**EXPLORING FIBROSIS IN BOVINE GROWTH HORMONE (bGH) TRANSGENIC MICE**

A Thesis

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**Abstract**

Fibrosis, a pathological process characterized by excess extracellular matrix (ECM) deposition, can occur in many internal organs and tissues in response to various stimuli. As fibrosis progresses, scarring occurs, which ultimately leads to tissue dysfunction and organ failure. Patients with acromegaly, a rare disease usually caused by a benign, GH-producing pituitary tumor, have been reported to have prominent ECM deposition and scarring in certain tissues, which is indicative of fibrosis. In bGH transgenic mice, which express high levels of bovine growth hormone, several tissues [white adipose tissue (WAT), heart, intestine, and kidney] demonstrate a fibrotic phenotype. However, there is no previous research that investigates various bGH tissues – particularly from mice derived from a single cohort – for fibrosis. Additionally, WAT fibrosis is associated with obesity and lipodystrophy, and seems to be particularly associated with excess GH. This study aims to investigate the role of different cell types and genes involved in the development and progression of WAT fibrosis and determine if fibrosis is increased in BAT, liver, quad, kidney, lung, and spleen of aged bGH mice. Results of this thesis included a striking observation of increased fibrosis in all bGH tissues examined. For WAT, decreases in fibrosis-associated RNA expression in 3-month-old bGH mice via qPCR analysis was only observed in the perigonadal depot and not the subcutaneous depot that has more prominent collagen deposition. Interestingly, we observed an intriguing increase in fibrosis-associated RNA expression in a population of adipose stem and progenitor cells in 6-month-old mice within subcutaneous bGH WAT. These results indicate a potential common GH-induced mechanism of fibrosis across bGH tissues and pave the way for future research into WAT fibrosis.

## Introduction

### *Extracellular Matrix*

The extracellular matrix (ECM) is a complex non-cellular protein network found in all tissue types.[1] It provides critical structural support for tissues and organs and works as an essential scaffolding around cells.[2] In addition to structural support, the ECM has the capacity to store and sequester growth factors and cytokines, serving as reservoir to influence their bioavailability. The ECM, despite being extracellular, influences many cellular functions, including growth and wound repair.[3] Of the various ECM proteins, collagens (28 types in total) are the major constituent of the ECM (particularly collagen I, II, and III); however, hundreds of molecules have been identified in the ECM, each with unique properties to influence tissue function.[4] Other common ECM proteins include proteoglycans, glycosaminoglycans, elastins, laminins, fibronectin, and other proteins and glycoproteins.[5]

In healthy organisms, the ECM is remodeled continuously.[1] New ECM proteins are constantly being synthesized; likewise, the ECM is degraded by various proteinases. Among the most critical to degradation are matrix metalloproteinases (MMPs), which are endopeptidases that are necessary for ECM breakdown and tissue remodeling.[6] There are over 23 MMP genes with specific substrates and biological effects, only some of which are known to target the ECM.[7] Tissue inhibitors of MMPs (TIMPs 1-4) also modify the ECM by inhibiting the enzymatic activity of MMPs.[8] Together, MMPs and TIMPs regulate ECM turnover and balance one another's activities.[6]

Most cells, commonly fibroblasts and endothelial cells, are signaled to produce new matrix components that remodel and repair the ECM (i.e. MMPs and TIMPs).[3,9] However, when the precise balance of the ECM is disrupted, ECM proteins accumulate and scarring results, which can lead to serious complications including organ failure and death.[10] The scarring resulting from increased deposition of ECM proteins in tissues is known as fibrosis.

***Fibrosis***

Typically associated with chronic inflammation, fibrosis is a progressive pathological process with many mechanisms and cell types implicated in its development.[11] Many stimuli can induce fibrotic pathways, including persistent infections, autoimmune reactions, and tissue injury.[12] As will be discussed in more detail below, obesity is known to induce adipose tissue remodeling to accommodate the increased lipid stores, which is also associated with chronic inflammation and fibrosis of the tissue. Most fibrotic diseases, such as cirrhosis and fibrosing interstitial lung disease, are connected to decreased quality of life and early mortality.[13,14]

Although once thought to be irreversible, some data suggest that fibrotic phenotypes may not be permanent. For example, natural killer cells can destroy cell cycle arrested hepatic stellate cells, the cell type responsible for excess ECM deposition in the liver. As natural killer cells target and destroy the senescent activated stellate cells, fibrosis is resolved.[15] Likewise, treatment of patients who have primary hypertension, left ventricular hypertrophy, and left ventricular diastolic dysfunction with an angiotensin-

converting enzyme inhibitor results in regression of myocardial fibrosis.[16] This regression is observed through decreased left ventricular collagen volume fraction and decreased myocardial hydroxyproline concentration (hydroxyproline is an amino acid unique to collagen that correlates with fibrosis levels.) These data demonstrate that, at least in some situations, fibrosis can be reversed.

*The ECM and Fibrosis in White Adipose Tissue* (WAT)

White adipose tissue (WAT) is found in discrete depots throughout the body and plays a critical role in energy homeostasis by storing and releasing lipids, depending on the caloric needs of other tissues. Energy is stored in adipocytes in the form of triglycerides in times of nutrient excess and is released as fatty acids and glycerol upon fasting.[17] In addition, WAT acts as an endocrine organ by synthesizing and secreting various hormones, enzymes, and cytokines, which are important to metabolic homeostasis and energy expenditure, among other processes.[18] Because of these attributes, changes in the constituents and quantity of WAT can lead to metabolic dysfunction.[19]

*The ECM in Normal WAT*

Two major populations comprise adipose tissue: adipocytes and the stromal vascular fraction (SVF). Adipocytes serve as a homogenous population of fat storage cells within the adipose tissue, while the SVF is a heterogenous mixture of non-adipocyte cells including fibroblasts, preadipocytes, mesenchymal stem cells, and endothelial

cells.[20] More than 80% of adipose tissue volume is made up of adipocytes, and the remainder of cells surrounding the adipocytes is the SVF.[21] These two cell fractions are surrounded by ECM. Both the adipocytes and the SVF contribute to the production of collagen VI, an ECM component that is particularly abundant in adipose tissue.[22] Type I collagen (collagen I) provides the main framework of WAT (found at higher levels in subcutaneous fat than visceral fat)[23], and type IV collagen makes up the basal lamina around the adipocytes.[24] Besides these specific collagens, most ECM proteins in WAT reflect those in other tissues (other collagens, fibronectin, laminins, etc.)[22] Most collagen is produced in the early stages of WAT differentiation.[25] However, the ECM in WAT is remodeled constantly as fat depots and adipocytes grow and shrink, typically with minimal ECM deposition and inflammation occuring.[26]

There are two extremes associated with the amount of WAT – obesity and lipodystrophy. As discussed in the subsequent sections, both extremes are associated with WAT fibrosis.

*Obesity and Adipose Tissue Fibrosis*

Obesity has various definitions, but the World Health Organization (WHO) describes it as "abnormal or excessive fat accumulation that presents a risk to health."[27] This fat accumulation in obesity is due to adipocyte enlargement and the formation of new adipocytes (known as hypertrophy and hyperplasia, respectively).[28]

The prevalence of obesity is steadily rising, with the occurrence of central obesity (a measure of visceral adipose tissue using waist circumference; more closely associated

with obesity-related health risks than BMI) predicted to reach 55.6% in men and 80.0% in women by 2030.[29] The constant increase in obesity rates is concerning given the health risks that occur alongside the condition. In fact, a study of 900,000 people found that each 5 $kg/m^2$ increase in BMI over 22.5-25 $kg/m^2$ is, on average, associated with a 30% increase in mortality.[30]

Many factors, including impaired metabolic health, contribute to the shortened lifespans of people with obesity. Individuals with obesity have greater incidence of heart disease, stroke, fatty liver, type 2 diabetes, certain types of cancer, and musculoskeletal disorders.[31] Both subcutaneous and visceral WAT are correlated with increased risk factors of metabolic dysfunction, but this correlation is more powerful for visceral adipose tissue.[32] Therefore, the quantity of visceral WAT is more strongly associated with an adverse metabolic risk profile than subcutaneous adipose tissue. For example, increased central (visceral) but not subcutaneous body fat is linked to insulin resistance.[33] The fat depots referenced can be found in Figure 1.

