were differentiated into bone marrow-derived macrophages as described (195). Following differentiation, macrophages were activated to M1, M2, and MMe activation states as described (107). After 3 X 5min washes with PBS, conditioned media was collected for 24 hours in serum-free low-glucose DMEM with antibiotics. Secreted TGF β -1 protein levels were measured with ELISA at the University of Michigan Cancer Center Immunology core facility.

Gene expression analysis

Total RNA was extracted using RNeasy Mini Kits (QIAGEN), and cDNA was generated from 0.5ug of RNA using high-capacity cDNA reverse transcription kits (Applied Biosystems). Power SYBR Green PCR Master Mix (Applied Biosystems) and the StepOnePlus System (Applied Biosystems) were used for real-time quantitative PCR. *Arbp* expression was used as an internal control for data normalization. Samples were assayed in duplicate, and relative expression was determined using the $2^{-\Delta\Delta CT}$ method. For microarray analysis, RNA was purified from FACS sorted viable "resident" ($CD45^+CD64^+CD11c^-$) and $CD11c^+$ ($CD45^+CD64^+CD11c^+$). Affymetrix microarray analysis (Mouse Gene ST 2.1) was performed as described in triplicate samples from lean and obese (HFD-fed for 16 weeks) (n=3 per group) (124). Pathway analysis was performed with DAVID (79, 80).

Statistical Analysis

Data are shown as mean ± standard error of the mean (SEM). Differences between groups were determined using two-tailed Student *t* tests and one-way ANOVA analysis with GraphPad Prism 6.0e software. $p < 0.05$ was considered significant.

Results

Diet-induced obesity increases adipose tissue ECM deposition in visceral fat depots

Since relatively few studies have evaluated how adipose tissue ECM changes with duration of high fat diet feeding over time, we performed a time course analysis of

C57/BL6 male mice fed either normal chow diet (ND) or HFD for 4 to 24 weeks. Body weights increased significantly with longer duration of HFD challenge **(Figure 3-1A).** Epididymal white adipose tissue (eWAT) weights increased with HFD and peak hypertrophy was reached at 12 weeks of diet challenge with a subsequent decline at later time points **(Figure 3-1B).** In contrast, subcutaneous inguinal white adipose tissue (iWAT) tissue weights peaked later after 16 weeks of diet challenge before stabilizing **(Figure 3-1C).**

To evaluate if collagen accumulation was a possible contributor to the differences in adipose tissue hypertrophy, we examined collagen deposition via picrosirius red total collagen stain at 12 and 24 weeks of diet **(Figure 3-1D).** HFD induced a qualitative increase in pericellular collagen staining around adipocytes in eWAT at 12 weeks that increased at 24 weeks. Also observed as an increase in smaller adipocytes reminiscent of new adipogenesis. In iWAT however, we did not observe a qualitative increase in collagen based on picrosirius red staining with obesity. This data suggests that in diet induced obesity models, total collagen density is a feature that appears in eWAT prior to iWAT and correlates with a limitation in adipose tissue hypertrophy.

Biochemical assessment of hydroxyproline was used on adipose tissue samples to quantify total collagen content in eWAT from animals fed HFD. Compared to age matched ND mice, 8 weeks of HFD induced a decrease in collagen content in eWAT. eWAT samples from mice fed a HFD for longer durations demonstrated a trend towards an increase in hydroxyproline content **(Figure 3-1E).** Hydroxyproline content in iWAT is lower after 16 weeks of HFD **(Figure 3-1F).** Gene expression analysis of fibrillar *Col1a1* and non-fibrillar *Col6a3* in eWAT demonstrated the induction of both genes after

8 weeks of HFD exposure that correlates with the histologic analyses **(Figure 3-1G).** Conversely, we observed a downward trend of expression of these ECM components in iWAT with HFD. These findings highlight the depot specific differences in the association between adipose tissue fibrosis and limitation in fat expansion.

Collagen expression decreases with adipocyte differentiation

Despite the evidence supporting increases in adipose tissue ECM gene expression and density in eWAT with obesity, the cellular sources of ECM production in adipose tissue are not clearly delineated. To investigate this in preadipocytes/adipocytes, we evaluated the dynamics of ECM gene expression in preadipocytes as they differentiate to lipid-laden adipocytes. 3T3-L1 preadipocytes were differentiated for 0, 3, 6, or 9 days and gene expression analysis was performed. During the course of differentiation, the expression of fibrillar collagen I (*Col1a1*) decreased as did the expression of lysyl oxidase (*Lox*), a key enzyme that crosslinks collagen and elastin fibers to mature the ECM **(Figure 3-2A)** (45). In contrast, the expression of non-fibrillar collagen VI (*Col6a3)* was more prominent in mature adipocytes compared to preadipocytes. These data suggest that undifferentiated preadipocytes express more fibrillar *Col1a1* and switch to express non-fibrillar *Col6a3* during differentiation.

To validate these results in primary tissues from mice, we evaluated ECM expression in stratified adipose tissue stromal vascular fraction (SVF) cells. We isolated populations of eWAT SVF cells from age-matched ND and HFD-fed mice using a combination of centrifugation and magnetic bead cell sorting **(Figure 3-2B).** This protocol allowed us to generate four fractions enriched for 1) mature adipocytes, 2)

$CD31^-$ $CD45^-$ preadipocytes, 3) lineage positive (Lin^+) $CD45^+$ leukocytes, and 4) $CD31^+$ endothelial cells. In ND-fed animals, the preadipocyte-enriched fraction expressed the highest levels of *Col1a1* at about a level ~20 fold higher than mature adipocytes **(Figure 3-2C).** After HFD challenge, the preadipocyte-enriched fraction express *Col1a1* at levels upwards of ~200X higher compared to mature adipocytes **(Figure 3-2D).** We also observed the induction of *Col1a1* expression in leukocytes and endothelial cell fractions with HFD. However, similar to ND tissues, the expression of *Col1a1* in leukocytes and endothelial cells was only a fraction of the levels expressed by preadipocytes.

We also examined *Col6a3* and *Lox* expression in these samples. In lean animals, the expression of *Col6a3* is highest in the leukocytes compared to the mature adipocyte fraction **(Figure 3-2E).** After DIO however, there was an induction of *Col6a3* in preadipocyte fraction and endothelial cells. Preadipocytes had the highest expression of *Col6a3* on a per cell basis. *Lox* is primarily expressed by leukocytes in lean animals. With DIO, *Lox* expression was suppressed in leukocytes and induced in the preadipocyte enriched fraction **(Figure 3-2F).** These data may implicate the $CD31^-$ $CD45^-$ preadipocyte-enriched fraction as the major adipose tissue stromal component responsible for the induction of ECM and ECM regulatory genes with obesity and potential key contributors to ECM deposition during the progression of obesity.

Microarray analysis demonstrates that preadipocytes are the major source of ECM gene expression in lean and obese adipose tissue

To better understand the contributions of adipose tissue SVF cells to the pro-fibrotic response to obesity, we used FACS to purify macrophages ($CD45^+$ $CD64^+$) and

preadipocytes ($CD31^-$ $CD45^-$ $Sca\text{-}1^+$ $PDGFR\ \alpha^+$) from eWAT and performed transcriptional microarray profiling **(Figure 3-2G)** (115) (216). By enriching for PDGFR α^+ cells by flow cytometry, we have eliminated other possible mesenchymal cells (e.g. PDGFR α^- pericytes that may have contaminated our previous $CD31^-$ $CD45^-$ fractions (114, 115). In lean mice, we compared the expression profile of "resident" ATMs ($CD45^+$ $CD64^+$ $CD11c^-$) and preadipocytes, the two most prominent SVF populations. Preadipocytes were significantly enriched for gene expression pathways involved in fibrinolysis, focal adhesion, ECM-receptor interactions, TGFß signaling, and integrin signaling **(Figure 3-2H).** Similar gene expression pathways were enriched in preadipocytes when we compared the gene expression profiles of $CD11c^+$ ATMs and preadipocytes in HFD fed mice (data not shown). This suggested that in both lean and obese settings, preadipocytes are the primary source of pro-fibrotic ECM gene expression.

To evaluate the qualitative effects of HFD on preadipocytes, we compared the gene expression profiles of preadipocytes purified from ND and HFD fed animals. This demonstrated a significant increase in gene pathways involved in ECM-receptor interactions, hematopoietic lineage, complement signaling, and cell adhesion components in preadipocytes from HFD fed mice **(Figure 3-2I).** Decreases in gene pathways involved in amino acid metabolism and steroid biosynthesis were observed in HFD preadipocytes. In summary, our microarray profiling further supports the role of preadipocytes as a major source of ECM component gene expression in lean and obese states and that DIO significantly activates the expression of ECM-receptor regulatory genes in preadipocytes.

Intracellular flow cytometry staining identifies collagen I$^+$ (ColI$^+$) preadipocytes

In order to directly assess fibrillar collagen I production in adipose tissue stromal cells, we utilized intracellular flow cytometry with anti-collagen I antibodies, a method developed to identify fibrocytes in pulmonary fibrosis (175). ImageStream confocal imaging of 3T3-L1 preadipocytes in suspension identified punctate intracellular ColI$^+$ staining vesicles that were independent of BODIPY$^+$ lipid droplets **(Figure 3-3A).** Flow cytometry analysis of 3T3-L1 preadipocytes demonstrated positive ColI staining compared to isotype and secondary antibody-only controls **(Figure 3-3B).** Immunofluorescence visualization of immature pro-collagen I and mature collagen I revealed similar punctate staining patterns consistent with the active production of collagen protein in preadipocytes that matches gene expression profiling (Fig 3B). Primary preadipocytes isolated from mouse eWAT also reveal ColI$^+$ cells using both flow cytometry and immunofluorescence detection methods **(Figure 3-3C).** Overall, we validate the use of flow cytometry to delineate collagen I expressing preadipocytes.

ColI$^+$ preadipocytes are expanded with HFD in visceral but not subcutaneous depots

Using our intracellular flow cytometry method, we set out to characterize collagen I expression in SVF cells in eWAT and iWAT with DIO. In both depots from ND and HFD-challenged animals, we observed ColI$^+$ cells in the three major stromal vascular fraction cell populations: endothelial cells (CD31$^+$), leukocytes (CD45$^+$), and preadipocytes (CD31$^-$CD45$^-$Sca-1$^+$, PDGFRα^+). In eWAT, only about 10% of CD45$^+$ leukocytes were ColI$^+$ and these did not change with DIO in either eWAT or iWAT, making leukocytes unlikely to be a significant source of collagen production with DIO

(Figure 3-4A,B). A population of ColI$^+$ endothelial cells (CD31$^+$ ColI$^+$) was identified in both ND and HFD mice, which amounted to ~10% of the SVF population. In eWAT the percentage of CD31$^+$ ColI$^+$ decreased with DIO. In contrast, the percent of PDGFRα^+ ColI$^+$ preadipocytes rose from ~60% in the lean animal to ~90% in the HFD animals demonstrating a correlation between ColI$^+$ preadipocytes and fibrosis measures in eWAT with DIO.

Next, we compared the percentage of ColI$^+$ cells as a percent of total SVF cells in the three major SVF populations in eWAT and iWAT. First, ColI$^+$ endothelial cells decrease from 7% to ~4% of the eWAT SVF with HFD, while in iWAT, ColI$^+$ endothelial cells increased significantly from 9% to 22% ($p<0.0005$) **(Figure 3-4B).** No significant changes in ColI$^+$ leukocytes were observed in eWAT or iWAT with HFD. In eWAT from lean mice, ColI$^+$ preadipocytes made up ~18% of the SVF. This proportion significantly increased to ~32% with HFD. In contrast, ColI$^+$ preadipocytes in the iWAT decrease with obesity. Representative eWAT and iWAT flow cytometry dot plots of preadipocyte populations are shown **(Figure 3-4C.)**

Flow cytometry permitted a quantitative assessment of per-cell collagen I content by assessment of mean fluorescence intensity (MFI). Comparing the three populations, CD45$^+$ cells were dimly stained (~750 MFI) compared to CD31$^+$ endothelial cells and preadipocytes (~8000-14,000 MFI) **(Figure 3-4D).** After HFD, endothelial cell MFI decreased while preadipocyte MFI increased significantly. Collectively, our results demonstrate 1) an induction of ColI expression in eWAT preadipocytes with DIO, and 2) a quantitative increase of ColI$^+$ preadipocytes with DIO.

Fat depot-specific differences in ColI$^+$ preadipocytes

Next, we set out to further understand how the progression of obesity affects the quantity of collagen-expressing preadipocytes over time. Mice were fed a HFD for 10, 20, and 30 weeks. In eWAT, the percentage of ColI$^+$ cells significantly higher than age matched ND controls regardless the duration of HFD challenge **(Figure 3-4E).** Between 10 and 20 weeks of HFD, the proportion of ColI$^+$ preadipocytes increases significantly in eWAT, but did not increase further after 20 weeks of HFD feeding. At all time points, the content of ColI$^+$ preadipocytes in iWAT was less than in eWAT. The proportion of ColI$^+$ preadipocytes in iWAT increases significantly at 10 weeks of HFD. Surprisingly, we observed a decrease in these cells after 20 weeks of HFD relative to age matched ND controls due to an increase in these cells in lean mice between 10 and 20 weeks of age. After 30 weeks of HFD, no differences between ND and HFD were found between iWAT ColI$^+$ preadipocytes **(Figure 3-4F).**

Adipose tissue secreted factors induce ECM expression in preadipocytes

We next set out to examine if adipose tissue secreted factors were sufficient to modulate ECM expression in preadipocytes. To address this, we designed an *in vitro* model where we collected conditioned media (CM) from eWAT and iWAT explants taken from ND and HFD-fed mice and then exposed 3T3-L1 preadipocytes to these media for 48 hours and followed with gene expression analysis. Compared to media only controls, ND eWAT CM was sufficient to induce the expression of many ECM and fibrosis-related genes including *Col4a1, Acta2,* and *Timp1* **(Figure 3-5A).** ND iWAT CM-treated samples also induced *Acta2* and *Timp1* expression but not *Col4a1*. HFD

eWAT CM induced *Col4a1* to a similar degree as ND eWAT, however *Col1a1*, *Col6a1*, *Col5a1*, and *Acta2* were induced by HFD eWAT CM to a greater extent than ND eWAT CM. HFD iWAT CM did not induce *Col1a1* expression and decreased expression of *Acta2* and *Timp1* relative to ND iWAT CM **(Figure 3-5B).** Flow cytometry MFI data further supported the ability of HFD eWAT CM can alone induce collagen I in these cells **(Figure 3-5C).** Together, findings are consistent with both our histology and flow cytometry data, suggesting that secreted factors derived from eWAT have a higher fibrogenic potential than those from iWAT.

We hypothesized that inflammatory chemokines and cytokines uniquely secreted by HFD eWAT explants, but not HFD iWAT explants, could explain the induction of collagen I expression in preadipocytes. To screen for potential factors, we performed a cytokine array analysis on the explant conditioned media to measure the relative amounts of 40 mouse cytokines secreted from similar weight adipose tissue explants from ND and HFD mice **(Figure 3-5 D,E).** Comparison of cytokines generated from ND and HFD mice revealed detectable expression of 22 cytokines above background in eWAT explants and 22 cytokines detected in iWAT. Surprisingly, most cytokines were lower in HFD explants relative to ND explants. IL-1 receptor antagonist (IL1RA) was one of the few cytokines identified as upregulated in eWAT and but not in iWAT as a potential pro-fibrotic factor. To test if IL1RA is sufficient to induce collagen production in preadipocytes, we treated 3T3-L1s with recombinant IL1RA. Flow cytometry analysis revealed that IL1RA was not sufficient to induce collagen expression. These data suggest that signaling mechanisms that regulate collagen expression in preadipocytes may not be chemokine-based.