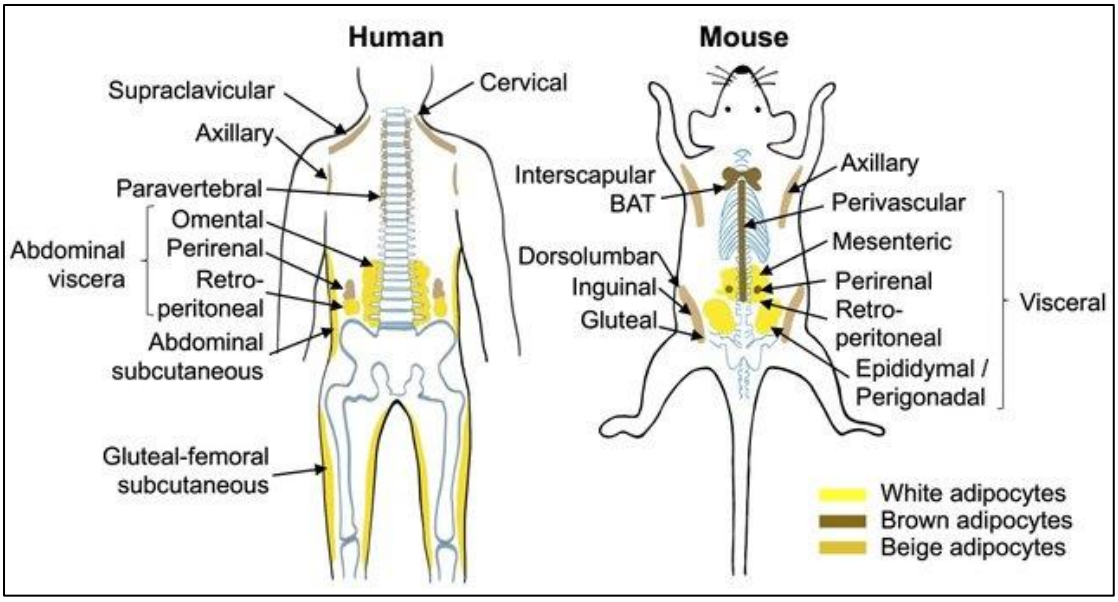


Figure 1: Distribution of fat tissues in the human and mouse. In human, adipose depots are divided into upper body subcutaneous (cervical, supraclavicular, axillary, paravertebral and abdominal subcutaneous), abdominal viscera, and lower body subcutaneous (gluteal-femoral subcutaneous). In mouse, adipose depots are generally distributed into anterior subcutaneous (axillary, BAT), visceral, and posterior subcutaneous (dorsolumbar, inguinal, and gluteal). Cheong, L.Y., Xu, A. Intercellular and inter-organ crosstalk in browning of white adipose tissue: molecular mechanism and therapeutic complications. *Journal of Molecular Cell Biology* Vol 13, Issue 7, 466-479 (2021). https://doi.org/10.1093/jmcb/mjab038

*WAT Fibrosis in Obesity*

WAT fibrosis is linked to advanced obesity—although the connection is complex and incompletely understood.[34,35] As the adipose tissue increases and adipocytes enlarge with obesity, ECM remodeling is critical for tissue health. Multiple pathways, cells, and molecules have been shown to influence WAT fibrosis in obesity as will be described below.

Immune Cells

In mouse and human studies, obesity is also associated with infiltration of immune cells and the release of proinflammatory cytokines in obese WAT. These immune cell types include macrophages,[36] mast cells,[37] T cells,[38] and B cells.[39] It is

thought that adipocyte hypertrophy and hyperplasia trigger inflammation and acute hypoxia, which results in the production of less flexible ECM, and ultimately ends in WAT fibrosis. However, whether inflammation and hypoxia precede fibrosis is debated, as detailed assessment of the appearance of fibrotic streaks in high fat fed mice appear prior to alterations in inflammation and infiltration of macrophages.[40]

### Transforming Growth Factor β (TGF-β) Signaling

Transforming growth factor β (TGF-β) signaling has been strongly implicated in fibrosis in many tissues, including obese WAT. TGF-β is produced in an inactive form by many cell types. It is cleaved and activated by proteases such as MMP2 and MMP9.[41] The downstream signaling pathway of TGF-β involves the Smad family of transcriptional activators and MAPK pathways.[42] Through SMAD-dependent suppression of peroxisome proliferator-activated receptor γ (PPARγ), the TGF-β family also inhibits adipogenesis.[43] The TGF-β family members regulate the induction of myofibroblasts, which are contractile cells that produce ECM, from adipose progenitor cells.[44]

A small population of immature cells within adipose tissue, known as adipose progenitor cells, are a major ECM-producing cell population in adipose tissue.[45] In human adipose tissue macrophages derived from obese subcutaneous WAT, TGF-β and MMP2 levels are higher, while MMP9 levels are lower than they are in macrophages from the non-obese group.[44] When human subcutaneous WAT progenitor cells are treated with TGF-β1, $\alpha$ -SMA, a myofibroblast marker, shows increased immunocytochemical staining. In addition, treatment of the progenitor cells with activin A, a member of the TGF-β family, leads to an up-regulation of myofibroblast transcription factor SNAIL.

Finally, myofibroblast transcription factors SNAIL and SLUG are expressed more in adipose tissue progenitor cells from obese individuals than non-obese individuals.[44]

Also, there are decreased TGF-β levels in collagen VI-deficient obese mice, as well as increased levels of a TGF-β inhibitor.[46] In these mice, SMAD2, SMAD3, and downstream TGF- β signaling mediators, show reduced phosphorylation states, suggesting lessened activation. Together, these data provide evidence that TGF-β induces fibrosis in obese subcutaneous WAT through the upregulated transcription of myofibroblasts. Collectively, these studies provide strong support for a role of TGF-β in WAT fibrosis with obesity.

Endotrophin

Endotrophin is a propeptide of type VI collagen (specifically the C5 domain of COL6α3). Collagen VI is ubiquitously expressed, but highly enriched in adipose tissue; thus, production of endotrophin levels is mainly influenced by amount of WAT. Endotrophin has been shown to be upregulated in obese mouse fat pads compared to those in lean littermates, and has also been implicated in tumor fibrosis.[47] Diet-induced obese transgenic mice that overexpress endotrophin have upregulated ECM components COL3α1 (the alpha 1 subunit of collagen 3) and COL6α1 in subcutaneous WAT.[48] Also, excess endotrophin increases the amount of ECM accumulation in subcutaneous WAT and brown adipose tissue (BAT) in endotrophin-overexpressing transgenic mice, implying that endotrophin stimulates fibrosis. This is supported by the discovery of increased crown-like structures—indicators of the proinflammatory process in WAT—in the WAT of the endotrophin-overexpressing transgenic mice.[48]

IL-13

Another molecule implicated in WAT fibrosis is interleukin 13 (IL-13). IL-13 is a pleiotropic cytokine largely produced by T cells (specifically Th2 $CD4^+$ T cells) that is established to mediate liver fibrosis.[49] Its role in high fat diet (HFD)-induced WAT fibrosis is unclear; however, one mouse study suggests that a "HFD induces the expression of IL-13 from non-Th2 $CD4^+$ T cells and may mediate the deposition of collagen to induce adipose tissue fibrosis."[50] If this is the case, then IL-13 is likely important in the progression of obesity-associated fibrosis although this has not been adequately explored to date.

Other Cells and Molecules Implicated in Obesity-Associated WAT Fibrosis

Several other molecules have been implicated in WAT fibrosis that is associated with obesity. These include: 1) Platelet-derived growth factor-α (PDGFα), which has been implicated in the proliferation of cells that promote fibrosis; 2) connective tissue growth factor (CTGF), a central mediator of tissue remodeling and fibrosis, and implicated recently in WAT fibrosis in humans;[51] 3) Toll-like receptor 4 activation;[52] and 4) specific immune cells, like macrophages.[53] Many studies have examined other progenitor cell populations and factors associated with obesity-associated fibrosis, although no single pathway, molecule, or cell type fully explains the phenomenon.

*Fibrosis in Adipose Tissue – Lipodystrophy*

Lipodystrophy syndromes are a collection of rare disorders that are characterized by the selective loss of adipose tissue resulting in the reduced ability to store lipids.[54]

Shared attributes of these diseases include ectopic steatosis (often fat accumulates in the liver[55]), severe insulin resistance, and dyslipidemia.[54,56] Lipodystrophy can be acquired or congenital. The fat loss can be "localized," impacting only specific areas usually due to trauma or medication injections (insulin, steroid, etc.);[57] "partial," impacting specific fat depots; or "generalized," impacting all fat depots. The severity of the metabolic dysfunction associated with lipodystrophy is typically associated with the degree of fat loss, with generalized lipodystrophy having the most significant metabolic abnormalities. Although not well studied in comparison to obesity-induced fibrosis, WAT in lipodystrophic conditions also appears to be fibrotic. The few studies that have evaluated the causes of lipodystrophy-induced WAT fibrosis are described below.

Integrins

Integrins are cell surface receptors that regulate the organization of the ECM, enable binding of cells to ECM components, and initiate intracellular signaling.[58] When the activity of integrins with β1 and β3 subunits is selectively blunted in adipose tissue, a lipodystrophic condition occurs with an age-dependent loss in adipose tissue mass, systemic insulin resistance, and adipose tissue inflammation.[59] Adipose tissue fibrosis also occurs. The appearance of WAT fibrosis when integrin activity is diminished and the fact that adipocytes are insulin-resistant suggests that there is cross-talk between integrin and insulin signaling pathways that leads to fibrosis in this lipodystrophic state.[59]

TGF-β

Transgenic mice that constitutively overexpress human TGF-β1 in liver, kidney, WAT, and brown adipose tissue (BAT) (targeted by the rat phosphoenolpyruvate

carboxylase gene, which is expressed in these tissues), show a lipodystrophy-like syndrome with adipose tissue fibrosis.[60] Because of this, TGF-β is likely implicated in the development of lipodystrophy-associated WAT fibrosis as well as obesity-associated WAT fibrosis (as discussed in the previous section).

### Other Factors Implicated in Lipodystrophy-Associated WAT Fibrosis

A few other studies have evaluated partial lipodystrophy syndromes. For example, the SVF of abdominal fat from HIV+ patients (that were treated with older nucleoside analogue reverse-transcriptase inhibitors, which result in partial lipodystrophy) contains more M1 (inflammatory) macrophages than healthy fat, suggesting chronic inflammation.[61] In addition, transcriptional profiles of subcutaneous adipose tissue of these HIV+ patients shows increases in inflammation-associated genes, including hypoxia-inducible factor (HIF)-1α and tumor necrosis factor (TNF)- α. Genes involved in the synthesis of fatty acids, triglycerides, and cholesterol also appear to be associated with a lipodystrophic phenotype and fibrosis,[62] as well as increased numbers of mitochondria and overexpression of uncoupling protein 1 (brown fat-like features).[63] Ultimately, because there are multiple forms of lipodystrophy, different mechanisms are likely responsible for fibrosis development.

### *Growth Hormone*

Growth hormone (GH) is a protein hormone that is produced in the anterior pituitary, a small gland located at the base of the brain. It is released in a pulsatile manner resulting from alterations in GH releasing hormone (positive control) and somatostatin (negative control), which are secreted by the hypothalamus.[64]

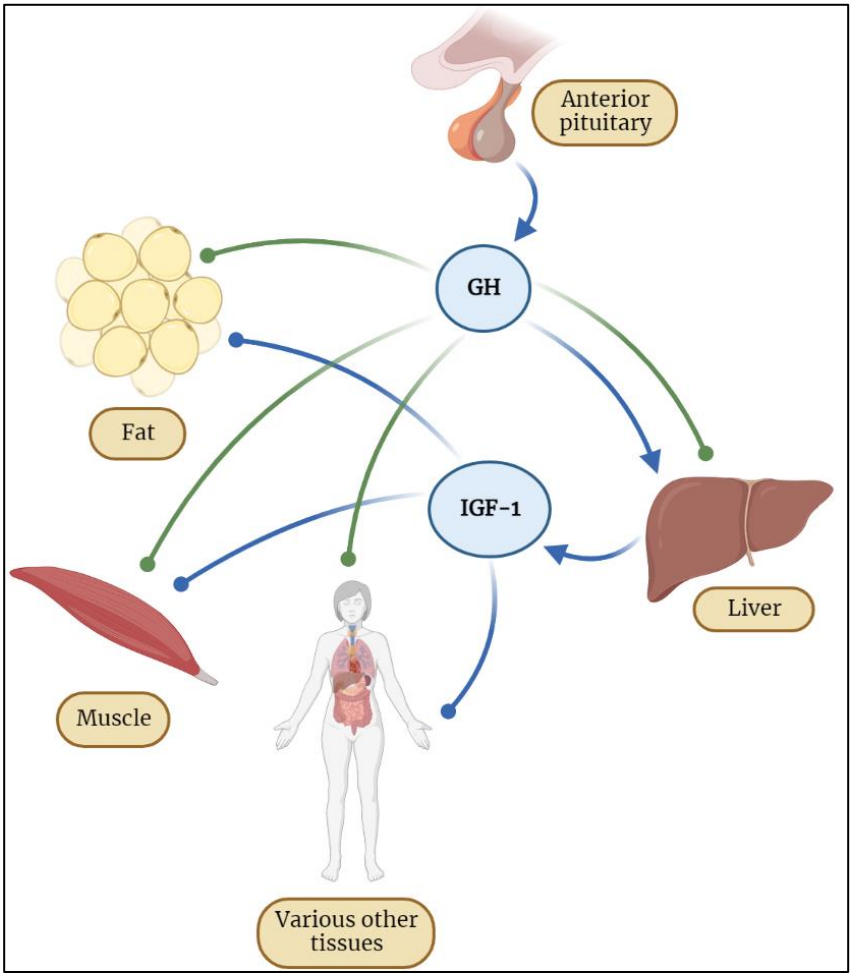


Figure 2: Simplified GH/IGF-1 pathway. GH, produced by the anterior pituitary, is stimulated by GHRH and inhibited by somatostatin. GH has direct effects on tissues including the liver, which releases IGF-1 in response to stimulation by GH. IGF-1 exerts effects on muscle, fat, and various other tissues, while also participating in a negative feedback loop: it inhibits GHRH and stimulates somatostatin. Arrows indicate an increase in activity, and lines ending in circles refer to a general effect on a tissue. Made in BioRender.

#### *GH Signaling Pathway*

Once it is released, GH binds to GH receptors found on various tissues, allowing GH to act directly on multiple organs.[65] GH receptors exist as transmembrane homodimers that bind GH at their extracellular domains and transduce signals through their cytoplasmic domains.[66] When GH binds to its receptor, it activates the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, in which JAK2 is activated and phosphorylates tyrosine residues in the GHR,

creating a binding site for STAT5a and STAT5b, where they are phosphorylated by JAK2.[67,68] When STAT is phosphorylated, it forms a dimer and is translocated to the nucleus, where it acts as a transcription factor.[67] While JAK/STAT is not the only signal transduction pathway initiated by the binding of GH to its receptor, it is a key pathway that regulates growth and metabolism.[69]

*GH and IGF-1*

GHR activation results in the expression of numerous GH-regulated genes. One significant outcome of GH signal transduction is the upregulated transcription of insulin-like growth factor 1 (IGF-1) – collectively referred to as the GH/IGF-1 axis. IGF-1, while made by most cells, is primarily released into circulation by the liver, which then exerts additional endocrine effects on muscle, fat, and various other tissues (Figure 2).[70] GH and IGF-1 also act as negative feedback in this axis. For example, IGF-1 inhibits GHRH (through decreasing GHRH-stimulated transcription of GH)[71] and stimulates somatostatin, thereby reducing the amount of GH produced when it is in high concentrations.[72] Thus, GH acts directly on tissues to alter gene expression[73] and indirectly via IGF-1, making it difficult to discern the tissue effects from IGF-1 action versus that of GH itself.[70] However, GH and IGF-1 have distinct and overlapping functions as elegantly demonstrated by Lupu et al (2001). Using IGF-I knockout, GHR knockout, and IGF-I/GHR double knockout mice, Lupu et al estimate that the GH/IGF-I axis accounts for 83% of postnatal growth with 14% attributed to GH alone, 35% to IGF-I alone, and 34% to overlapping GH/IGF-I function while 17% is unrelated to the GH/IGF-I axis.[74]

*Effects of GH on Physiology*

As the name implies, GH plays a critical role in somatic growth. In addition to supporting growth, GH is important in the regulation of nutrient metabolism, including glucose homeostasis, proteolysis, and lipid and mineral metabolism.[75,76] In WAT, GH increases lipolysis, resulting in release of free fatty acids—and inhibits lipogenesis, resulting in a loss of WAT mass. In other tissues, such as muscle, GH causes a rise in protein synthesis and promotes insulin resistance, sparing glucose as an energy source.[76] Thus, individuals with higher GH levels are relatively lean with an increase in muscle tissue and a decrease in fat mass. Obesity is also linked to low GH secretion.[77]