TGF β-1 as a potential signaling mechanism for pro-fibrotic preadipocyte activation

We next set out to identify other putative pro-fibrotic factors secreted by HFD eWAT that might regulate expression of ECM components in preadipocytes. Our microarray data implicated TGFß signaling pathways in preadipocytes to fibrosis. The source(s) of TGFß-1 in adipose tissue are not fully understood. We first measured *Tgfb1* gene expression in total eWAT from lean and obese animals and observed a significant induction of *Tgfb1* with DIO **(Figure 3-6A).** We then measured TGF β-1 protein levels in conditioned media from eWAT explants from lean and obese animals. ELISA analysis reveals higher TGF β-1 protein produced from HFD eWAT explants **(Figure 3-6B),** but not in iWAT explants (data not shown). To identify the primary regulated source of *Tgfb1* in both lean and obese adipose tissue, we separated adipocytes and the stromal vascular fraction cells from eWAT by magnetic immunoaffinity isolation as in **(Figure 3-2B)**. We observed that both leukocytes and endothelial cells are major sources of *Tgfb1* regardless of diet, and the expression levels in both populations decreased with HFD **(Figure 3-6C).** To determine the contribution of ATM *Tgfb1* expression, we performed gene expression analysis of FACS sorted ATMs ($CD11b^{+}F4/80^{+}$) and the non-ATM ($CD11b^{-}F4/80^{-}$) from eWAT SVF cells. Gene expression analysis revealed that ATMs express *Tgfb1* at higher levels than non-ATMs in lean and obese mice **(Figure 3-6D).** Together, our data suggest that ATMs are a main source of *Tgfb1* in eWAT.

Alternatively activated macrophages induce collagen I in primary preadipocytes

To understand how macrophage activation states influenced TGFß-1 expression, conditioned media from M0, M1, M2, and MMe macrophages were assessed for TGFß-1

production via ELISA **(Figure 3-7A)** (107). M1 macrophages had decreased TGFß-1 production compared to unstimulated M0. While M2 and MMe activation both significantly induced TGFß-1 compared to unpolarized M0 macrophages, the highest levels of TGFß-1 were secreted by M2 alternatively activated macrophages.

Given these observations, we set out to investigate if TGFß-1 signaling was required for activating a pro-fibrotic preadipocyte phenotype. We exposed primary preadipocytes derived from mouse eWAT to macrophage-conditioned media (M0, M1, M2, MMe) with or without the TGFß-1 signaling inhibitor, SB431542 and measured collagen I protein expression by flow cytometry. CM from M2 macrophages was the strongest inducer of collagen I production **(Figure 3-7B)** compared to M0, M1 and MMe-CM. The effect of M2-CM on collagen I induction was lost with the addition of the TGFß-1 signaling inhibitor SB431542. This suggests that the potent effects of M2 macrophages induced collagen I via a TGFß-1 receptor signaling pathway.

Our data suggests that TGFß-1 as a macrophage derived signal that contributes to a pro-fibrotic phenotype in preadipocytes. However, this is somewhat at odds with the decrease in *Tgfb1* seen with HFD and the induction of pro-inflammatory M1/MMe activated $CD11c^+$ ATMs with obesity. We next considered the possibility that altered signals in resident $CD11c^-$ ATMs with obesity may be an underappreciated source of pro-fibrotic signals. We compared gene expression profiles of resident $CD11c^-$ ATMs in eWAT from lean and obese animals **(Figure 3-7C).** Microarray analysis reveals elevated expression of gene pathways related to ECM-receptor interactions, focal adhesion, and lysosome pathway in resident macrophages from HFD mice. Overall, these data suggest

that resident ATMs from obese animals are unlike those of lean animals and are characterized by the induction of pro-fibrotic gene expression profile.

Discussion

Along with adipocyte hypertrophy, the progression from a lean to an obese state includes the dynamic reorganization of the adipose tissue ECM and takes the form of fibrosis with chronic obesity (203). The progression to fibrosis in many tissue settings has been proposed to occur in four major phases: injury, activation of effector cells, dynamic deposition and crosslinking of ECM, and insufficient ECM resorption that promotes the accumulation of insoluble fibril-associated collagens (182). In this study, we have focused on understanding the sources of ECM production in adipose tissue with obesity and the mechanisms that contribute to phases 2-4 of the above paradigm in relation to obesity status and adipose tissue depots.

Our findings shed light on the sources of ECM in adipose tissue, and highlight the differences in fibrotic potential between fat depots in mouse models of diet-induced obesity. The increased density of the ECM in fibrotic adipose tissue increases the mechanical rigidity of the tissue and limits adipocyte function as an efficient nutrient store (202, 203). Our data in a model of diet-induced obesity support this as we observed that adipose tissue hypertrophy in eWAT peaks in conjunction with an increase in fibrotic gene expression, ECM accumulation in adipose tissue, and an increase in the quantity and degree of expression of ColI in preadipocytes. iWAT lags behind eWAT in terms of ECM accumulation and ColI $^{+}$ preadipocyte accumulation suggesting tissue specific

regulation. Our results are consistent with other studies (7), but provide stronger evidence that adipose tissue stromal cells contribute significantly to these changes.

Our quantitation of hydroxyproline did not show a robust increase in collagen expression as did the picrosirius red histology staining and gene expression data. Few studies have used hydroxyproline quantitation in assessments of collagen content in adipose tissues; one study in particular performs an additional normalization step to account for this inconsistency (65). A possible explanation of why our hydroxyproline and histology/gene expression data is not aligned is due to the increase in lipid content in adipocytes that comes with obesity and may decreases total protein content in the input tissue substrate. Additionally, fibrosis develops heterogeneously in pericellular space; therefore averaging out hydroxyproline content per mg of tissue may obscure the findings.

In obese mice and humans, gene expression of *Col1a1* has been consistently reported, but the source of regulated expression of collagen I is unresolved (97, 214). Collagen I is a major insoluble fibril-forming component of the ECM in all tissues and is acutely induced by stimuli such as hypoxia and TGFß1 in fibroblasts (57, 65). Groups have also reported PDGFR α signaling activation in promoting a pro-fibrotic mechanism and in blocking the adipogenic program in perivascular cells (86). Together, our data that further points to preadipocytes and their expression of fibril-forming collagen I is in agreement with published findings.

We have employed methods to identify ColI$^+$ preadipocytes to establish that preadipocytes are a principal source of fibrillar collagen I. While all major cellular populations in adipose tissue (mature adipocytes, endothelial cells, and leukocytes)

express ColI$^+$ to some degree, preadipocytes were the largest ColI$^+$ population in lean states and were the only ColI$^+$ population significantly induced with caloric excess. DIO also stimulated preadipocytes to increase their collagen I production on a per cell basis. Reflecting the increase in fibrosis in visceral depots, ColI$^+$ preadipocytes were enriched in eWAT compared to subcutaneous iWAT depots, but increased in both with HFD induced obesity. A potential limitation in intracellular flow cytometry is in the detecting of cells that may have ingested collagen such as seen in pulmonary fibrocytes (100). Our data suggests that the detection of pro-collagen I in preadipocytes along with the gene expression profiling from FACS sorted cells make this possibility unlikely for preadipocytes. An additional caveat is that we based our observations of collagen expression on an immature form of collagen, which does not necessarily reflect the true nature of fibrotic collagen that has undergone the post-translational modifications and crosslinking to render it insoluble.

Total collagen content measured by complementary methods indicated that at per milligram of tissue, iWAT from lean animals contains more collagen I than in eWAT. HFD induced collagen accumulation and a fibrotic profile in eWAT but not in iWAT. Uniquely, our data reveal that after HFD, the largest population of ColI$^+$ cells in iWAT are CD31$^+$ endothelial cells and not preadipocytes as we observed in eWAT. This suggests that a major differentiator between eWAT and iWAT in the control of fibrosis may be the capacity to degrade fibrillar collagen. This may vary with duration of HFD stimulation as we observed that *Col1a1* gene expression was not substantially induced until after 8 weeks of HFD. This correlated with the observation of lower adipose tissue collagen content in eWAT with shorter (6-8 weeks) HFD exposure. These data

altogether further reveal differences in adipose tissue depot fibrogenic potential, chronic inflammation, and influence on metabolism.

Conditioned media from eWAT or iWAT explants revealed that eWAT, but not iWAT, induced *Col1a1* expression in 3T3-L1 preadipocytes. Cytokine arrays identified IL-1RA a candidate that was induced in eWAT but not iWAT with HFD, however this cytokine was not sufficient to induce *Col1a1*. This led us to consider other secreted molecules as possible mediators of preadipocyte fibrosis such as TGFß-1. TGFß-1 plays a complex role in metabolism in that it plays numerous functions in development, growth, and wound repair (208). TGFß-1 is implicated in ECM remodeling, as it can inhibit matrix metalloproteinase ECM degradation activity, which could further promote ECM accumulation (167). At the physiological level, TGFß-1 plays a regulatory role in insulin transcription, and blocking TGFß-1 signaling can protect against obesity-induced insulin resistance (236). TGFß-1 can inhibit adipogenesis via a Wnt-independent, Smad3-mediated pathway in preadipocytes (37, 38, 215).

TGFß and associated signaling pathways have in the past been investigated in human preadipocytes, however we believe this study produced insufficient results in a study of only a single fibrosis marker (24). Our current study further implicates TGFß-1 in fibrosis as we investigate its role in the expression of synthesized intracellular fibril-forming collagen type I.

Inflammation is also associated with fibrosis in pre-clinical studies and is essential for adipose tissue expansion and remodeling (232). ATM depletion or inactivation attenuates fibrosis (221). In our studies, we observed higher levels of *Tgfb1* gene expression in tissues from obese animals and that ATMs are a main source of *Tgfb1*.

Our gene expression data suggests that the increase of *Tgfb1* expression in whole tissue is most likely due to a quantitative increase in ATM content and not in per cell expression with obesity. We replicated macrophage activation states normally present in lean and obese animals using an *in vitro* model in order to investigate how ATM activation influenced preadipocyte ECM expression. Previous studies have only evaluated quiescent or M1 activated macrophage signaling to preadipocytes (96). Despite the association between $CD11c^+$ ATM accumulation with obesity and fibrosis, we observed that M1 and MMe polarized bone marrow-derived macrophages did not induce significant collagen protein expression in preadipocytes compared to M2 macrophage conditioned media. This agrees with other reports that implicate M2 macrophages in ECM remodeling via TGFß-1 to promote ECM accumulation (167, 198). Potential approaches to support this model include generating mice with macrophage-specific deletion of TGFß-1.

Together, we propose a model where after dietary challenge, resident "M2" ATMs, but not pro-inflammatory "M1" or metabolically activated "MMe" ATMs, promote a pro-fibrotic phenotype in preadipocytes. In support of this, our microarray studies demonstrate significant activation of ECM-receptor pathways in resident ATMs between lean and obese mice.

In summary, our observations reveal that preadipocytes are major producers of fibrillar type I collagen. Because adipose tissue fibrosis in the pathological sense has few explicit criteria, it still remains unclear at what threshold point or at what scale the increased fibrotic deposition begins to have negative effects on metabolism. In addition, there remain significant gaps between our understanding of adipose tissue ECM remodeling in clinical and pre-clinical models. In mice, the link between fibrosis,

macrophage inflammation, and insulin resistance is fairly uniform. In clinical populations, the relationship between fibrosis and metabolic disease is more complex in line with variation between the relationship between obesity and metabolic health (1, 54, 105, 108, 203). Our studies suggest that the function and pro-fibrotic state of preadipocytes may contribute to this variation and may be a future direction of research to unravel this problem.

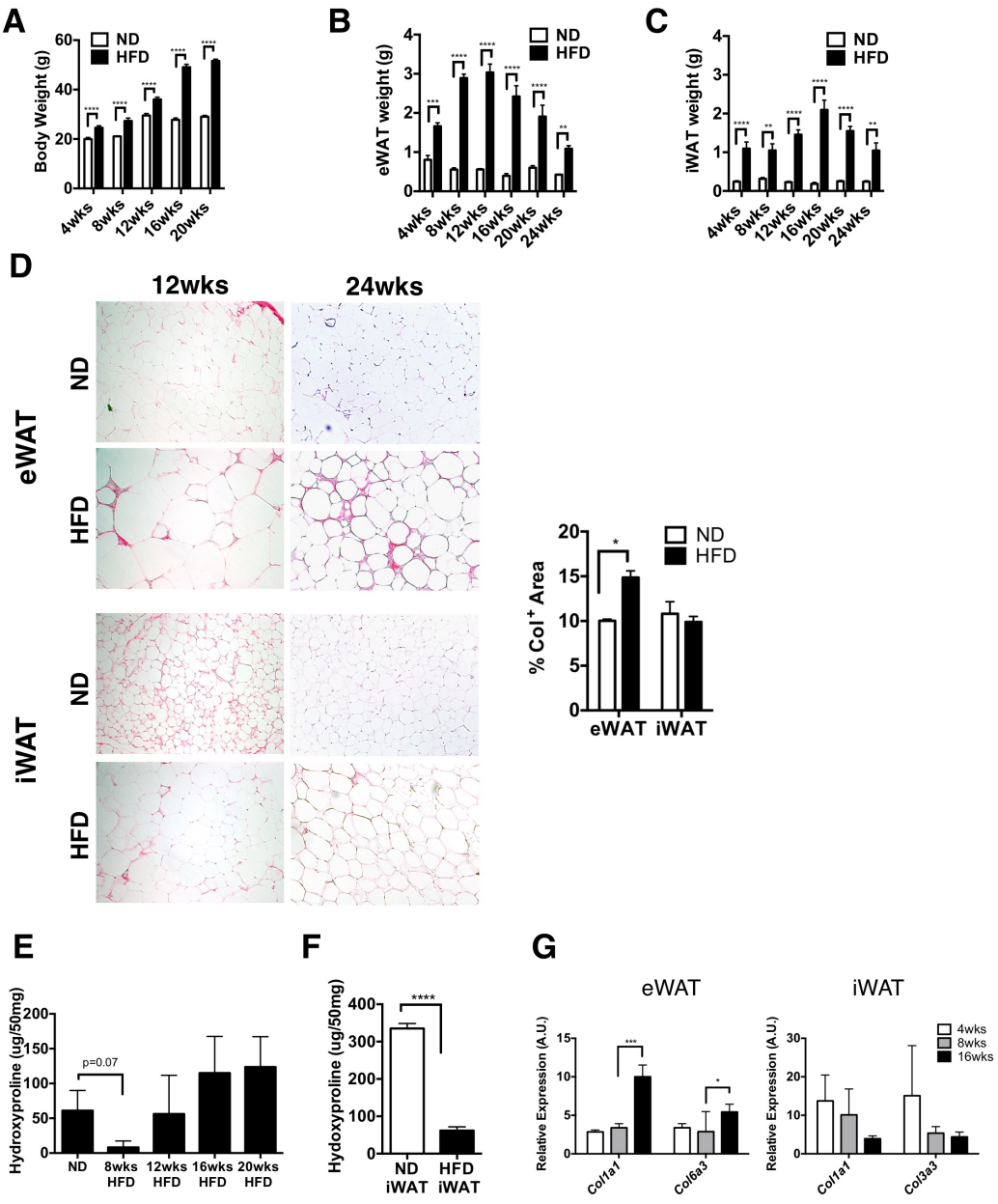

Figure 3-1. Obesity increases ECM density in visceral adipose tissue depots.
(A) Body weight, and eWAT and iWAT fat pad weights (B-C) were measured from ND and HFD mice challenged 4, 8, 12, 16, 20, or 24 weeks of HFD. (D) Picrosirius red histological stains from ND/HFD eWAT and iWAT tissues at 12 weeks and 24 weeks of diet, with quantification of %Col^{+} staining area at 24 weeks of HFD (scale bar = 200 μ M). (E) Total eWAT collagen content shown from time course HFD, ND sample is from adult mouse at 16 weeks of age. (F) Total collagen content shown from iWAT tissue of ND or 16 weeks HFD diet. (G) Gene expression analysis of eWAT and iWAT at 4, 8, or 16 weeks of HFD, each time point is represented as fold change over age-matched ND controls. Samples represented as fold change over age-matched lean controls. (mean ± SEM; n=4 per group for all graphs, *p<0.05, **<0.01***0.001, ****0.0001)