*Depot-Specific Effects of GH Action on WAT*

Although GH has an overall lipolytic effect, it affects individual fat depots differently. For example, patients with acromegaly (who therefore experience excess circulating GH and IGF-1, as will be discussed in more depth below) have decreased visceral and subcutaneous adipose tissue compared to healthy subjects, but increased intermuscular adipose tissue.[78] However, untreated patients with Laron Syndrome, which is a condition characterized by GH insensitivity and consequent IGF-1 deficiency due to dysfunctional GH receptors, exhibit marked increases in subcutaneous fat.[79]

The depot-specific differences of GH action on WAT are best studied in mouse lines in which multiple fat depots can be sampled simultaneously and studied. For example, adipocyte-specific GHRKO (AdGHRKO) mice, which lack functional growth hormone receptors exclusively on adipocytes, have marked depot specific effects. That is,

the subcutaneous WAT depot is more drastically impacted by the removal of GHRs in AdGHRKO mice, with an almost 200% increase in depot mass and significantly larger adipocytes when compared to littermate controls.[80] Perigonadal depots show similar, albeit less pronounced, trends in depot mass and cell size. Likewise, the global GHR gene disrupted mouse, a mouse line similar to humans with Laron syndrome, also shows the most significant alterations in subcutaneous WAT and the least in perigonadal depots.[81] Using various mouse lines with altered GH action, differences between adipose tissue depots have been found in immune cell infiltration,[82] adipokine production,[83] differentiation of preadipocytes,[84] gene expression,[85] and senescence.[86] In addition, WAT fibrosis is decreased in subcutaneous depots in AdGHRKOs and other mice with decreased GH action, but not significantly altered in perigonadal depots.[80,81]

### *Acromegaly*

Acromegaly is a rare, chronic disease characterized by excess GH production in adulthood, typically resulting from a non-cancerous pituitary tumor called an adenoma.[87] As a result of increased GH (and therefore IGF-1) levels, patients with acromegaly have disproportionate skeletal, tissue, and organ growth. Symptoms include thickening of the skin, enlarged bones in the face and digits, swelling of the ventricles of the heart, swelling of the kidney and other organs, sleep apnea, and respiratory and cardiac failure, among others.[88] Acromegaly is well-reported to result in cardiovascular disease and decreased life expectancy,[87] and treatment involves surgical removal of the adenoma (if possible), radiotherapy, and/or reduction or inhibition of GH through pharmacological means.[89] When excess GH is secreted during childhood, a condition known as gigantism

results.[90] This is due to open epiphyseal growth plates in the long bones allowing for linear growth, which causes tall stature. The root cause of gigantism and acromegaly are the same (excess GH production); however, gigantism occurs in childhood, while acromegaly appears in adulthood. There can also be overlap between the symptoms of these conditions.[91] As might be expected based on the known functions of GH, acromegaly and gigantism are associated with reduced WAT mass but decreased insulin sensitivity.[92]

Acromegaly is associated with increased fibrosis in several tissues. For example, autopsies performed on twenty-seven patients with acromegaly reveal that 85% have myocardial interstitial fibrosis.[93] Patients with acromegaly also have been reported to have increased liver fibrosis[94] as well as thyroid nodules thought to result from nodular fibrosis.[95] Thickened skin is a key characteristic of the disease, and the skin of people with acromegaly contains dense infiltration of glycosaminoglycan deposits, which are a class of ECM biomolecules.[96] As further support of the capacity of GH to promote tissue fibrosis, GH has been shown to stimulate the production of collagens in various tissues, including skeletal muscle.[97,98] In addition, people with acromegaly have elevated serum markers of collagen turnover and degradation (procollagen type I amino-terminal propeptide, procollagen III amino-terminal propeptide, and type I collagen degradation product) and fibroblast activation protein (FAP), which correlate with the severity of disease.[99] However, it should be noted that while GH in excess is correlated with fibrosis, it is not clear 1) whether the excess GH or IGF-1 is the contributing factor and 2) whether GH causes fibrosis or is an outcome of overall metabolic dysfunction in this condition. In addition, fibrosis is commonly assessed only in a single tissue in these acromegaly

studies. Thus, it is not clear whether fibrosis progresses in a similar manner or time frame throughout these tissues or if fibrosis onset occurs in a tissue-specific manner.

### *bGH Mice*

In our laboratory, we have used bovine GH (bGH) transgenic mice to assess the relationship between GH and adipose tissue. These transgenic mice were created by linking the first exon and intron of the bGH DNA to a metallothionein promoter (MT1) and injecting into the pronucleus of C57BL/6J mouse embryos.[100] MT1 is ubiquitously expressed in almost all organs.[101] Mice with this transgene have high levels of constitutive bGH expression which increase two- to ten-fold upon induction by heavy metals (which are present in high levels in organs such as the liver and kidneys.)[102] bGH mice have chronically elevated serum levels of GH, IGF-1, and insulin, which result in giant, lean mice.[103] Many features of these mice, including their insulin resistance, leanness, and short lifespans, mimic those of people with gigantism/acromegaly,[103] making them a mouse model for the disease.

Interestingly, young bGH mice have relatively more fat mass than littermate controls,[104] which is thought to be due to GH or IGF's ability to promote preadipocyte proliferation and differentiation. However, by 4 months in males and 6 months in females, the bGH mice become leaner than controls, likely due to the increased lipolysis due to high levels of GH.[104] The absolute fat mass of bGH mice remains consistent over their lifetime (on a standard rodent diet), while nontransgenic controls show an increase in fat with advancing age. Subcutaneous, epididymal/parametrial, retroperitoneal, and

mesenteric adipose tissue depots weigh much less in 54-week-old bGH mice than controls in both male and female mice.

The bGH mice appear to experience greater fibrosis. That is, male bGH mice have increased fibrosis in select tissues, including adipose tissue (measured in mice 3-6 months of age),[103] kidneys (3-12 months),[105] small intestine (4 months),[106] and heart (older than 12 months)[105] as shown (Figure 3). As seen in Figure 3, adipose tissue demonstrates a depot-specific pattern of fibrosis in bGH mice, with more prominent fibrosis in the subcutaneous depot. However, to date, most of these studies that have shown increased fibrosis in bGH mice have utilized different ages, sexes or genetic background strains and typically do not evaluate fibrosis in different tissues within the same animals but rather are focused on a single tissue.

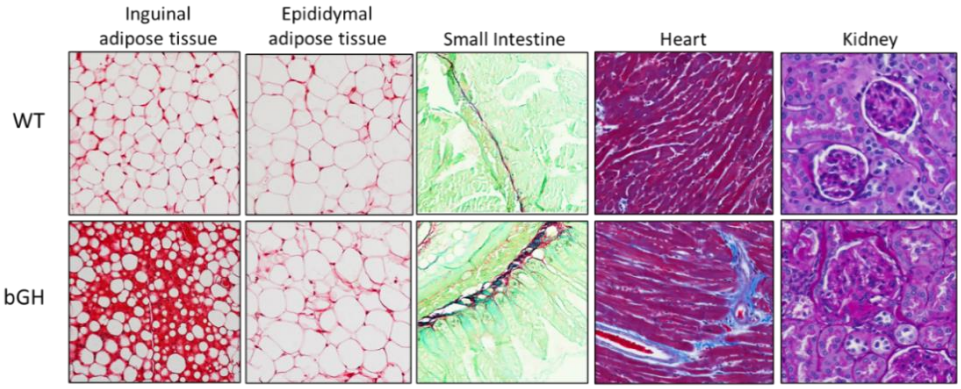


Figure 3: Histology of assorted tissues in aged (10-13-month-old) male bGH and WT control mice. White adipose tissue (WAT) from inguinal or epididymal fat was stained with Sirius red (a non-specific red collagen stain), Swiss rolls of the small intestine were stained using Sirius Red/Fast Green, heart was stained with Masson's Trichrome (stains connective tissue blue), and kidney sections were stained with Periodic Acid Schiff Stain (stains connective tissue purple-magenta color). Kopchick, J.J., Basu, R., Berryman, D.E., *et al.* Covert actions of growth hormone: fibrosis, cardiovascular diseases and cancer. *Nature Reviews Endocrinology* 18, 558-573 (2022). https://doi.org/10.1038/s41574-022-00702-6

**Specific Aims**

1. Evaluate the role of adipocytes vs. other cell types in the development of fibrosis in adipose tissue of bGH mice

To uncover the mechanism(s) through which GH induces fibrosis in adipose tissue, we will separate the adipocytes and the stromal vascular fraction (SVF) and analyze each of these components for their role in the initiation and progression of fibrosis using qPCR. We will look at genes for collagens, MMPs, and TIMPs in bGH and control mice. In addition, we will investigate subpopulations of adipose tissue using single nuclear sequencing. Together, these data may highlight genes, signaling pathways and cell types involved in the development of fibrosis in adipose tissue.

2. Determine if fibrosis is a common characteristic of tissues in older bGH mice

The goal of this aim is to determine if the fibrotic phenotype seen in WAT of bGH mice is detectable in other tissues in 13-month-old bGH mice or if bGH induces fibrosis in a tissue-dependent manner. If we find evidence of fibrosis in tissues other than WAT, then excess GH may work through a common mechanism to induce fibrosis in multiple tissues, which could be responsible for the negative health outcomes of this mouse line (reduced lifespan, organ dysfunction, et cetera). Fibrosis will be determined by evaluating collagen deposition histologically and via hydroxyproline assays (hydroxyproline is a collagen-specific amino acid). We will investigate brain, liver, heart, kidney, spleen, lung, and skeletal muscle in the same aged mice to determine incidence of fibrosis when tissues are exposed to high GH levels.

## Methods

### *Aim 1: Evaluate the role of adipocytes vs. other cell types in the development of fibrosis in adipose tissue of bGH mice*

To discern which cell types of adipose tissue (adipocytes or components of the SVF) are responsible for the development of fibrosis, we dissected WAT from subcutaneous and perigonadal depots from 3-month-old male bGH and WT mice (n=7). The mature adipocytes were separated from the other cell types (referred to as the stromal vascular fraction) via standardized collagenase procedures from our lab[82] and published protocols.[107] Briefly, samples were treated with 1.1 mg of collagenase type I per gram of WAT for tissue digestion. After incubation at 37° C for 45 minutes in a shaker, samples were filtered through mesh and centrifuged at 1000 rpm for 10 minutes to isolate the SVF cells from the adipocytes.

To isolate adipocyte and SVF RNA from the subcutaneous and perigonadal WAT depots mentioned above, a QIAzol™ Lysis Reagent (QIAGEN) and an RNeasy Lipid Tissue Mini Kit (QIAGEN) were used after homogenizing tissues in the Precellys 24 Tissue Homogenizer (Bertin Instruments). Following RNA isolation, cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Then, qPCR was performed using Power SYBR™ Green PCR Master Mix (Applied Biosystems), primers for fibrosis and select ECM-associated genes (i.e., *Col1a1, Col3a1, Col4a1, Col5a1, Col6a1, Col11a1, Timp1, Timp3, Mmp2, Mmp9, Mmp13,* and *Mmp14*), along with established reference gene primers (*Actb* and *B2m*). Analysis of gene expression was carried out in a QuantStudio 3 Real-Time PCR System (Applied Biosystems).

Relative RNA levels were calculated using two methods: the delta-delta Ct (cycle threshold) method[108] and the modified Pfaffl method.[109] The modified Pfaffl method is based on the principle that the "difference in quantification cycle value between samples … is transformed into relative quantities using the exponential function with the efficiency of the PCR reaction at its base."[109] This method also permits the use of multiple reference genes, allowing for reliable measurements of subtle differences in expression, unlike the Pfaffl method. The delta-delta Ct method also permits the use of multiple reference genes.

Two mice from the WT group and two mice from the bGH group were excluded from the qPCR analysis for the following reasons: their RTC wells had Ct values within 5 cycles of the experimental wells and/or the experimental duplicate wells resulted in Ct values differing by more than 0.5. These suggest poor-quality cDNA. Because of this, the sample size of the qPCR data decreased to n=5 for both WT and bGH groups.

Additionally, we analyzed data from single nucleus sequencing on inguinal subcutaneous WAT to analyze gene expression of select cell types within WT versus bGH mice. These data were already available in the laboratory for inguinal WAT from 3 WT or 3 bGH male mice at 6 months of age. The WAT from these mice were pooled together (150 mg/mouse) and sent to SingulOmics. Nuclei were isolated from the adipose tissue, and single cell gene expression libraries were constructed using the 10x Genomics Chromium system. The libraries were paired-end sequenced on an Illumina Novaseq to a depth of approximately 200 million reads per sample. Per the company, clean reads were analyzed using Cell Ranger v6.1.2 with the mm10 mouse reference genome. The predominant cell type in each cluster was determined using SingleR 1.10.0. Cell

populations of WAT were distinguished based on their shared gene characteristics. Expression data from Emont et al. was used as the reference annotation.[110] The single nuclear data for each of the genes quantified by qPCR (i.e., *Col1a1, Col3a1, Col4a1, Col5a1, Col6a1, Col11a1, Timp1, Timp3, Mmp2, Mmp9, Mmp13,* and *Mmp14*) were analyzed in each identified cell population of WAT. This bioinformatics work was done in collaboration with a research scientist in the laboratory.

***Aim 2: Determine if fibrosis is a common characteristic shared among other tissues in older bGH mice***

Using 13-month-old male bGH mice and littermate controls (n=4), tissues (brain, heart, kidney, liver, quadriceps, spleen, lung, and BAT) were stained with Sirius Red (also known as picrosirius red) and Fast Green contrast to histologically assess levels of fibrosis. For histological analyses, dissected tissues were fixed in 10% PBS buffered formalin and then embedded in paraffin and sectioned by AML labs in St. Augustine, FL. Then, the slide-mounted tissues were stained at our lab with the following procedure.

First, the sections were cleared in 3 two-minute passes through xylenes. Then, they were rehydrated in two-minute washes of decreasing grades of ethanol (100%, 100%, 95%, and 70%) followed by water. Slides were then immersed in 0.1% Sirius Red solution (dissolved in 1.3% picric acid) for 20 minutes and rinsed in water. Next, the slides were immersed in 0.04% Fast Green + 0.1% Sirius Red for 30 minutes. After being rinsed in water, the sections were dehydrated for one minute each in three increasing grades of ethanol (95%, 100%, 100%.) Slides were cleared in xylene and left to dry, and

then cover glass was fixed to each slide with Permount™ mounting medium (Fisher Chemical.)

Next, the tissues were imaged, and the degree of collagen staining was quantitatively assessed. Pictures of 10 non-overlapping fields (X200) were taken per tissue depot per mouse. ImageJ was used to isolate the red and green layers of the images and measure the percent of the field taken up by tissue and the percent of the field that was stained red. For each image, the percent stained red was divided by the percent of the field taken up by tissue, which gave us a proportion of red to total tissue (% picrosirius red stained area). Representative images were chosen based on image quality and proximity of the image's percent picrosirius stained red area to the average percent picrosirius red stained area for that genotype.

We also investigated tissue hydroxyproline in old male bGH mice and littermate controls (n=10.) Hydroxyproline from liver (12-, 13-, and 15-month-olds) and lung (11- and 15-month-olds) was investigated, and brain, heart, kidney, quad, spleen, and BAT hydroxyproline will be analyzed in the future. To quantify contents of hydroxyproline in tissues, a procedure previously described was used.[111] 50-100 mg of tissue was hydrolyzed in HCl and heated at 110°C for 24 hours. Then, 1:10 dilutions of the samples were made using supernatant and buffer (composed of citric acid monohydrate, glacial acetic acid, sodium acetate trihydrate, sodium hydroxide, and water). Hydroxyproline standards of 25, 12.5, 6.125, 3.125, 1.56, 0.78, and 0.39 ug/mL were made alongside the samples. Next, a 0.05M chloramine T solution was added to all samples and standards and incubated for 20 minutes. Following this step, perchloric acid was added and samples were incubated for 5 minutes, and then 20% p-dimethylaminobenzaldehyde in 2-

methoxyethanol was added. The samples and standards were placed in a 60 °C water bath for 20 minutes. Then, the samples and standards were transferred to a 96 well spectrophotometry plate. The absorbancy of the solutions was determined spectrophotometrically at 550 nm using a standard curve. The hydroxyproline data were generated for only liver and lung (10 bGH and 10 WT males).