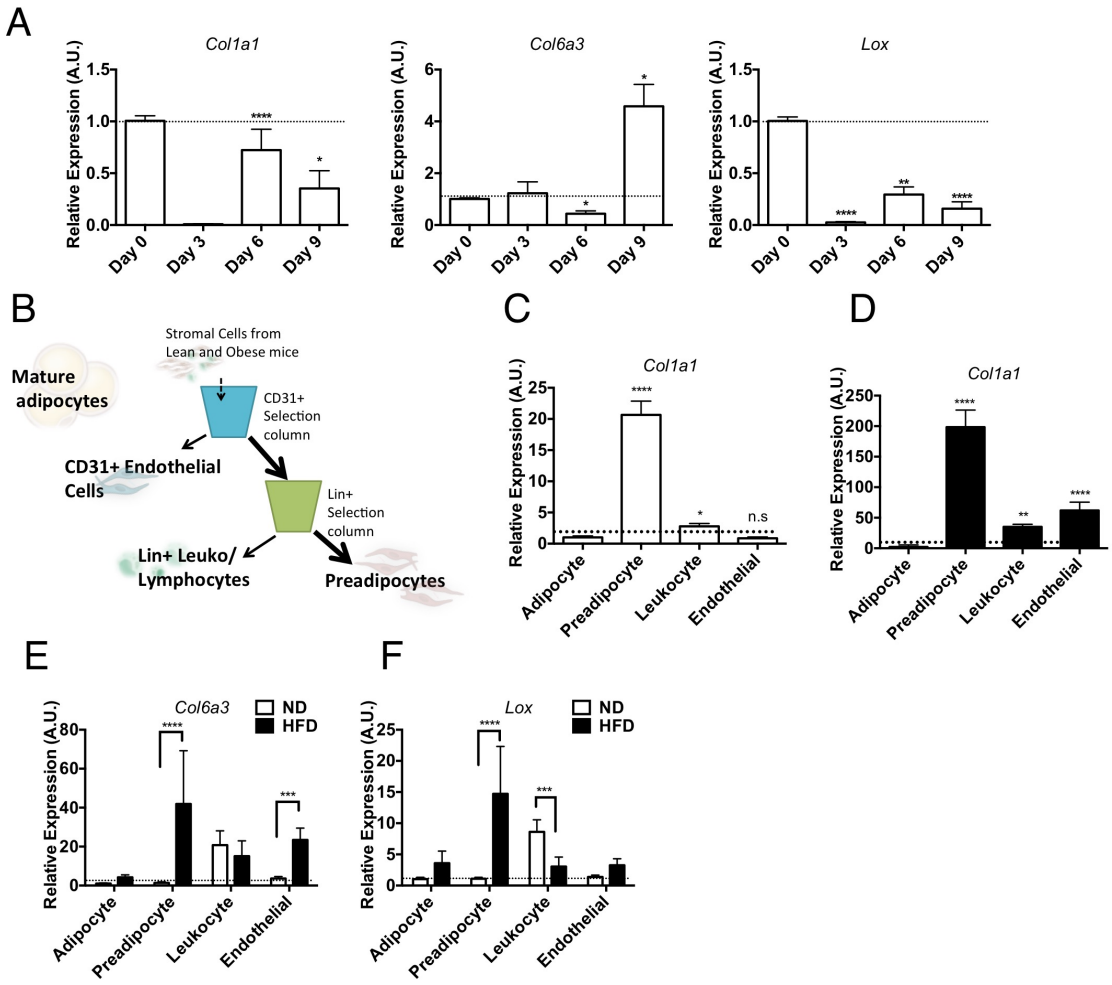

Figure 3-2. Preadipocytes are major source of collagen type I in adipose tissue.
(A) Time course 3T3-L1 preadipocyte cell line differentiation and gene expression analysis, all data points normalized to Day 0 time point. (B) Magnetic microbead cell fractionation strategy is shown. (C-F) Gene expression analysis on sorted populations from eWAT, from ND and 16wk HFD age-matched animals. All data normalized to internal adipocyte fraction values. (mean ± SEM; n=4 per group for all graphs, *p<0.05, **<0.01***0.001, ****0.0001)

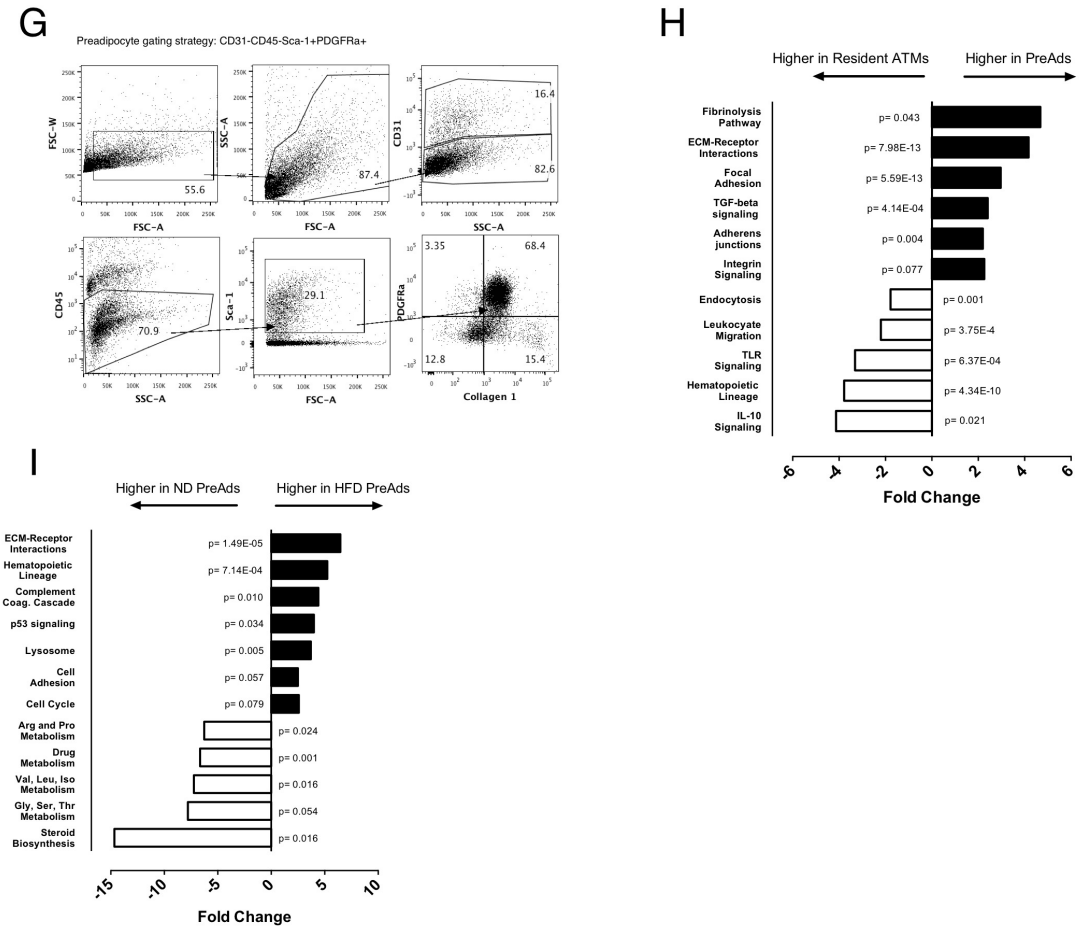

Figure 3-2 continued.

(G) Gating strategy for preadipocytes is shown ($CD31^{-}CD45^{-}Sca\text{-}1^{+}PDGFRa^{+}$). (H-I) A visual representation of microarray analysis data showing fold change of gene expression cluster families (mean ± SEM; n=3-4 per group, *p<0.05, **<0.01***0.001, ****0.0001)

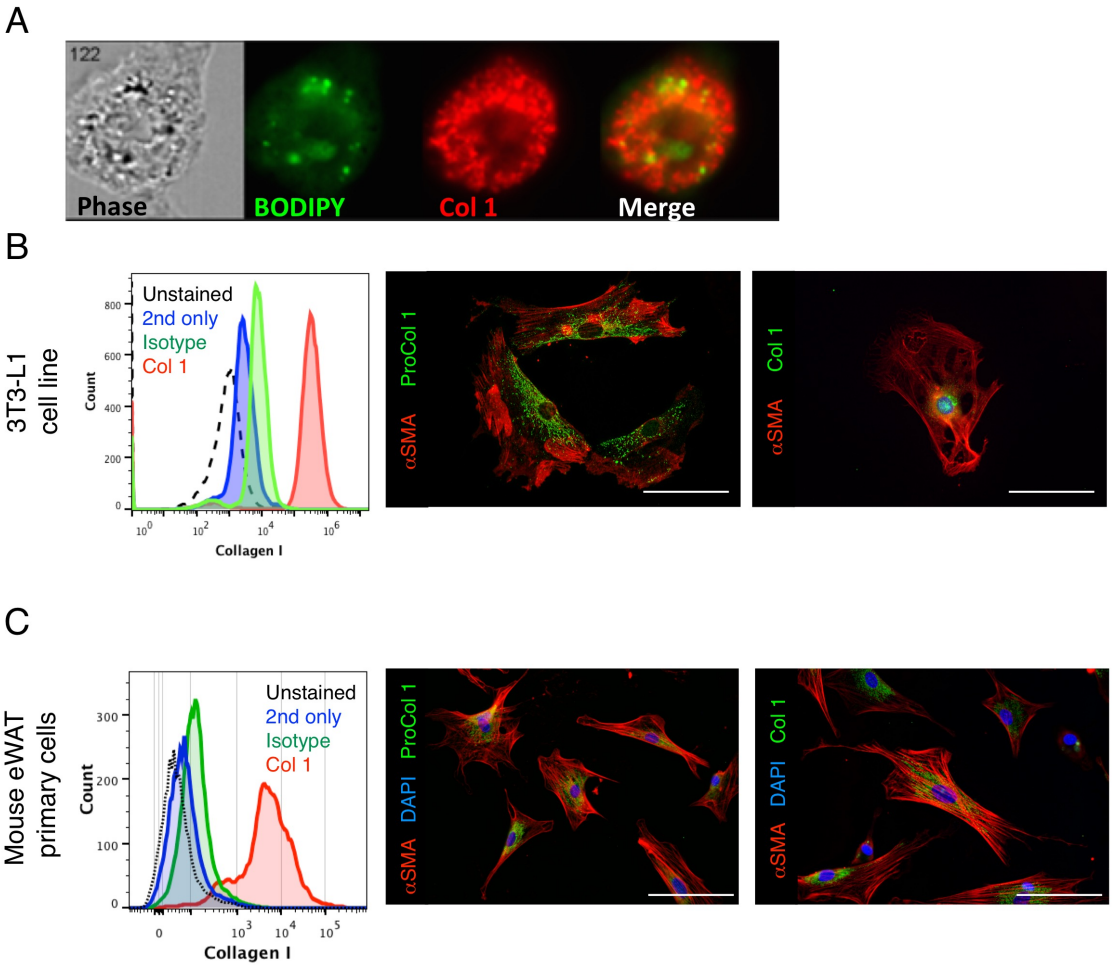

Figure 3-3. Validation of collagen type I detection in preadipocytes. (A) Image Stream cytometer visualization of 3T3-L1 cells in suspension stained with lipid stain (BODIPY) in green and Col 1 in red. (B) Intracellular Collagen I flow cytometry staining controls, immunofluorescence pro-collagen I and mature collagen I stains (green), and actin cytoskeleton (red) are shown on 3T3-L1 cells. (C) Primary mouse preadipocyte flow cytometry and immunofluorescence stains are shown (scale bar = 100 μ M).

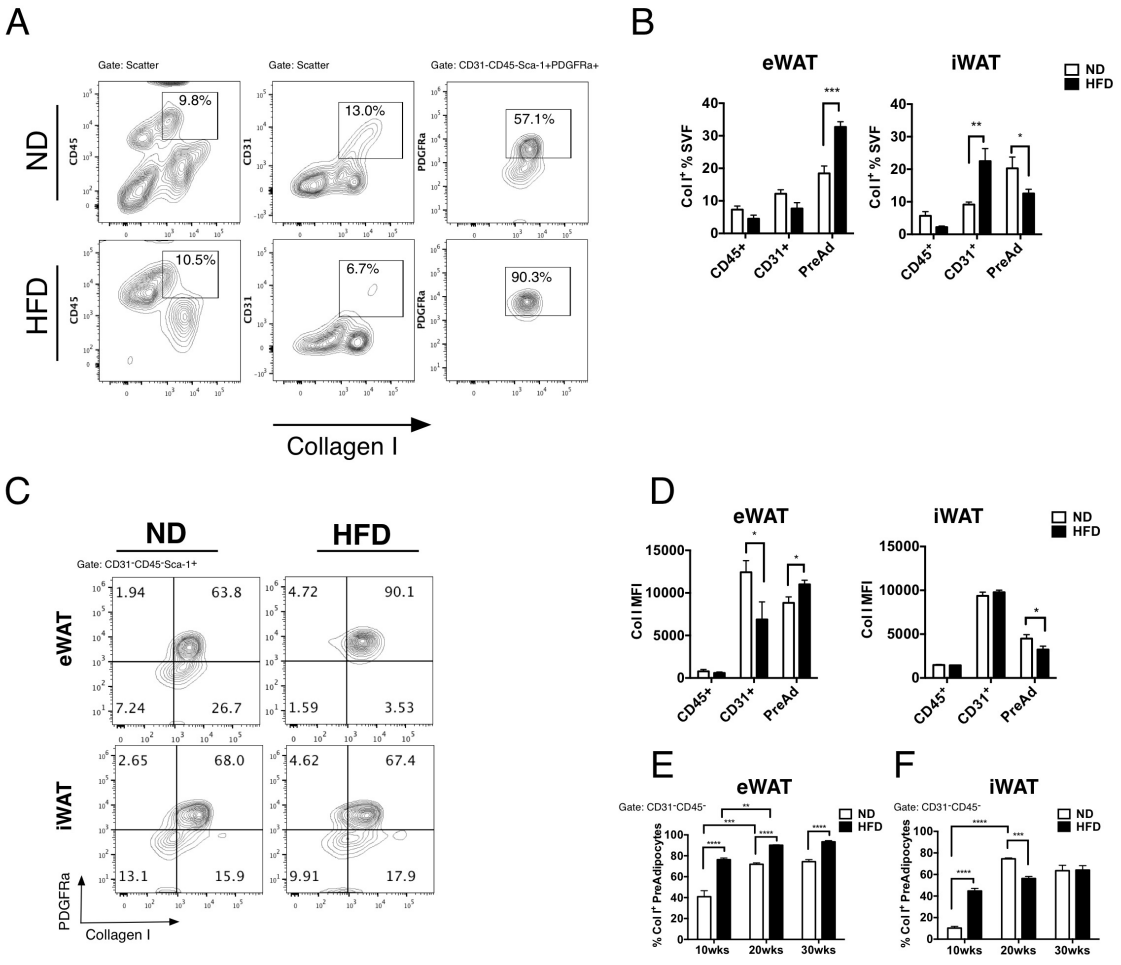

Figure 3-4. ColI$^+$ preadipocytes are induced in visceral adipose tissue depots with diet-induced obesity. (A) Flow cytometry stains reveal Collagen expression in major adipose tissue stromal populations in ND and HFD eWAT. (B) Populations of ColI$^+$ populations were examined in eWAT and iWAT after 16 weeks of HFD challenge. (C) Representative dot plots of preadipocyte populations and Collagen I expression from eWAT and iWAT from ND and HFD-fed animals. (D) Median fluorescence intensity (MFI) of Collagen I stain is shown. (E-F) Populations of gated CD31$^-$CD45$^-$ ColI$^+$ preadipocytes from eWAT and iWAT are shown at 10, 20, and 30 weeks of HFD challenge (mean ± SEM; n=4 per group, *p<0.05, **<0.01***0.001, ****0.0001).