### *Statistics*

All statistical analyses, excluding those for single nuclear sequencing (described above), were performed using GraphPad Prism 8. Normality of data was assessed using the Shapiro-Wilk test. To assess the differences between control and bGH mice, Mann-Whitney tests were performed. Differences were considered significant when $P < 0.05$.

## Results

### *qPCR Results*

Building on previous data that showed an increase in WAT fibrosis in adult bGH mice but no change in expression of key ECM genes in whole tissue, we evaluated the expression of ECM-related genes in younger male mice (when the fibrotic cascade appears to be initiated) and in the SVF versus the adipocyte fraction. For these analyses, high-quality RNA could not be isolated for the adipocytes despite repeated attempts. This is likely because the adipocyte RNA could not be preserved properly during its separation from the SVF. The high lipid content of this fraction also interferes with RNA extraction.[112] Thus, adipocyte fraction samples were excluded from qPCR and subsequent analysis. SVF samples were analyzed for *Col1a1, Col3a1, Col4a1, Col5a1, Col6a1, Col11a1, Mmp2, Mmp3, Mmp9, Mmp13, Mmp14, Timp1,* and *Timp3* expression by qPCR. Using these data, we also compared two different methods (delta-delta CT and Pfaffl) currently used in the laboratory for qPCR analysis.

The gene *Mmp13* provided unusable results using both delta-delta Ct and modified Pfaffl analysis methods. The Ct values for each sample fell in the 35-40 range, indicating that the gene measured is fluorescently detected after 35 cycles, at which point the results are not dependable. Because of this, any potential differences between WT and bGH groups were not reliable.

With delta-delta Ct analysis of the qPCR results from the SVF of perigonadal WAT, four genes exhibited significant differences in their expression levels between WT and bGH mice. *Col1a1, Col3a1, Col6a1,* and *Timp1* are expressed less in bGH tissue

compared to WT tissue (Figure 4). When the SVF of subcutaneous WAT was analyzed via delta-delta Ct, there were no significant differences in gene expression between bGH and WT mice (Figure 4).

Delta-Delta Ct Results

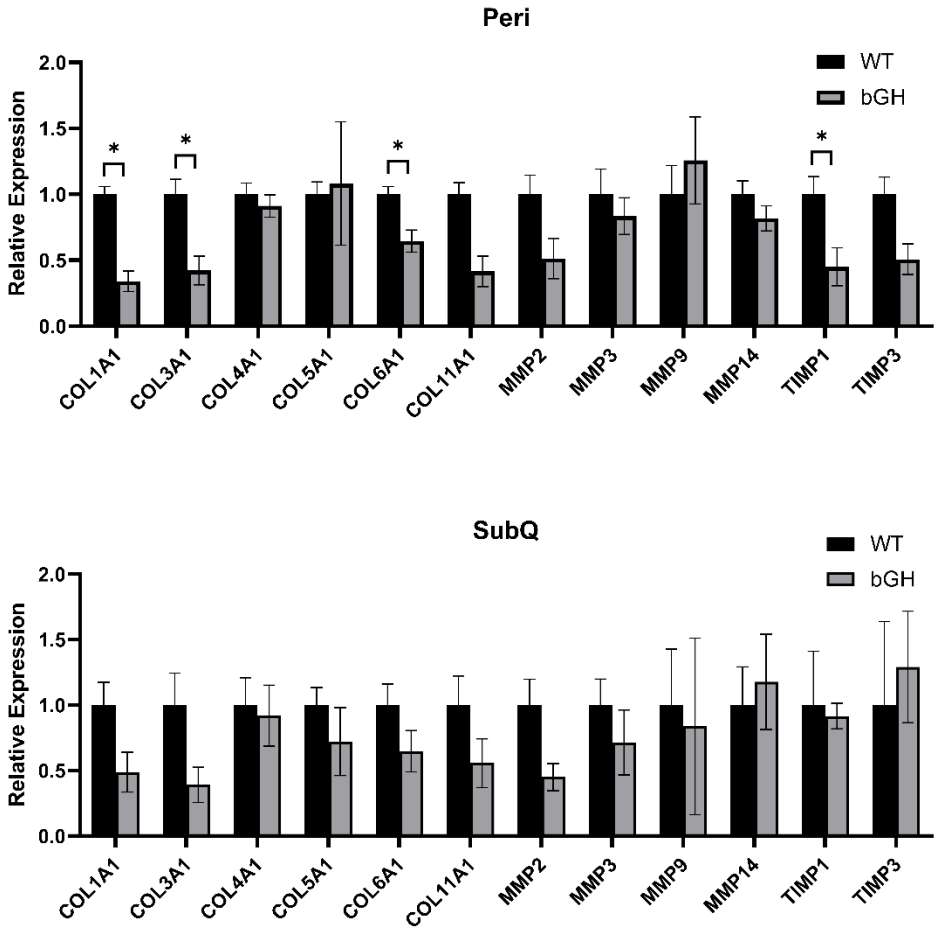


Figure 4) Relative mRNA expression in the SVF of perigonadal visceral (top) and subcutaneous (bottom) WAT in 3-month-old male WT and bGH mice (n=5) using delta-delta Ct analysis.

## Modified Pfaffl Results

With a modified Pfaffl analysis of the qPCR results from the SVF of perigonadal WAT, four genes exhibited significant differences in their expression levels between WT and bGH mice. *Col1a1*, *Col6a1*, *Col11a1*, and *Timp1* were expressed less in bGH tissue compared to WT tissue (Figure 5). When the SVF of subcutaneous WAT was analyzed via modified Pfaffl, there were no significant differences in gene expression between bGH and WT mice (Figure 5).

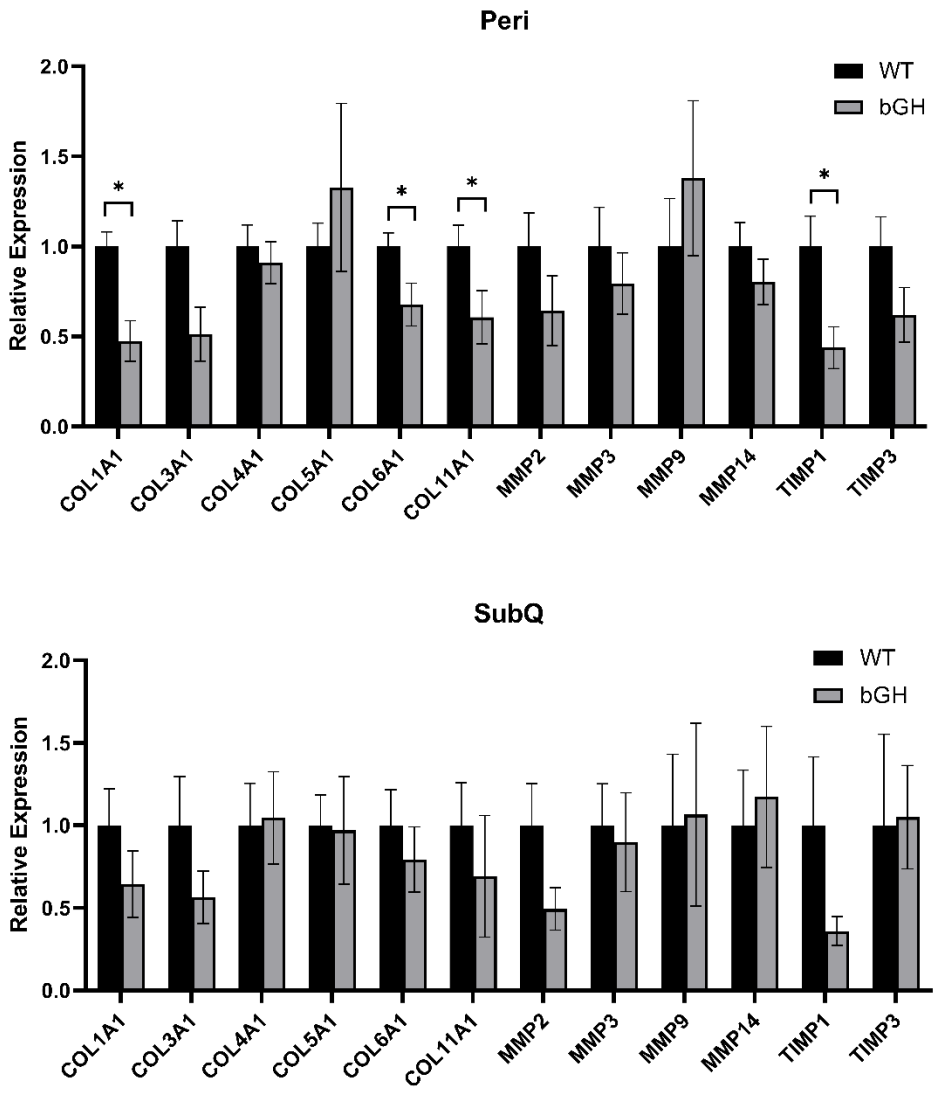


Figure 5) Relative mRNA expression in the SVF of perigonadal visceral (top) and subcutaneous (bottom) WAT in 3-month-old male WT and bGH mice (n=5) using modified Pfaffl analysis.

### *Single Nuclear Sequencing Results*

Through previously generated data from single nuclear sequencing, the RNA expression in multiple cell types was examined. Analysis was performed on the same

genes that were investigated for qPCR analysis: *Col1a1, Col3a1, Col4a1, Col5a1, Col6a1, Col11a1, Mmp2, Mmp3, Mmp9, Mmp13, Mmp14, Timp1,* and *Timp3.* The cell types identified include adipocytes, two subpopulations of adipose stem and progenitor cells, two subpopulations of B cells, dendritic cells, endothelial cells, macrophages, T cells, and three unidentified groups denominated "other cells 1," "other cells 2," and "other cells 3."

Of the twelve cell groups that were distinguished, only the cells labeled "other cells 3" showed significant differences in gene expression between the inguinal subcutaneous WAT of WT and bGH mice. In this group of cells, the gene expression of *Col1a1, Col3a1, Col4a1,* and *Col5a1* is significantly higher in bGH mice than littermate controls (Figure 6). Additionally, the gene expression of *Mmp14* is higher in bGH mice compared to littermate controls (Figure 7).

When the unidentified cells were analyzed more closely, it was revealed that most of the cells were mouse adipose stem and progenitor cells (ASPCs), specifically showing gene expression that correlated with mASPC4 cells (Figure 8).

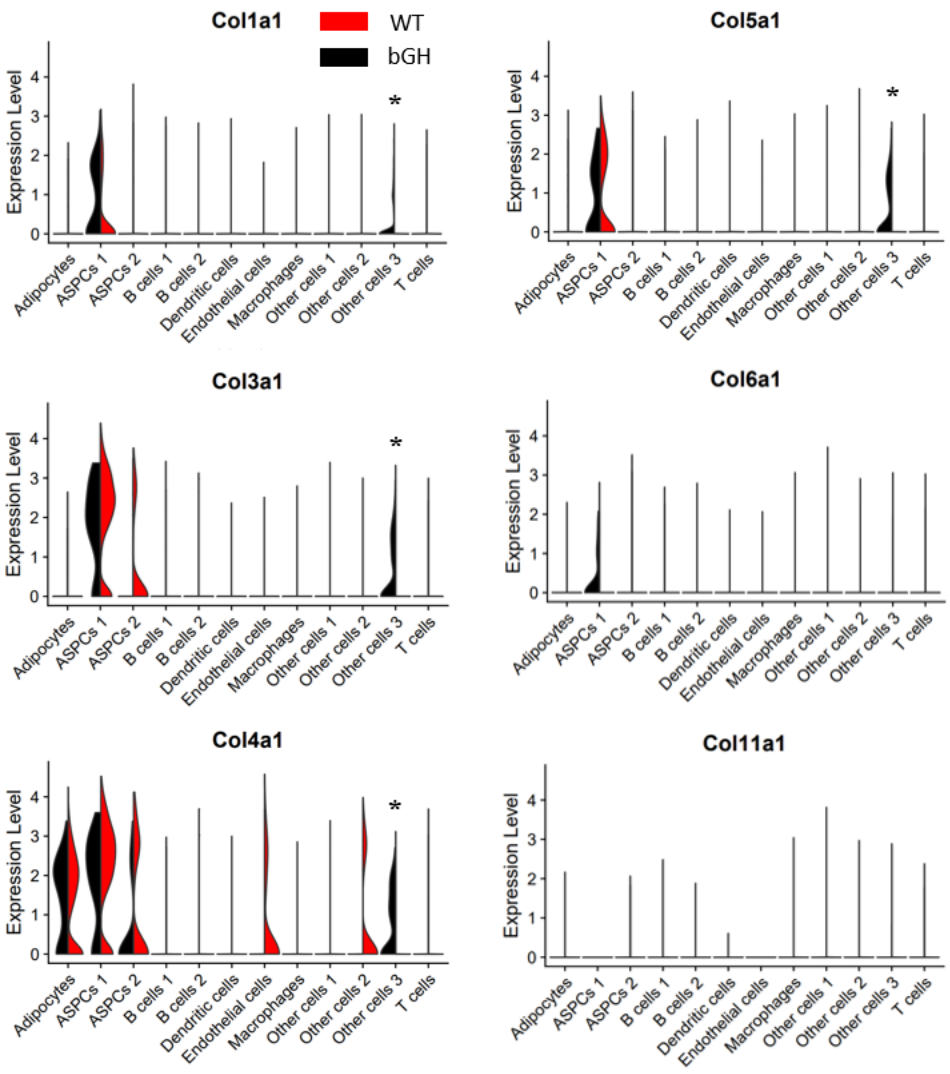


Figure 6) Violin plots showing cell type specific expression level of various collagen genes in subcutaneous inguinal WAT of 6-month-old male bGH and WT mice (n=3). Cell group "other cells 3" demonstrated significantly higher expression in genes *Col1a1*, *Col3a1*, *Col4a1*, and *Col5a1* in bGH mice than in WT mice.

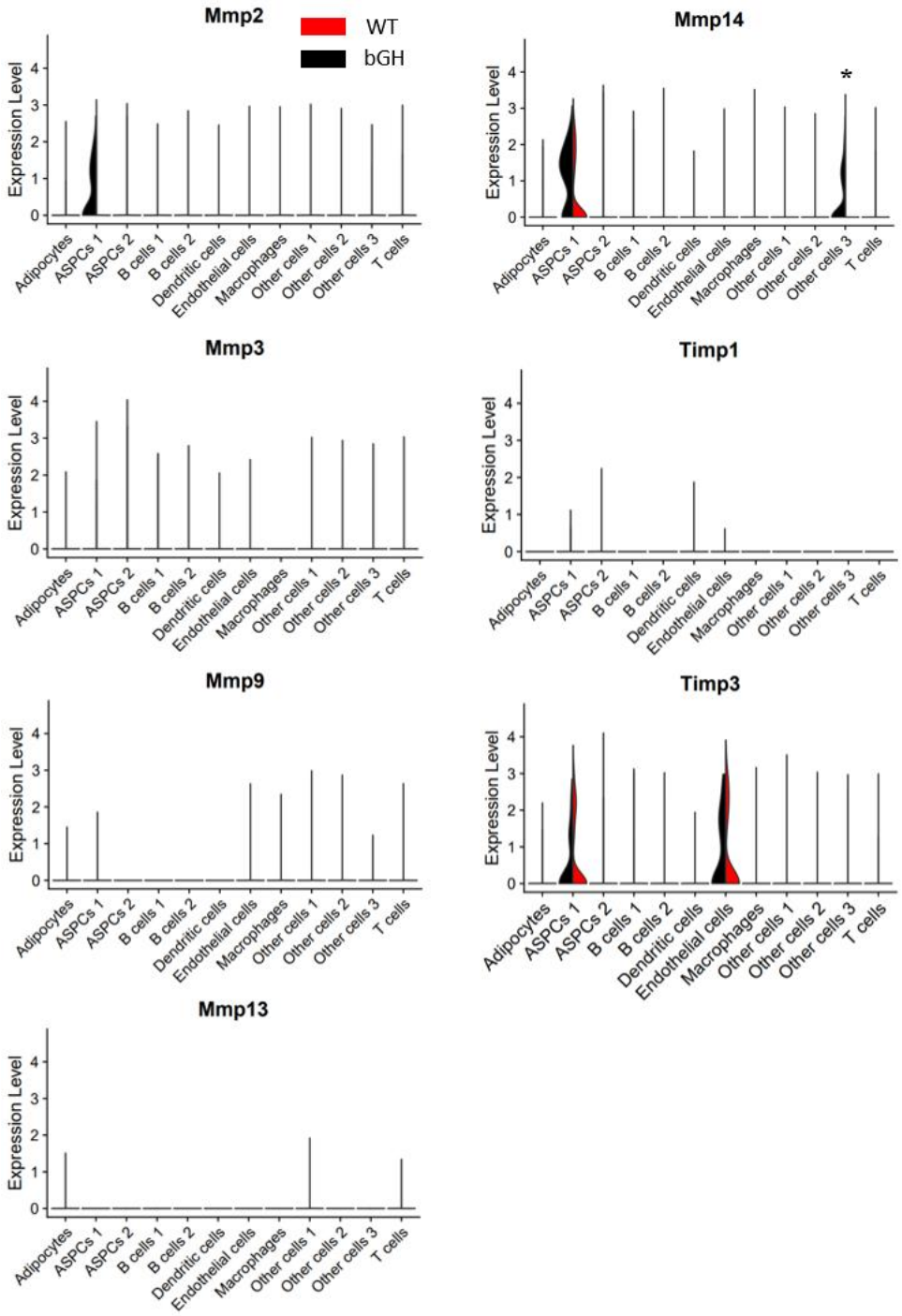


Figure 7) Violin plots showing cell type specific expression level of MMP and TIMP genes in subcutaneous inguinal WAT of 6-month-old male bGH and WT mice (n=3). Cell group “other cells 3” demonstrated significantly more *Mmp14* expression in bGH mice than in WT mice.