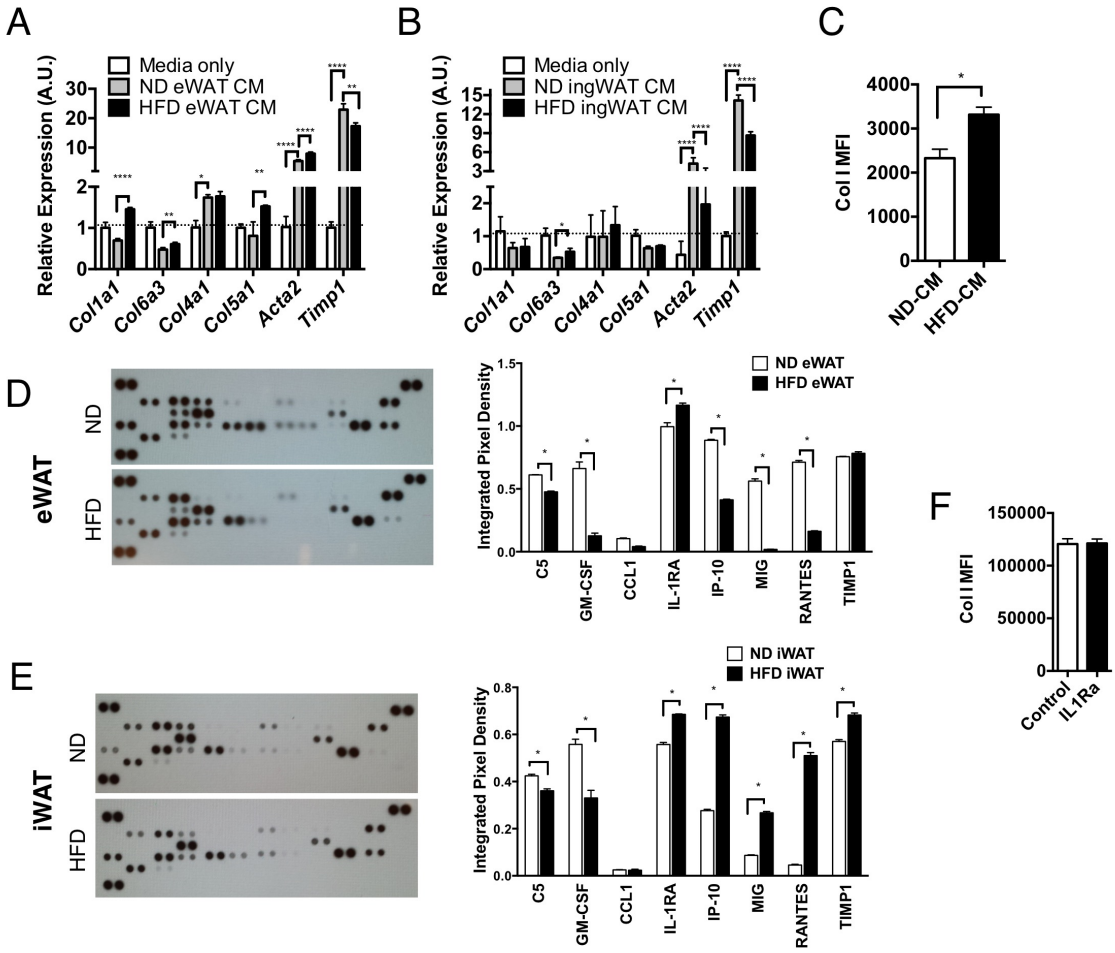

Figure 3-5. Obesity-associated factors from visceral adipose tissue explants induce collagen type I expression in preadipocytes. (A-B) Gene expression analysis of fibrosis markers from 3T3-L1 preadipocyte cells exposed to eWAT or iWAT explant conditioned media. (C) Collagen I MFI from 3T3-L1s exposed to ND and HFD eWAT explant conditioned media. (D-E) Cytokine array blots are shown from eWAT and iWAT explant conditioned media. (40wks HFD with ND age-matched lean controls). (F) Intracellular Collagen I MFI flow cytometry stain from 3T3-L1s treated with cytokines. (mean ± SEM; n=24 per group, *p<0.05, **<0.01***0.001, ****0.0001)

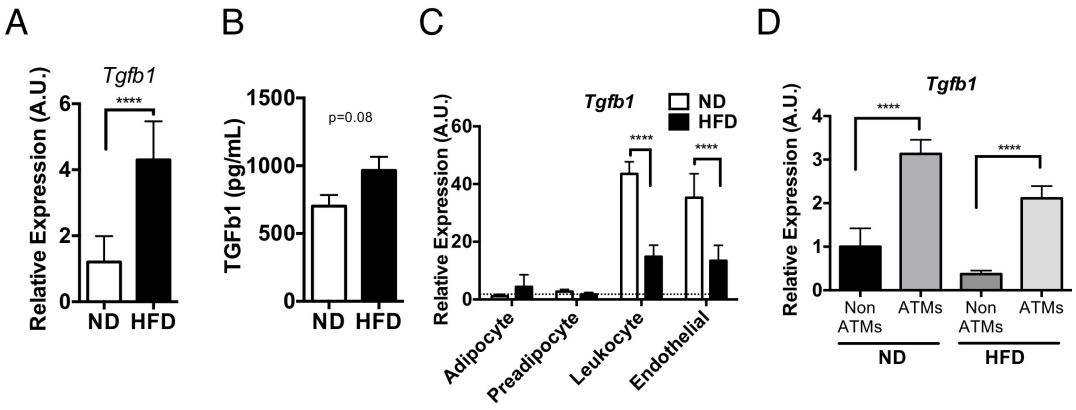

Figure 3-6. Adipose tissue macrophage-derived TGF β -1 is induced with obesity
(A) Gene expression of *Tgfb1* in eWAT after 20 weeks of HFD. (B) ELISA quantitation of TGF β -1 protein levels in eWAT conditioned media. (C) Gene expression of *Tgfb1* in microbead-sorted stromal populations. (D) Gene expression analysis on FACS-sorted ATMs and Non-ATMs shown from ND or 16 week HFD-fed animals. (mean ± SEM; n=3-4 per group for all graphs, *p<0.05, **<0.01***0.001, ****0.0001)

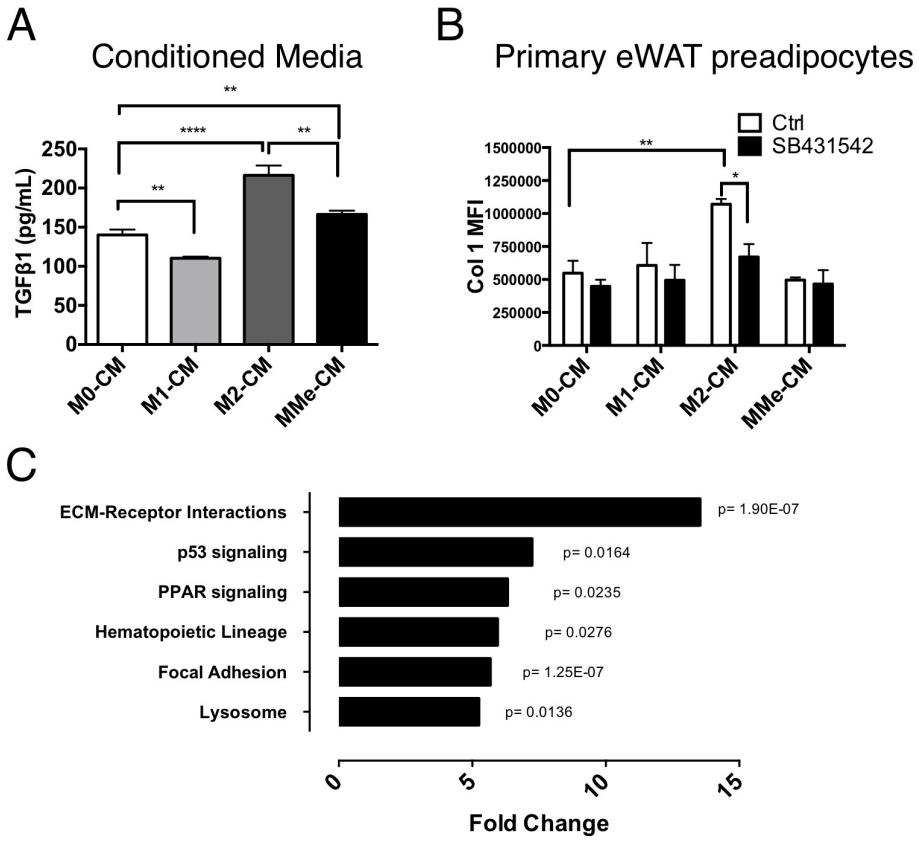

Figure 3-7. Alternatively activated macrophage-derived factors induce collagen type I expression in preadipocytes via TGFß signaling. (A) Quantified TGF β -1 levels secreted from activated macrophages (B) Collagen I MFI from primary mouse preadipocytes treated with activated macrophage conditioned media and TGF β -1 signaling inhibitor. (C) Gene family fold change increase comparing resident macrophages from lean and obese eWAT. (mean ± SEM; n=3-4 per group, *p<0.05, **<0.01***0.001, ****0.0001).

Chapter 4

Adipose Tissue Fibrosis After Weight Loss is Related to Retention of Pro-fibrotic Preadipocytes

Abstract

Obesity alters the architecture of the ECM and is associated with excessive ECM accumulation in the form of adipose tissue fibrosis. Weight loss can improve metabolism and decrease adipose tissue inflammation, however in many cases, the risk of cardiovascular and metabolic disease remains high in formally obese individuals. The reasons for this are unclear, but one potential mechanism is that obesity induces permanent changes in adipose tissue function that do not resolve with weight loss. This hypothesis is supported by reports that suggest that adipose tissue fibrosis is sustained in in patients after bariatric surgery induced weight loss. To examine the mechanisms behind this effect, we have utilized an animal model of weight loss to investigate how the ECM is reorganized with loss of fat mass. We observed that ECM accumulation increases dramatically after weight loss, and that insulin sensitivity remains abnormal despite normalization in body weight by removal of high fat diet feeding. Endothelial cells and preadipocytes are major sources of ECM components type I collagen and elastin. $ColI^{+}$ preadipocyte content increases in gonadal fat pads and remain elevated in quantity with

weight loss. In contrast, the quantity of ColI^{+} preadipocytes return to normal levels in subcutaneous adipose tissue with weight loss. The per cell expression of ColI and Elastin are decreased with weight loss, suggesting that the signals inducing ECM production are dampened. Gene expression analysis supported a general decrease in pro-fibrotic gene expression in eWAT with weight loss. Candidate cytokines were identified by protein array, and were found to be associated with restoration of a low fibrotic environment with weight loss. These data suggest that the sustained presence of adipose tissue fibrosis after weight loss in eWAT does not relate to a sustained high level of ECM production and may relate to a decrease in ECM degradation machinery. Our findings have implications to sustained impairment in adipose tissue function after weight loss.

Introduction

The ECM in adipose tissue plays an important role in the regulation of adipogenesis, adipocyte function, and adipose tissue architecture (39, 44). Adipose tissue expansion requires orchestrated interplay between numerous cell types (177, 186, 200). With the development of obesity, an increase in ECM density is widely observed which is hypothesized to reduce structural plasticity of adipose tissue and contributing to metabolic dysfunction (53, 74). This plasticity is integral to metabolic function of adipose tissues, as the inability to remodel the ECM is correlated with aberrant insulin sensitivity and metabolic disease (53, 197, 202). However, some studies suggest fibrosis may have a protective effect on metabolism by restricting adipocyte hypertrophy (9, 10, 203). Given the importance of healthy adipose tissue expansion and contraction in metabolic health

we are interested in understanding how the adipose tissue ECM is remodeled in different nutrient settings. Understanding how adipose tissue is ECM remodeling in weight loss in particular is of high priority given the common problem of substantial weight regain after initial weight loss (WL) interventions. Understanding the long-term changes obesity has on adipose tissue may also aid in the understanding why the risk of cardiovascular disease does not diminish despite the resolution of obesity (177, 237).

During WL, the ECM undergoes massive reorganizations in architecture in response to a rapid decrease in adipocyte size and quantitative change in stromal cell populations (29, 106). Evidence suggests that adipose tissue function and architecture do not return to normal in patients that have undergone bariatric surgery, which may be due to a permanent disruption of ECM architecture generated by chronic obesity (53, 54). Studies have shown that diabetic patients have stiffer and more fibrotic subcutaneous adipose tissues and lose less weight after bariatric surgeries, providing further support for the ECM flexibility-metabolism paradigm (1, 174). One of the most striking observations is that ECM accumulation appears to persist even in obese patients that have undergone WL (53, 54, 135). Gene expression studies of humans that have undergone bariatric surgery reveal altered expression of ECM components after WL (53). Data that integrate both metabolic and histological studies from clinical and pre-clinical models are limited as few, if any, studies have investigated effects of WL in a longitudinal manner (1).

The persistence of inflammation adipose tissue after is hypothesized to be a major contributor to ECM deposition, and several studies have supported a link between the two processes (54, 186, 199). Adipose tissue from subcutaneous regions of formally obese patients have an inflammatory profile more similar to obese patients (29). There have

also been conflicting studies reporting that metabolic perturbations remain in formally obese mice as well as humans (3, 44). Animal models of short-term weight-loss reveal the recruitment of ATMs, which appears to have functions in initiating ECM remodeling and in the metabolism of excess lipids (54, 106, 110, 231). In human models of surgery-induced WL, there is a decrease of inflammatory ATM activation and an overall decrease of ATMs (29, 44).

There are three major gaps in knowledge of how WL alters the adipose tissue ECM. First, the cellular components in adipose tissue that produce ECM materials with weight loss have yet to be fully delineated. Second, the connections between the qualitative and quantitative changes in stromal populations, and the increase in ECM accumulation that is observed after WL are not understood. Third, mechanisms of cell-cell and cell-matrix communications that influence ECM component production are also not well understood. Filling these gaps in knowledge has the potential to identify modifiable pathways to either amplify weight loss responses or potentiate to increase resolution of adipose tissue inflammation in the weight-reduced state.

In this work, we fill in these gaps with an investigation that integrates metabolic data with in-depth observations of histological findings and investigations into changes in adipose tissue stromal cells with WL relating to ECM production. Our animal model studies reveal that, along with the inability for insulin sensitivity to normalize after WL, the ECM becomes denser after WL. While the quantitative increase in preadipocytes induced by HFD are sustained after prolonged weight loss, the per cell expression of ECM proteins such as collagen and elastin are decreased. This suggests that the

sustained level of adipose tissue fibrosis seen after weight loss is not due to ongoing activation of ECM production.

Materials and Methods

Animal Studies

C57BL/6J Male mice were fed ad libitum normal diet (4.5% calories from fat; PMI Nutrition International) or high fat diet (60% calories from fat; Research Diets) starting at 6 weeks of age for durations as described. WL was induced by replacing high fat diet, with ad libitum normal diet for duration described. Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed after a 6-hour fast. For GTT, mice were injected IP with D-glucose (0.7 g/kg). For ITT, mice were injected IP with human insulin (1 unit/kg). For both GTT and ITT, blood glucose concentrations (mg/dl) were measured 0, 15, 30, 45, 60, 90, and 120 min after injection. Animal procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan and were conducted in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals.

Total collagen assessment

Total collagen staining was performed on formalin-fixed paraffin-embedded tissue sections using the Picrosirus Red Stain Kit (Polysciences, Inc. Warrington, PA) or by Masson's Trichrome stain performed by the Tissue Core of the University of Michigan Comprehensive Cancer Center. Images were captured using an Olympus

inverted microscope fitted with an Olympus DP27 camera. Image J software (NIH) was used to measure density. Hydroxyproline content was measured using the Hydroxyproline assay kit from QuickZyme Biosciences (Leiden, Netherlands, dist. Cedarlane Labs Burlington, NC).

Flow Cytometry

Adipose tissues were dissociated as previously described (36). For intracellular collagen staining, cells were incubated in Fc Block then stained with surface antibodies (Affymetrix eBioscience, San Diego, CA) and Live/Dead viability stain (Thermo Fisher Scientific-Life Technologies, Carlsbad, CA) for leukocytes (Live/Dead$^-$CD64$^+$, CD45$^+$) preadipocytes (Live/Dead$^-$CD31$^-$CD45$^-$Sca-1$^+$, PDGFR α^+) and endothelial cells (CD31$^+$) for 45 min at 4°C. Briefly, intracellular stains were performed using FOXP3 Fix/Perm buffer (BioLegend, San Diego, CA) followed by 15 min block with 0.5% goat serum, a 1hr incubation with 0.5 μ g of rabbit Collagen Type I antibody (Rockland, Limerick, PA) or a-Elastin antibody (abcam, Cambridge, UK), three washes, and 30 min incubation with 0.2 μ g of goat anti-rabbit AlexaFluor 647 antibody (Thermo Fisher Scientific-Life Technologies, Carlsbad, CA). Flow cytometry performed on FACSCanto II Flow Cytometer or BD Accuri C6 (BD Biosciences, San Jose, CA.) and analyzed with FlowJo data analysis software (Ashland, OR).

Explant tissue culture and conditioned media

Tissue explants were cut to 150mg pieces and cultured in serum-free AIM-V AlbuMAX (BSA) media for 48 hours at 37°C (Thermo Scientific-Life Technologies

Carlsbad, CA). For ELISA and Cytokine Array, tissues were cultured in serum-free DMEM with antibiotics for 48 hours 37°C. Explant conditioned media was pooled and assessed with the Proteome Profiler Cytokine Array (Panel A) (R&D systems, Minneapolis, MN). Immunoblots were quantified after black/white equilibration for integrated pixel density with ImageJ. Secreted TGFβ-1 protein from tissue explants were measured with ELISA at the University of Michigan Cancer Center Immunology core facility.

Gene expression analysis

Total RNA was extracted using RNeasy Mini Kits (QIAGEN), and cDNA was generated from 0.5ug of RNA using high-capacity cDNA reverse transcription kits (Applied Biosystems). Power SYBR Green PCR Master Mix (Applied Biosystems) and the StepOnePlus System (Applied Biosystems) were used for real-time quantitative PCR. *Arbp* expression was used as an internal control for data normalization. Samples were assayed in duplicate, and relative expression was determined using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

Data are shown as mean ± standard error of the mean (SEM). Differences between groups were determined using two-tailed Student *t* test analysis with GraphPad Prism 6.0e software. $p < 0.05$ was considered significant.

Results

Extracellular matrix deposition in adipose tissue is not removed after short-term weight loss

While there are various published models of WL in mice and humans, few include observations of tissue sizes, metabolism, and analyses of ECM architecture after WL in both major visceral and subcutaneous fat pads. We performed a short time course WL analysis of obese HFD fed C57/BL6 male mice induced to lose weight by replacing the HFD with ND for 2 weeks. First, body weights decreased in the 2 week WL group (**Figure 4-1A).** Fasting glucose and insulin levels show significant improvement within two weeks of WL compared to HFD fed control mice. The weights of epididymal (eWAT) and subcutaneous inguinal (iWAT) adipose tissue depots increased significantly in the HFD group and decreased after the diet was switched, but did not return to normal diet weights after two weeks of WL **(Figure 4-1B).** We explored adipose tissue ECM architecture of eWAT via Trichrome Collagen stain, and Verhoeff van Gieson's Elastin stain, where we observed an increase in ECM density after HFD. WL did not result in any significant changes in collagen or elastin material staining in eWAT with this short term WL paradigm.

Weight loss increases density of the ECM

The above result suggests that longer WL intervention may be required to return adipose tissue to a normal ECM architecture. To examine this we used a longer term WL paradigm by examining age-matched lean animals (ND, 20 weeks), obese animals (HFD,

20wks), and a set of weight loss animals (WL) that was placed on HFD for 12 weeks, followed by 8 weeks of calorie restriction via feeding of ND chow. Body and tissue weight measurements reveal an increase in weights with HFD, and subsequent decrease in weight with WL **(Figure 4-2A).** Metabolic assessments revealed that glucose tolerance normalizes after 8 weeks of WL, however insulin tolerance tests reveal that animals are less insulin sensitive **(Figure 4-2B)** compared to age matched ND mice.

Histological observations of adipose of eWAT tissues via Picrosirius total collagen stain revealed an increase in ECM accumulation and adipocyte hypertrophy after HFD as previously observed. After 8 weeks of WL, there was a significant qualitative increase in the accumulation of collagen based on Picrosirius red staining **(Figure 4-2C).** We assessed collagen content by hydroxyproline quantitation from eWAT samples. No significant changes in hydroxyproline were noted between ND and HFD groups, however WL led to a significant increase in eWAT hydroxyproline content which is in agreement with histological findings **(Figure 4-2D).** There was a statistically significant difference between groups as determined by one-way ANOVA ($F, (2, 25) = 8.060$, $p=0.002$) and Kruska-Wallis test shows a significant difference between diet ($p=0.0036$).

Collagen I$^+$ adipose tissue stromal cells increase after weight loss

Next, we set out to investigate the cellular changes in adipose tissue stromal cells that may account for the increased ECM deposition and gene expression changes observed after WL. In the previous chapter, we identified three distinct stromal cell types that express fibrillar type 1 collagen (ColI). Endothelial cells and preadipocytes were the

most active collagen-expressing populations; therefore we hypothesized that these populations may be responsible for the excess ECM deposition observed after WL.

We measured the prevalence and expression levels of two prevalent ECM components, Collagen I and Elastin, in endothelial and preadipocyte populations. Mice were fed either a ND (20 weeks), HFD (20 weeks), or put through a WL regimen (12 weeks HFD followed by 8 weeks ND). Quantitation of endothelial cell ($CD31^{+}$) and preadipocyte ($CD31^{-}CD45^{-}Sca1^{+}PDGFRa^{+}$, PreAds) populations was performed with flow cytometry. As a percentage of the SVF, eWAT endothelial cells did not change with HFD or WL **(Figure 4-2E).** PreAds (%SVF) increase with HFD and remain elevated with WL. Two-way ANOVA (ordinary) reveals significant effect of diet ($F(2, 18) = 20.32$, $p<0.0001$), and Tukey's multiple comparison test reveals significant differences between ND vs. HFD and ND vs. WL ($p<0.05$). Normalization to adipose tissue weight (cell/g) demonstrates that WL increases the overall density of endothelial cells and PreAds in eWAT relative to both ND and HFD fed controls. iWAT had a different pattern. As a percentage of SVF, iWAT endothelial cells and PreAds increase with HFD and return to levels similar to ND with WL **(Figure 4-2F).** Normalized to adipose tissue weight, there was a significant increase in iWAT endothelial cells with WL similar to eWAT. No significant differences were seen in the density of PreAds with HFD or WL. This demonstrates depot specific differences in stromal cell remodeling with WL. Accumulation of PreAds in eWAT increases with WL while in iWAT, preAds change their number in proportion to fat mass with WL.

In the previous chapter, we discussed and validated the use of a technique to quantitate intracellular fibrillar type 1 collagen producing cells. We applied this technique

to investigate how weight gain and loss affects the number of cells that produce collagen I and to examine the expression levels of collagen at a per cell basis. A representative flow cytometry plot is shown of eWAT and iWAT preadipocytes gated as a percent of $CD31^{-}CD45^{-}Sca1^{+}$ cells **(Figure 4-3 A,B).**

In eWAT, the percent of $ColI^{+}$ endothelial cells in the SVF decreases with obesity, and returns to levels similar to ND mice with WL **(Figure 4-3C).** The quantity of $ColI^{+}$ PreAds increased significantly with HFD and demonstrated a small but significant further increase with WL. When normalized to fat pad weight, WL increased the density (cells/gram of adipose tissue) of both endothelial cells and PreAds compared to both ND and HFD conditions **(Figure 4-3D).** In iWAT, $ColI^{+}$ endothelial and PreAds increased significantly with HFD, then decreased dramatically after WL to return to levels similar to ND mice **(Figure 4-3E).** When normalized to iWAT mass, no significant differences in the density of collagen expressing cells in the iWAT between the ND, HFD or WL groups **(Figure 4-3F).**

We next evaluated per cell expression of ColI by median fluorescence intensity (MFI). HFD decreased collagen I expression in endothelial cells, and did not increase with WL in eWAT **(Figure 4-3G).** In eWAT PreAds increased collagen expression with HFD, and this decreased after WL to a level below what was observed in lean mice. In iWAT, an increase in collagen expression was only observed in endothelial cells after WL **(Figure 4-3H).** Overall this demonstrates that per cell expression of ColI in eWAT PreAds is decreased WL although their content in remains high. This also demonstrates depot specific differences in the capacity of stromal cells to alter their quantity with WL – a more robust capacity to return endothelial cell and PreAd levels to normal in iWAT.

Elastin1^{+} adipose tissue stromal cells increase after weight loss

Another prevalent ECM component relatively unexplored in adipose tissue is elastin. We applied a similar strategy as our intracellular collagen I stain to measure production of alpha elastin in endothelial and PreAds populations. Control staining demonstrated specificity of our elastin flow cytometry experimental controls **(Figure 4-4A).** Analysis of SVF cells revealed that the majority of PDGFRi^{+} PreAds express elastin in both eWAT (~90%) and iWAT (~75%) of CD31^{-}CD45^{-}Sca1^{+} cells **(Figure 4-4B).**

In eWAT from ND mice, ~10% of endothelial cells expressed elastin (Eln^{+}) and ~20% of the PreAds were Eln^{+} **(Figure 4-4C).** HFD did not alter the quantity of Eln^{+} endothelial cells but the quantity of Eln^{+} PreAds doubled. This increase in Eln^{+} PreAds was sustained after WL in eWAT. Normalizing to fat pad weight (Eln^{+} cells/g), there are no significant changes in Eln^{+} endothelial cells or PreAds with HFD in eWAT. WL induces a significant increase in Eln^{+} cells in both populations in eWAT.

In iWAT, HFD significantly increased the quantity of Eln^{+} endothelial and PreAds as a %SVF. WL induced a similar decrease in Eln^{+} cells to levels similar to lean mice **(Figure 4-4D).** Normalizing to fat pad weight, the density of Eln^{+} endothelial cells increased after WL and no significant differences were seen in Eln^{+} PreAds between groups. Examining elastin MFI, endothelial cells express elastin at higher levels than PreAds in eWAT but not iWAT **(Figure 4-4E).** In both depots, elastin MFI increases with HFD and decreases after WL for both endothelial cells and PreAds.

Weight loss decreases gene expression of ECM components and markers of fibrosis

To assess global changes in ECM components and regulatory genes at the tissue level, we analyzed gene expression in whole eWAT samples from ND, HFD, and WL mice. HFD induced expression of *Col1a1* and *Col6a3* **(Figure 4-5A).** Markers of fibrosis such as *Spp1, Postn,* and *Tgfb1* were also induced, however only *Spp1* reached statistical significance **(Figure 4-5B).** Increases in expression of tissue inhibitor of matrix metalloproteinase family genes (TIMPs) are consistent with the generation of a pro-fibrotic environment with HFD and a downregulation of ECM degrading enzymes **(Figure 4-5C).** A decrease in the levels of *Mmp2* and *Mmp9* after both HFD and WL suggests a decrease in the degradation of the basement matrix. An increase of *Mmp12* and *Mmp14* and subsequent decrease after WL suggest active ECM degradation was promoted. We also measured the makers of brown/beige adipocytes and noted few changes in *Pgc1a* and a strong induction of *Ucp1* after HFD. *Ucp1* decreased after WL, but was elevated compared to ND lean animals **(Figure 4-5D).** Overall, gene expression analyses reveal that WL decreases gene expression of ECM remodeling factors in eWAT. This suggests that the increase in collagen staining in WL mice indicates persistent of previously deposited ECM components and not the ongoing generation of a pro-fibrotic environment.

Secreted factors from weight loss tissues identify weight-dependent cytokine and growth factor changes

To screen for cytokines that change with weight loss that may relate to a sustained pro-fibrotic setting, we performed a cytokine protein array screen for 40 cytokines on

conditioned media from ND, HFD, and WL explants **(Figure 4-6A).** 20 cytokines were detected above background. The cytokines elevated in HFD and WL samples but not in ND samples were IL-1 receptor antagonist (IL-1RA) and, monocyte chemotactic protein 5 (MCP-5, also known as CCL12) **(Figure 4-6B).** Cytokines that were restored to the level of ND mice were MIP1A, RANTES, and TNF α .

The cytokine array does not measure growth factors previously implicated in fibrosis such as TGF β -1. Therefore, we assessed levels of total TGF β -1 in eWAT ND, HFD, and WL explant conditioned media. ELISA results suggested TGF β -1 levels are elevated in HFD and WL conditioned media, however only the levels in the WL samples reached significance **(Figure 4-6C).** This suggests that sustained TGFß-1 may contribute to sustaining a pro-fibrotic environment in eWAT with weight loss. Lastly, to assess if TGFß-1 signaling is sufficient to induce expression of ECM components, we added recombinant TGFß-1 to the 3T3-L1 preadipocyte cell line. TGFß-1 significantly induced gene expression of *Cola1*, *Col6a3*, and *Spp1* **(Figure 4-6D).**

Discussion

In the current study, we sought to understand why metabolic perturbations remain in formally obese patients. To investigate these observations, we focused our attentions to filling in gaps in the published literature by evaluating the dynamics and reorganization of adipose tissue ECM architecture after WL. Overall, our findings demonstrate in mouse models of WL, that normalization of fat pad weights does not revert the adipose tissue ECM to its pre-obese status. Our most surprising finding was that an increase of

fibrotic ECM accumulation is observed after an 8 weeks WL intervention that normalized body and tissue weight. This is most prominently seen in the eWAT and not in iWAT. We have validated this finding via multiple and complementary analyses such as by histological methods (Picrosirius, Verhoeff's, Trichrome) and by biochemical methods (total tissue hydroxyproline). This may be due to the dynamic ability of eWAT to adapt expand in response to metabolic demand.

We focused on investigating the mechanisms by which ECM accumulation is sustained or increased with WL. The quantity of ColI$^+$ and Eln$^+$ stromal cells increase dramatically in eWAT after WL, and that the main producers of these compounds are endothelial cells and PreAds. Despite this increase in cell quantity, both endothelial cells and PreAds decreased per cell expression of these components after WL. Gene expression analysis supports this observation as gene expression of ECM components decrease in whole eWAT adipose tissue samples with WL.

Overall, our data is consistent with the model that the increased quantity of pro-fibrotic PreAds induced by HFD in eWAT remain after WL and retain pro-fibrotic characteristics (ColI and Eln production). However these characteristics are dampened relative to the HFD situation. The persistence of adipose tissue ECM is likely due to a combination of persistent pro-fibrotic PreAds population and an inability of the ECM degradation machinery to remodel the adipose tissue fibrosis back to a normal state. Subsequently, we have not analyzed ECM degradation capacity with WL. It is possible that suppression of ECM degradation is more important than the induction of new ECM production.

Since endothelial cells are such a small population of the SVF (~10%) it is unlikely this population plays a significant causative role in the pathogenesis of fibrosis. Our data align with other reports implicating PreAds as as pro-fibrotic cells under inflammatory conditions, however we have taken further steps in providing data showing that these cells can also produce ECM components (74, 96). Elastin is an ECM component that remains understudied, and to our knowledge, our work is one of the few that explores the elastin matrix in adipose tissues (5, 14). Our analysis revealed that populations and production of elastin increases in PreAds with diet-induced obesity, and much like collagen, expression of elastin also decreases after weight loss.

We observed significant adipose tissue depot specific changes after WL. For most of our stromal measures (e.g. quantity of $Col1^+$ PreAds), the effects of HFD were not readily reversible in eWAT and were reversible in iWAT. This was apparent in similar content of iWAT stromal cells when normalized to adipose tissue weight. This suggests that iWAT depots have a more robust capacity to dynamically decrease the stromal cell induction as seen with HFD. It may also relate to the pro-fibrotic changes induced primarily in the eWAT that may limit the ability of PreAds to return to levels similar to a lean state. We postulate that the decrease in PreAd content in iWAT is due to an exhaustion of the PreAd pool, which may not be the case in eWAT.

Adipose tissues are rich with a variety of ECM compounds, such as fibrillar and non-fibrillar collagens, fibronectins, laminins, and proteoglycans. A limitation in our study is that we did not explore how these additional components are deposited or degraded. An important next step in the evaluation of ECM architecture in the context of fibrosis is the analyses of crosslinking enzymes such as Lysyl oxidase and

Transglutaminase, and matrix assembly elements (Decorin, Periostin,) that function to reinforce ECM rigidity. We postulate that higher degrees of ECM crosslinking may result in the inability of enzymes to degrade and remodel the architecture. Along with this, further investigation of the activities of degradation enzymes like Matrix metalloproteinases (MMPs), A disintegrin and metalloproteinase (ADAMs), Plasminogen activator inhibitor (PAI-1), and Cathepsins may answer questions relating to how ECM components are degraded.

Additional missing information in our study relates to the mechanism of WL, which may lead to multiple changes in metabolism/inflammation. HFD has been shown to be pro-inflammatory even with short term feeding, therefore, it is possible that the removal of the HFD stimulus independent of WL may be sufficient to alter inflammation and the ECM. Additionally, we cannot rule out that the intracellular collagen or elastin content we measured using flow cytometry is due to uptake rather than deposition. Further studies will be required to better understand the fate of the ECM components (i.e. fibril-formation and crosslinking) that are produced.

Our current model is that persistent fibrosis in adipose tissue is dependent upon the induction of a pro-fibrotic environment driven by diet-induced obesity. Here we propose a model, where the development of obesity induces both the expansion of preadipocytes, and also their expression of ECM components **(Figure 4-7).** With weight loss, we hypothesize that widespread lipolysis recruits and activates ATMs. Increases in the maturation and crosslinking of the ECM via crosslinking enzymes such as Lysyl Oxidase, increase the abundance of insoluble fibers, and degradation of ECM components lags, imprinting an imbalance of ECM deposition and degradation. Together,

this increase in fibrosis limits the physical flexibility of the ECM, perturbs adipocyte metabolism, and decreases insulin sensitivity.

Our study highlights a striking observation in the remodeling of the ECM architecture after WL. Even after this caloric restriction, insulin sensitivity was decreased, and we believe this finding is linked with our observation of increased fibrotic ECM accumulation after WL. The next viable question for the future is to understand how metabolic stress, such as lipolysis, influences gene expression of signals that promote the depot-specific persistence of PreAds. Studies point to PDGF signaling as a possible fate-determining mechanism which may direct this persistence (86, 163).

Similarly, due to their unique microenvironment, exposure to inflammatory and growth factors, PreAd fate might be skewed toward a pro-fibrotic phenotype. Longitudinal studies that measure both ATM and PreAd populations are required to understand the dynamics of these population shifts, especially considering that short-term WL models in animals dramatically increase ATM populations, while human long-term weight loss models of reveal the opposite (29, 44, 106). The subject of future studies will be to resolve these variables and more closely analyze ATM-PreAd communications in a comprehensive collection of metabolic contexts and non-inflammatory conditions in order to understand their roles in influencing ECM architecture.

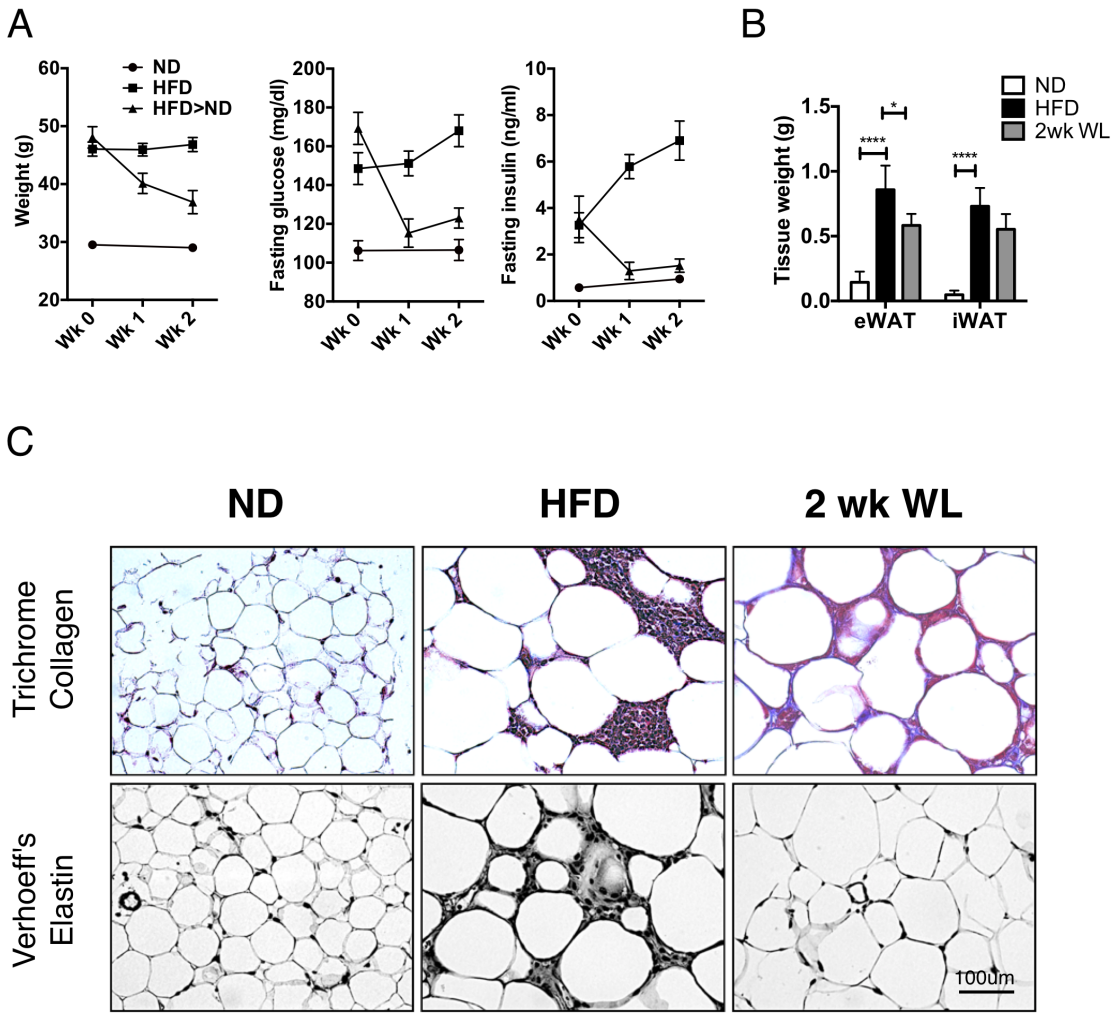

Figure 4-1. Fibrotic ECM composition is sustained after short-term weight loss. (A) Body weight, fasting glucose, fasting insulin, with (B) visceral epididymal (eWAT) and subcutaneous inguinal (iWAT) fat pad weights are shown for studies in this figure. (C) Trichrome collagen stain and Verhoeff's Elastin stain on sectioned eWAT. Scale bar = 100uM. n=3-4 per group. (*p<0.05, **<0.01***0.001, ****0.0001).

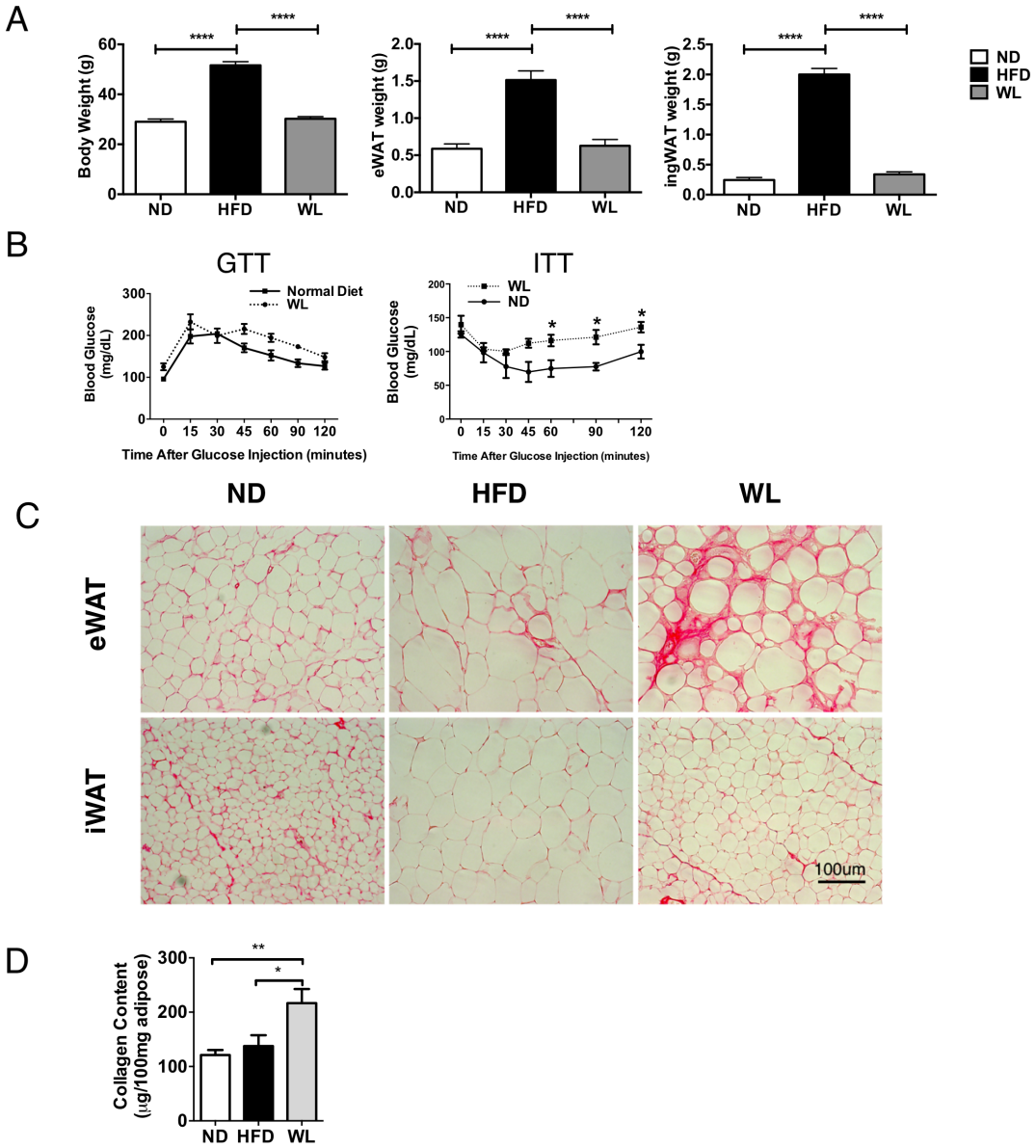

Figure 4-2. Insulin sensitivity decreases as ECM accumulation increases after long-term weight-loss. (A) Body and tissue weights are shown after weight loss. (B) Glucose and insulin tolerance tests (GTT, ITT, respectively). (C) Picrosirius stains of eWAT and iWAT from ND (20wk ND) HFD (20wk HFD), and WL (12wk HFD, 8wk ND) animals. Scale bar = 100uM. (D) Total collagen content and collagen as a percent of total protein.

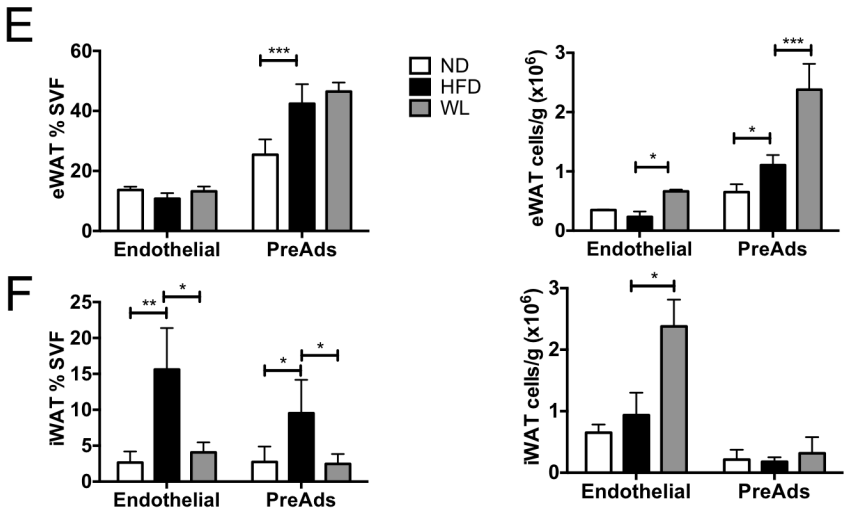

Figure 4-2 continued. (E). Density of endothelial and preadipocytes (PreAds) per gram of tissue and as a percent of stromal vascular fraction (SVF) at ND, HFD, and WL. n=4 per group. (*p<0.05, **<0.01***0.001, ****0.0001).

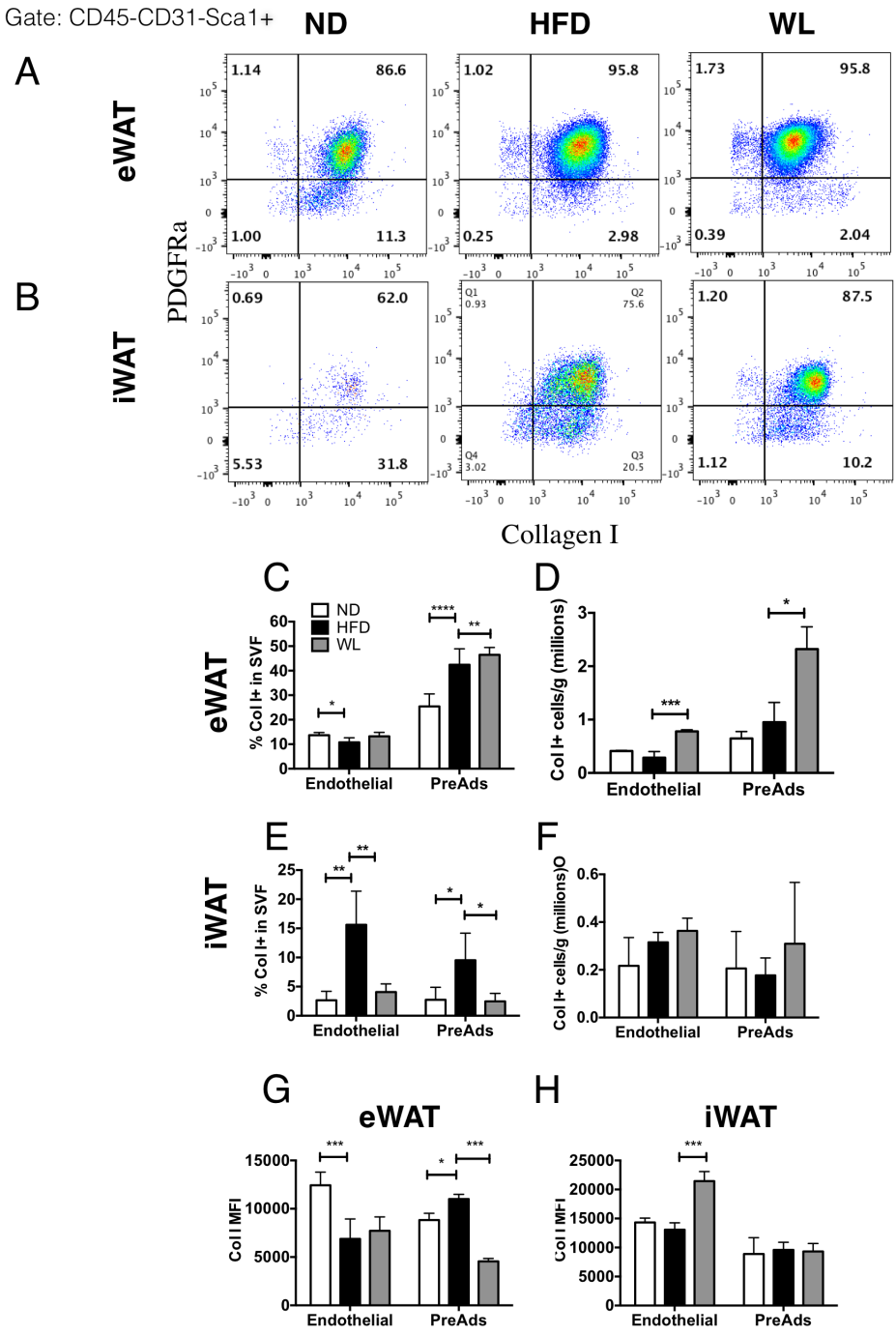

Figure 4-3. Endothelial cells and preadipocytes are major collagen type I-expressing populations in the stromal vascular fraction. Flow cytometry results, gated on CD45$^-$ CD31$^-$Sca1$^+$ PDGFR α^+ preadipocytes that express collagen I (ColI$^+$) in eWAT (A) and iWAT (B). ColI$^+$ endothelial cells and PreAds are shown as a percent of SVF (C,E) density of ColI + cells per gram (D, F) and median fluorescence intensity (MFI) (G, H) in eWAT and iWAT respectively. (*p<0.05, **<0.01***0.001, ****0.0001).

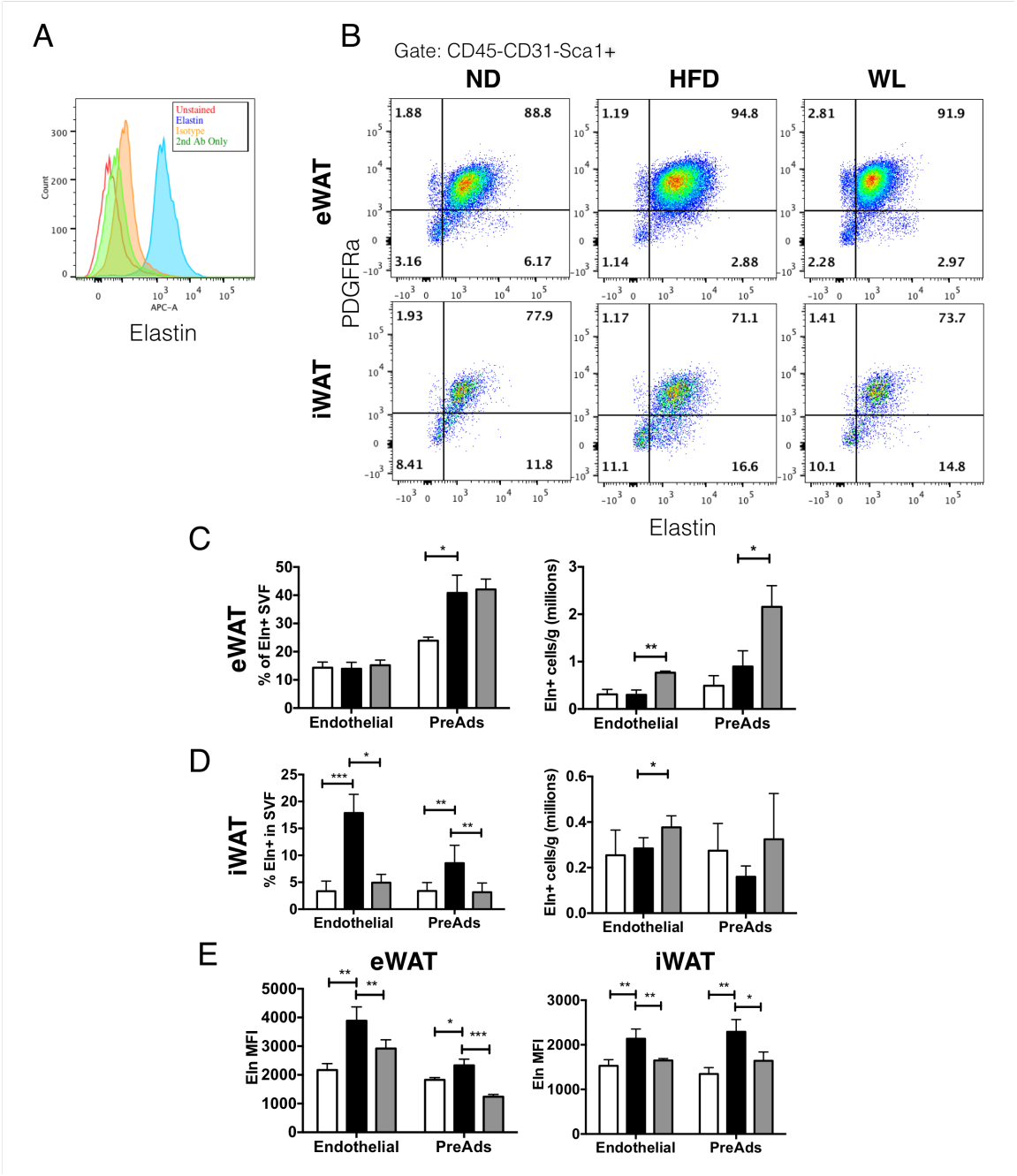

Figure 4-4. Endothelial cells and preadipocytes express elastin. (A) Elastin flow cytometry experimental controls. (B) Flow cytometry results, gated on $CD45^{-}CD31^{-}Sca1^{+}$ PDGFR α^{+} preadipocytes that express elastin (Eln^{+}) in eWAT and iWAT. (C, D) Measurements of Eln^{+} endothelial and PreAds as a percent of SVF and as density of Eln^{+} cells per gram of tissue. (E) Elastin staining intensity MFI in eWAT and iWAT. n=4 per group. (*p<0.05, **<0.01***0.001).

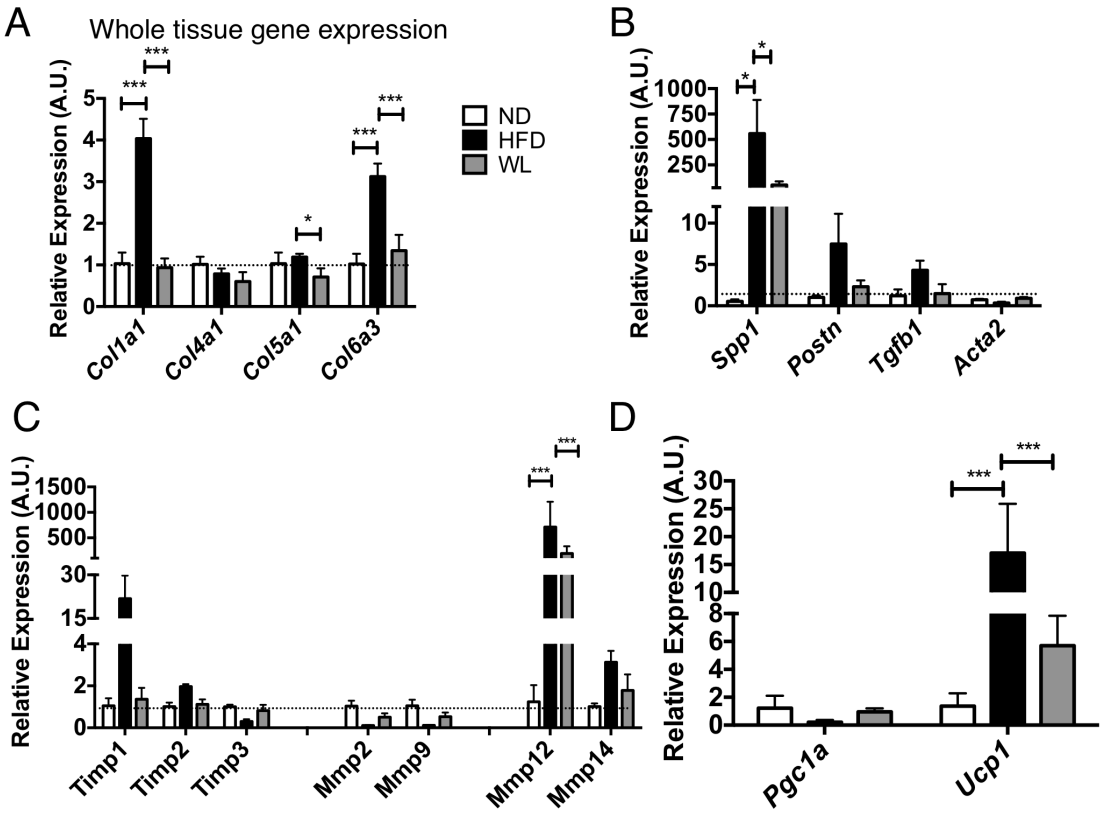

Figure 4-5. Gene expression of ECM components and remodeling factors after weight gain and weight loss. Gene expression of (A) ECM components, (B) markers of fibrosis, (C) remodeling enzymes, and (D) markers of browning. n=4 per group. (*p<0.05, **<0.01***0.001)

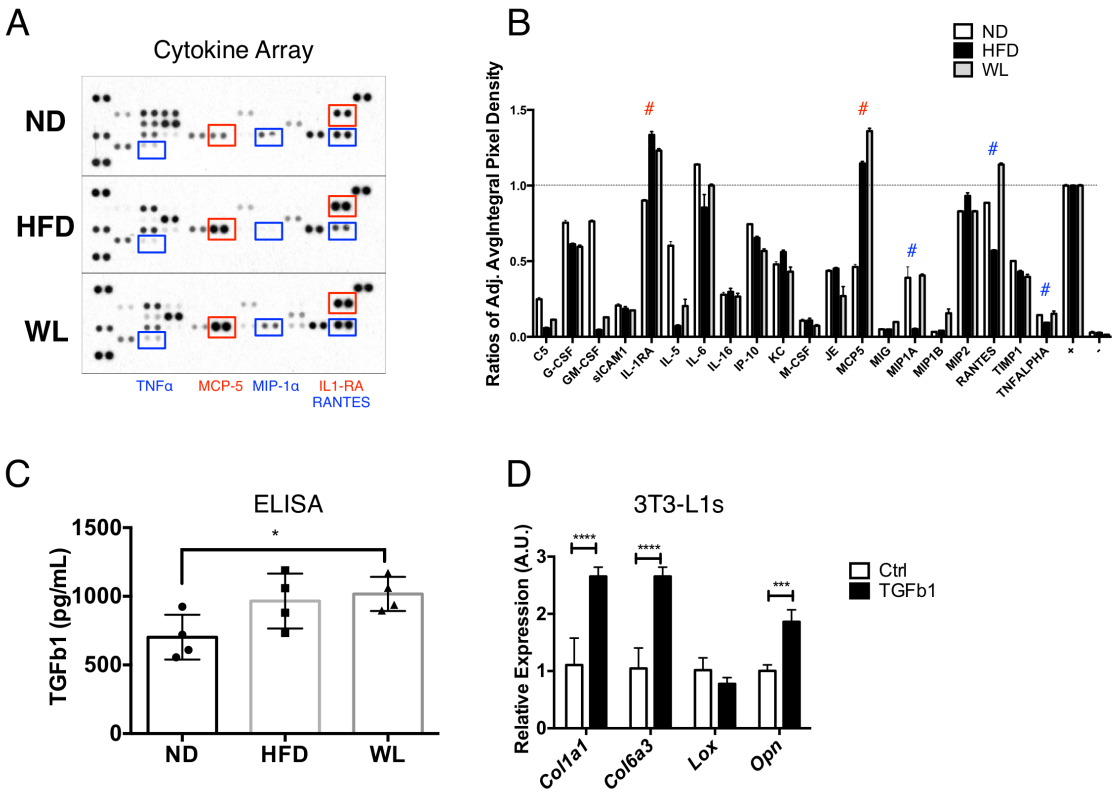

Figure 4-6. Secreted factors from weight loss tissues identify weight-dependent cytokine and growth factor changes. (A) Cytokine array membrane is shown, proteins increasing with obesity are highlighted in red boxes (IL-1RA and MCP-5) and proteins decreasing with obesity and increasing after WL in blue boxes (TNF α, MIP1 α, RANTES). (B) Ratios of quantified pixel density from cytokine array, IL1-RA and MCP5 shown as blue and red arrows respectively. (C) ELISA analysis of TGF β-1 in conditioned media from ND, HFD, and WL eWAT tissue explants. (D) Gene expression of fibrosis markers from 3T3-L1s treated with TGF β in. n=4 per group. (*p<0.05, **<0.01***0.001, ****0.0001).

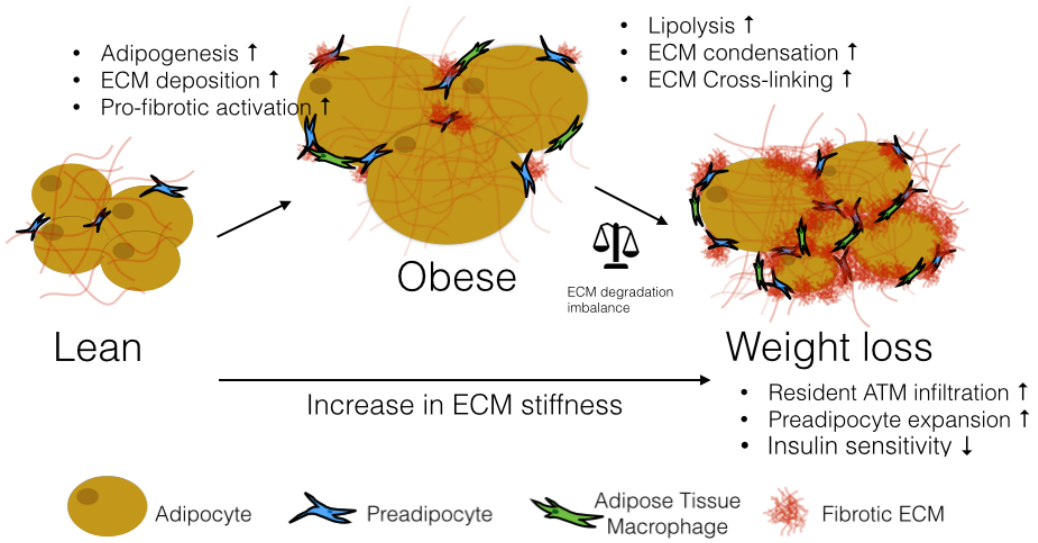

Figure 4-7. Model of ECM accumulation after weight loss. The progression from the lean to the obese states results in new adipogenesis, adipocyte hypertrophy, and the activation of extracellular matrix (ECM) production in preadipocytes. In the obese state, Adipose tissue macrophages (ATMs) secrete factors that activate ECM production and proliferation by preadipocytes. With weight loss, adipocytes shrink in size via lipolysis, which recruits and activates additional ATMs and stimulates expansion of preadipocyte populations. ECM forms insoluble fibers that are created by crosslinking enzymes. A decrease in the relative ECM degradation results in an imbalance, which favors the permanence of ECM components. Additionally, mechanical stress imposed by a fibrotic ECM limits adipocyte energy regulation and decreases insulin sensitivity.

Chapter 5

Conclusions, Limitations, Future directions, and Clinical Relevance

Conclusions

Adipose tissue functions as the main nutrient storage organ, which is capable of rapid adaptation to metabolic demand. In settings of over nutrition, adipocyte hypertrophy and new adipocyte formation serve to increase fat mass. With negative energy balance adipocytes releasing its stored nutrients in the form of triglycerides to meet metabolic demand and adipocytes decrease in size. Disturbances in the efficiency of these remodeling events can disrupt the normal homeostatic function of adipose tissue and over time can contribute to systemic insulin resistance. In this scenario, with adipose tissue dysfunction, the energy stores that were once managed efficiently by adipose tissue are not well contained, resulting in ectopic energy storage in tissues such as liver and muscle (202, 203).

While there are many potential contributors to the metabolic disease with obesity, these projects have focused on ECM deposition and fibrosis as a mediator of adipocyte dysfunction. The adipose tissue ECM normally functions to physically support the structure of the tissue and provide the mechanical and cell adhesion cues that influence cell fate. The ECM provides required cues which are needed to drive adipocyte growth

and differentiation, however, excessive ECM accumulation negatively influences development and metabolism (165). In vitro studies show that growing adipocytes in dense fibrous material-laden ECM results in an increased inflammatory response and inhibition of differentiation (154, 173).

ECM remodeling, as we have described as the balance between the deposition and degradation of the ECM along with post-translational modifications, is important to support normal adipose tissue expansion. The composition of the matrix itself has the potential to influence adipocyte function and can influence whole-body metabolism upon high fat diet challenge. Studies have shown that reducing the capacity of an adipocyte to degrade its pericellular ECM via a lack of functional matrix metalloproteinase (MMP) inhibits adipocyte hypertrophy (41, 42). A lack of MMP inhibitors can on the other hand, increase adipose tissue expansion (87). Similar findings were shown in animal models lacking a particular ECM component, Collagen VI, in which animals gained excessive weight, had more hypertrophic adipocytes, but were still metabolically healthier (97).

While there is a vast amount of literature reporting the negative effects of inflammation and adipose tissue fibrosis in the pre-clinical rodent models, inflammatory pathways are also required in adipocytes for normal metabolic homeostasis (232). Therefore, a careful balance on the level of inflammation is required in the adipocyte microenvironment for normal physiological expansion and ECM remodeling.

Key findings in the degradation of the adipose tissue extracellular matrix

In this work, we approached the ECM degradation/deposition paradigm in the context of adipose tissue fibrosis with three different models. In our first approach, we

hypothesized that the depletion of one key elastin-degrading enzyme, MMP-12, would increase the accumulation of ECM components, namely elastin, and result with metabolic perturbations during the development of obesity. We identified an elastin matrix forming a mesh-like network around all adipocytes. We also determined that that MMP-12 in adipose tissue is primarily produced by ATMs, however in whole-body MMP-12 deficient animals, we observed no observable difference in elastin matrix architecture or in metabolism. Our data suggests that MMP-12 does not play a major role in influencing ECM remodeling or whole body metabolism. Other elastin-degrading enzymes exist, however very little is known regarding their specific role in reorganizing the Elastin matrix in adipose tissues. It is also possible that elastin inhibitors such as α1-antitrypsin play a prominent role in elastin regulation (229). Neutrophil elastase (NE) has been shown to promote adipose tissue inflammation and promote insulin resistance with DIO (205). Mice deficient of NE are resistant to diet-induced obesity, insulin resistance, inflammation, and fatty liver (130). Additionally, mice deficient of NE do not develop diet-induced obesity-mediated adipose tissue fibrosis. Taking together, we speculate that elastin degradation subunits may promote inflammation and metabolic dysfunction. However, it is still unclear if NE has roles specific to elastin ECM degradation in adipose tissue, or if its roles are limited to mediating inflammation. Another elastin degrading enzyme Matrix metalloproteinase-7, (MMP-7) or matrilysin has also been implicated in obesity and weight loss. Formally obese patients increase circulating levels of MMP-7 post bariatric surgery, which may indicate an induction of remodeling or degradative activity of ECM architecture with weight loss (180). An alternative explanation to this may be that MMP-7 may be facilitating the activation of other interrelated MMPs,

thereby activating a cascade of ECM remodeling. Further studies investigating adipose tissue specific NE or MMP-7 activities may elucidate mechanisms relating both to Elastin matrix architecture remodeling and inflammation.

Preadipocytes produce key extracellular matrix components related to fibrosis

In Chapter 3, we set out to understand the mechanisms of the deposition of a key insoluble ECM component, fibrillar collagen type I. Our results implicate preadipocytes as the primary regulated source of collagen type I production induced with DIO primarily in the gonadal fat pads and not subcutaneous fat pads. Microarray analysis and intracellular protein detection corroborated these findings. Several studies have suggested that preadipocytes take on a pro-fibrotic phenotype in obesity triggered by macrophage inflammatory cues, but no studies have provided convincing evidence that these cells produce ECM proteins *in vivo* (25, 96).

ATMs and preadipocytes co-localize in ECM-dense regions in crown-like structures. While some studies have examined the influence of classically activated (M1) macrophages on preadipocyte function, the influence of the full range of macrophage activation states on preadipocyte ECM production are unknown (24, 25, 198). Considering that ATMs were a major source of TGFβ-1, we hypothesized that macrophage activation states played a major role in the regulation of type I collagen expression. In vitro experiments revealed that alternatively activated resident "M2" macrophages, not classically activated "M1" or metabolically activated macrophages "MMe" induced expression of fibrillar type I collagen. Using a small molecule inhibitor,

we determined that TGF β -1 signaling was required for resident macrophages to produce the pro-fibrotic effect in primary preadipocytes.

Our data suggests that preadipocytes are the main source of fibrillar type I collagen expression, and that preadipocyte-macrophage communications leading to pro-fibrotic activations are TGFβ-dependent. This is in agreement with findings in other fibrotic diseases as scleroderma animal models have also implicated TGFβ signaling in the pathogenesis of this form of subcutaneous adipose tissue fibrosis (162). Further, both a systemic blockade and a genetic depletion of TGFβ-dependent Smad3 signaling attenuated the development of fibrosis and prevented glucose intolerance (236). These findings further suggest that TGFβ signaling is required for tissue homeostasis and may be a potential target to treat obesity and diabetes by attenuation of adipose tissue fibrosis.

Several important questions remain. While we are able to measure increases in collagen type I expression, it is still unclear if preadipocyte derived collagen type I is required for the development of adipose tissue fibrosis given the wide range of other collagens that are produced in adipose tissue and induced at the gene expression level with obesity (74). Additionally, we have not investigated the production or the role other non-collagen ECM components, which may also have the potential to influence adipocyte maintenance, turnover, and tissue inflammation, as was shown by Khan et al (97). There is also the potential that accumulations of only certain types of collagens, fibrillar or even non-fibrillar, may lead to local inflammation and metabolic dysfunction. We made attempts at proving the importance of preadipocyte Collagen type I experimentally in mice by using cre-lox technology. To generate an inducible deletion of *Col1a1* gene in preadipocytes we crossed *Pdgrfa-creERT* transgenic mice with a *flox*ed *Col1a1*

transgenic mouse (93). We were unable to show efficient excision of the transgene after inducing recombination with tamoxifen. This model suffered several technical limitations that included mosaic expression of the transgene and leaky Cre expression in the absence of tamoxifen induction. This is a general weakness in the field as no currently available transgenic models are able to specifically modify gene expression uniformly in preadipocytes (16, 88).

Weight loss induces a dynamic increase in adipose tissue fibrosis

In Chapter 4, we set out to understand the dynamics of ECM reorganization with weight loss. Human studies suggest that adipose tissue fibrosis appears to persist and may even increase after weight loss (53, 54, 135). In contrast, exercise may attenuate the development of diet-induced adipose tissue fibrosis (95). Therefore, we set out to understand the pathogenesis of weight loss induced adipose tissue fibrosis in animal models. We observed a significant increase in collagen accumulation in visceral fat and lower insulin sensitivity in animals that underwent weight loss compared to weight matched lean mice. While there were more preadipocytes in weight loss mice, the per cell expression of intracellular collagen and elastin was decreased compared to obese mice. This suggests that ongoing ECM deposition may not the primary mechanisms behind the increase in ECM accumulation with weight loss, but that there may be a long-term persistence in the inability to degrade adipose tissue collagen once it is deposited and cross-linked with diet-induced obesity.

These findings reinforce the multi-faceted nature of the regulation of the adipose tissue ECM and that our focus on collagen production alone may not be sufficient to get a

full picture of how ECM is remodeled. ECM remodeling requires tight regulation of elements that produce, organize, deposit, and degrade ECM components. Our investigations into the sources of fibrillar ECM components reveal that preadipocytes are major sources of type I collagen and elastin, which supports previous studies into pro-fibrotic preadipocytes (96). Currently we are limited by the availability of assays to reliably measure ECM degradation in and around adipocytes in the context of adipocyte hypertrophy or regression. This is an overall weakness in the field that we have made attempts to address. Unpublished experiments we performed examined changes in collagenase activity zymography with variable results potentially due to the harsh conditions required to remove lipids from adipose tissue prior to analysis. Cellular assay of ECM degradation of fluorescence collagen matrices identified macrophage-dependent collagen degradation, however this was not quantitative. Most studies published to date depend solely on gene expression analysis for assessment of collagen degradation or deposition, but the significant post-translational modification of the ECM make such measure unreliable in our experience to date.

Such more advanced measures are required to get a better understanding of the longitudinal changes that regulate adipose tissue ECM accumulation with weight loss. Currently, it is not clear at which point the density of the ECM increases and how to differentiate this from a stable ECM network that condenses as adipocytes shrink in size. We feel that our studies are consistent with the model that weight loss might trigger ECM degradation at a slow rate that does not overcome the large amount of ECM that has been deposited with initial weight gain. Published data on ECM status after weight loss in animals is limited, therefore additional studies investigating ECM architecture at the gene

expression level, biochemical level, and histological level are in need. A potential alternative approach for future studies include the use of de-cellularized adipose tissue to study and quantify ECM structure and protein content (28, 164).

Limitations

Our animal models and experimental techniques have several limitations of which we are aware. One major limitation is the use of diet-induced obesity models that employ a high fat diet chow composed of 60% calories from animal lard. While high fat consumption is one potential contributor to human obesity, there is evidence that carbohydrate excess is more relevant to metabolic disease than fat excess (46, 212). We might have observed difference effects on adipose tissue ECM composition, preadipocyte expansion, and macrophage accumulation if high carbohydrate diets were employed.

Another limitation, which is not well documented in the field but is acknowledged, is the large regional variability of ECM accumulation between and within tissues in animals. For example in the gonadal fat pads we have primarily focused on, fibrosis and ATM accumulation are highly concentrated in the rostral "tip" of the fat pad and decrease in the caudal "base". This may relate to blood flow at the tip region, which may be limited compared to the base. Another significant challenge in our experiments and in the field is normalization of stromal cell and other measures in tissues that are changing 100-300% with dietary manipulation. For example, histology in obese mice with very large adipocytes may underestimate the amount collagen in adipose tissue and may not correctly represent the true quantity and structure of the ECM with obesity.

In addition, normalization for biochemical assays, such as hydroxyproline and total protein content assays, have weaknesses due to the increase in lipid content per mg of total tissue input. We have observed that normalizing to total protein, or tissue weight may not be feasible methods of comparing ECM content in adipose tissues. We postulate that total DNA content may similarly not be feasible for ECM content comparisons because the cellularity and stromal cell content increases in adipose tissues from obese animals. As previously mentioned, fibrosis development is not homogenous in nature; therefore alternative approaches to measuring total collagen accumulation need to be considered for future studies. Such possible strategies may include the de-cellularization of an entire eWAT fat pad, followed by normalization of total protein content per input mg, and by hydroxyproline total collagen content. This method may remove the risk of regional variability of ECM content, yet may still adequately represent the total collagen content per unit of total protein.

The majority of our studies focus on the epididymal peri-gonadal fat pads (eWAT) in mice, which has been shown to be the most pro-inflammatory and fibrotic in many studies. Such a depot is not prominent in humans and there is a valid concern in the field that eWAT may not represent a true visceral adipose tissue depot. Still, in animal studies, the eWAT is highly responsive to metabolic stress, inflammation, and has been studied extensively for its ECM content. We note that the issue of which visceral fat depot is the most deleterious in humans remains an open question, as omentectomy has not proven to have significant effects on improving metabolic control (51, 120).

Our methods for studying intracellular collagen content via flow cytometry also come with caveats. At this time, we are unable to completely exclude the possibility that

the intracellular collagen or elastin we are detecting is taken up from the environment. In the pulmonary fibrosis literature, $ColI^{+}$ fibrocytes were recently definitively proven to derive from injected collagen type I and not from endogenous production (175). While our controls and gene expression data argue that under static conditions, Collagen I positivity is a measure of intracellular production, there may be other circumstances where a combination of production and uptake contribute to this signal. We have made attempts to use pro-collagen antibodies to support this finding but these were not technically feasible with the reagents currently available. Future use of antibodies that detect degraded collagens type I may be useful measure. Along the same lines, a weakness of our studies is that we are currently unable to investigate the eventual fate of the ECM components after production by the preadipocytes. Steps between production and deposition include protein folding, post-translational modification, and crosslinking, which we are currently unable to measure *in vivo*. Alternative strategies using GFP-labeled collagen or elastin may prove useful to assess these events.

In our studies investigating mechanisms of the pathogenesis of adipose tissue fibrosis, we identified TGF β -1 as a likely mediator of ATM-preadipocyte communication. Our studies however, did not investigate the activation state of TGF β -1 when performing conditioned media experiments. As previously mentioned, TGF β -1 is expressed in multiple latent forms, and further activation by proteolytic cleavage by other ECM processing enzymes such as matrix metalloproteins is required for signaling to occur. Future investigations of activation states may be performed with currently available antibodies designed to detect TGF in its active and latent forms.

Future directions

While the understandings of the physiology of adipose tissues have increased dramatically over the last decade, there is still much that is not understood. There are several outstanding questions that extend from the work presented in this dissertation.

How is the adipose tissue ECM degradation machinery regulated?

Most published studies, including our own, focus mostly on markers and elements of ECM composition, however few have studied the ECM degradation machinery and its regulation by weight gain and loss. Plausible future studies include the use of transgenic models deficient of multiple but related degradation enzymes to understand both the roles of the enzymes, and also their own internal specific ECM substrate.

What role do growth factors play in adipose tissue physiology?

We, and others, have implicated macrophage-derived TGF β -1 signaling as being required for the induction of primary preadipocytes toward a pro-fibrotic phenotype. Additional studies investigating the prevalence of TGF β -1 in its active form in adipose tissues as well as its form when secreted from whole explants will be necessary to understand the degree to which this mode of signaling is active. In addition, groups have also implicated PDGFR α^+ cells and PDGF signaling in adipose tissue specifically, in the initiation of fate-determining and fibrogenic mechanisms (86). Investigations into these two growth factors and signaling mechanisms may shed light into the physiology and metabolic regulation of adipose tissues.

What is the fate of newly synthesized ECM and what is the importance of crosslinking?

While we were able to detect increases in staining of intracellular ECM components such as collagen type I and elastin, we were unable to determine their final form or destination. Future studies will be required to understand if those components end up in insoluble fibrillar form, or if they are degraded. Additionally, investigating the role of crosslinking enzymes such as Lysyl Oxidase, the Lysyl Oxidase Like family of enzymes (LOXL1-4), and Transglutaminases (TGM2) would provide key information regarding the maturation and formation kinetics of insoluble ECM fibers (2). This could be performed using available small molecule LOX inhibitors. Additionally, there is evidence that of TGFβ-mediated LOX induction, which indicates that TGFβ may have roles in ECM production as well as ECM cross-linking (189).

What measures best define adipose tissue fibrosis?

Currently there is no strict definition of criteria that defines ECM tissue as fibrotic. In the future, more comprehensive quantitative methods will be required in order to convincingly compare studies. Possible approaches include de-cellularizing the entire fat pad, normalizing total hydroxyproline content to total DNA, or to an average total cell density, which would require knowledge of number of adipocytes and adipocyte size. Ideally, future studies can better quantify fibrotic accumulations by measuring amounts of insoluble ECM materials collected after tissue dissociations.

What is the role of obesity-induced inflammation in ECM deposition and degradation?

Our cytokine array studies suggest that inflammatory cytokines may not be direct inducers of fibrosis in preadipocytes. However there is still much left to investigate considering the prevalence of ATMs and their varying activation states with obesity and weight loss. ATMs are recruited to CLSs, which are also prevalent fibrotic areas, which indicates that inflammation may be an initial step in the activation of a pro-fibrotic ECM architecture reorganization (114, 115). Investigating the role of obesity-induced inflammatory cytokines in activating phenotypes in ATMs is necessary, and can be done with animal models deficient of activation receptors (TLR 4 deficient, CD11c deficient, IL4 deficient) or those deficient of inflammation of all together (RAG knockout, NOD).

Clinical Consequences of adipose tissue fibrosis

Is fibrosis bad, or good?

While the majority of pre-clinical studies support the model that adipose tissue fibrosis is pathogenic, there is evidence that it may also be metabolically protective in humans. Visceral adipose tissue fibrosis may impart protective effects on metabolic disease by limiting adipocyte hypertrophy and promoting the formation of smaller adipocytes, which are associated with less inflammation (9, 10). Patients with larger adipocytes have a more disrupted metabolic profile (9). Studies show that patients with smaller adipocyte sizes, due to fibrotic accumulations that restrict adipocyte hypertrophy, have improved blood plasma lipid profiles (203). In fibrotic visceral adipose tissues, lower levels of circulating triglycerides were found; consequently it is not out of the

question that fibrosis may impart protective effects to some degree (203). In contrast, fibrosis in subcutaneous adipose tissue depots may have the opposite effect and lead to preferential accumulation of nutrient stores and adipocyte hypertrophy in visceral fat depots. Therefore, we speculate that adipose tissue ECM may be a regulator of body fat distribution between visceral and subcutaneous depots. Support for this hypothesis comes from GWAS studies demonstrating association between many genes that control mesenchymal cell function, angiogenesis, and adipogenesis and body fat distribution (59, 193, 194).

While adipose tissue fibrosis shows fairly uniform responses in mouse models of obesity, there are a variety of reasons why clinical studies still lack substantive evidence in either direction of adipose tissue fibrosis pathogenesis. Current studies vary in the sense that some studies only measure fibrosis in one adipose tissue depot, only study gene expression, or are solely based on correlative studies (73,143,192). Studies investigating causative effects of fibrosis in adipose tissue, that include in depth examinations of metabolism do not currently exist, this remains a noteworthy barrier to research in this field. Overall, the association between adipose tissue fibrosis, inflammation, and metabolic dysfunction is not clearly delineated in human studies.

Reversibility of fibrosis

Currently, there is little evidence that fibrosis is reversible. Studies have investigated the results of weight loss on fibrosis that occurs in the liver, however results are mixed. Studies of weight loss using bariatric surgeries also remain controversial, as some patients are poor responders to surgery (1). Some studies however, describe a

decrease in expression of ECM components after weight loss surgeries (55, 101). A study of gene expression and histology of adipose tissues of the same patients before and two years after surgery report no decreases in fibrosis (29).

Concluding remarks

In closing, fibrosis is regarded as a hallmark of dysfunctional adipose tissue. At the start of our studies the primary source of ECM production in obesity and the mechanisms that drive it were unknown. Our studies contribute to the field by implicating ATM-preadipocyte communication as a potential modifiable node in the link between obesity and adipose tissue ECM deposition that may be targeted to improve metabolic health. Further studies are required to better relate our findings clinical settings to better understand associations between fibrosis, adipose tissue stiffness, and metabolism.

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