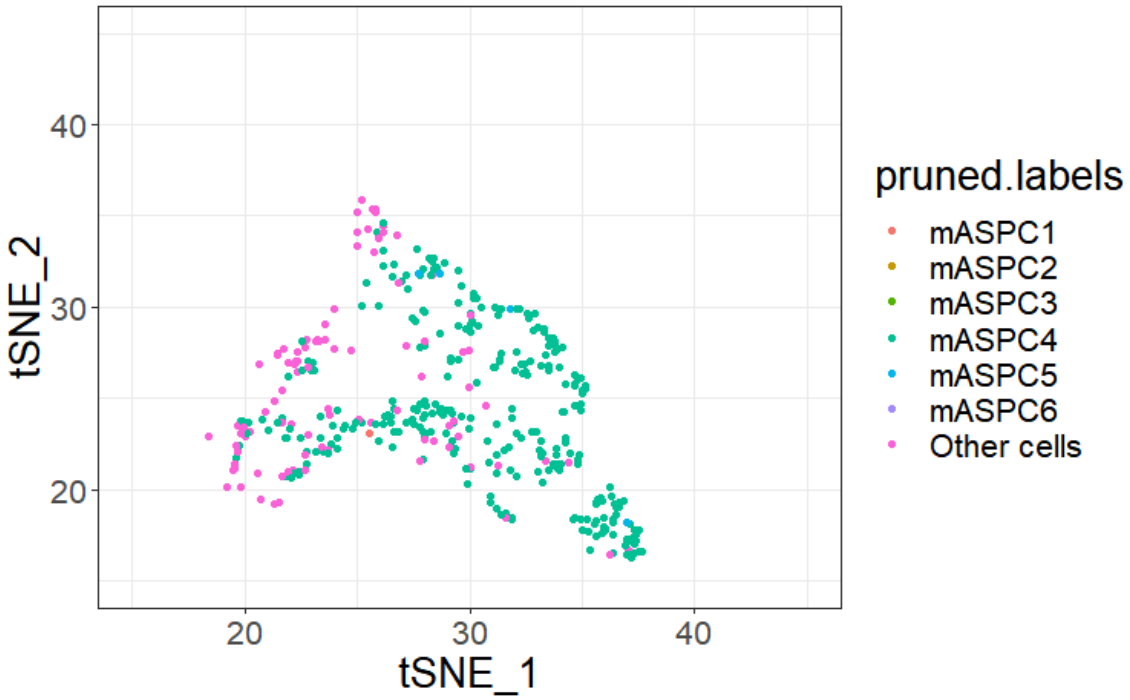


Figure 8) Further analysis of cell type with significant changes in collagen and MMP gene expression referred to “other cells 3” in figures 6 and 7.

***Collagen Staining and Quantification Results***

Various tissues [brain, heart, BAT, liver, skeletal muscle (quadriceps), kidney, lung, and spleen] from bGH and WT mice were stained with picrosirius red and fast green, and then percent picrosirius red stained area was quantified. Brain tissue did not tend to pick up any picrosirius red stain, so it was excluded from the final analysis. Additionally, heart showed inconsistent staining, so it was also excluded from the final analysis. Qualitative increases in red staining were visible in bGH BAT, liver, quad, kidney, lung, and spleen compared to WT tissues. When quantified, the BAT, liver, quad, kidney, lung, and spleen of bGH mice showed a significant increase in percent picrosirius

red stained area (Figure 9). These findings are consistent with the qualitative data obtained from image observation.

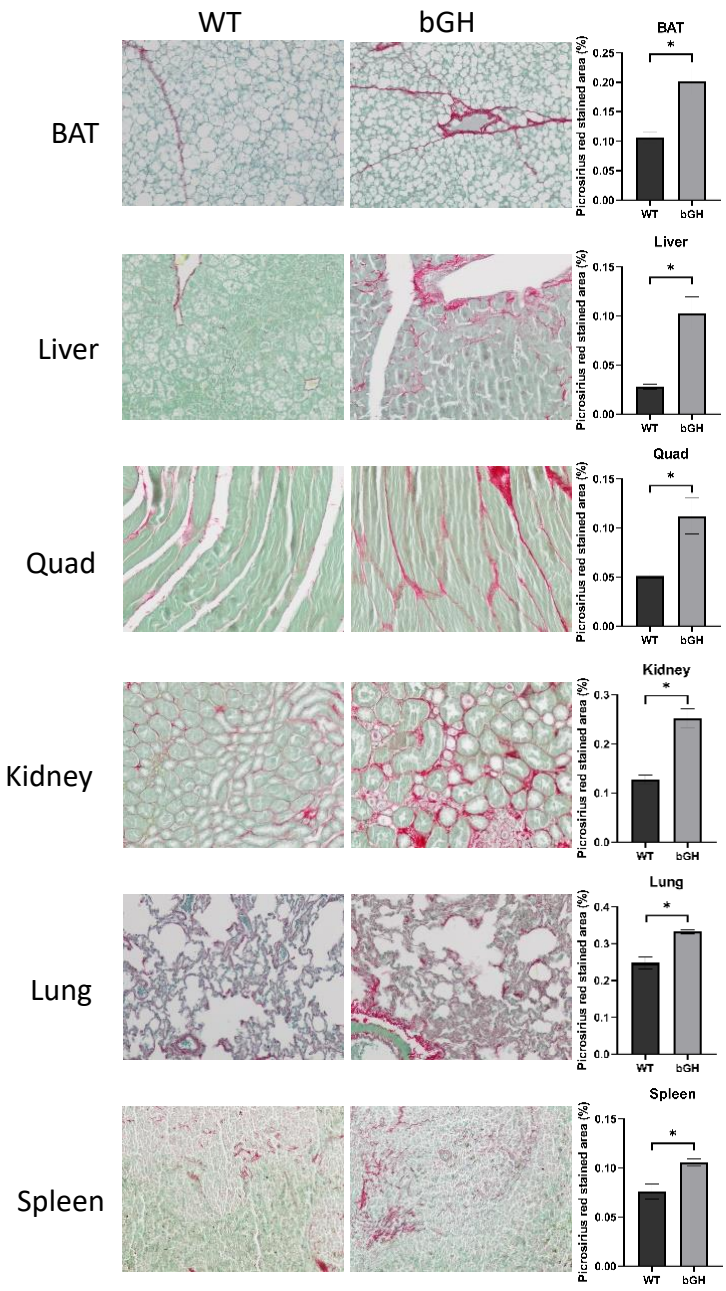


Figure 9) Images of various tissues from 13-month-old male bGH and WT mice (n=4) stained with picrosirius red and fast green at 200X magnification. The BAT, liver, quadriceps, kidney, lung, and spleen of bGH mice had significantly increased picrosirius red stained area (%) compared to these tissues in WT mice.

***Hydroxyproline Results***

Hydroxyproline was quantified in the liver and lung of older male bGH and WT mice to determine the relative amount of collagen in the tissues. In liver, 6 WT mice had optical density (OD) values below the lowest concentration of the standard curve, suggesting that these samples need to be re-analyzed. Thus, our sample size for hydroxyproline concentration in the liver of WT mice decreased to n=4. Both the liver and the lung of bGH mice showed significant increases in hydroxyproline concentration when compared to tissues from littermate controls (Figure 10).

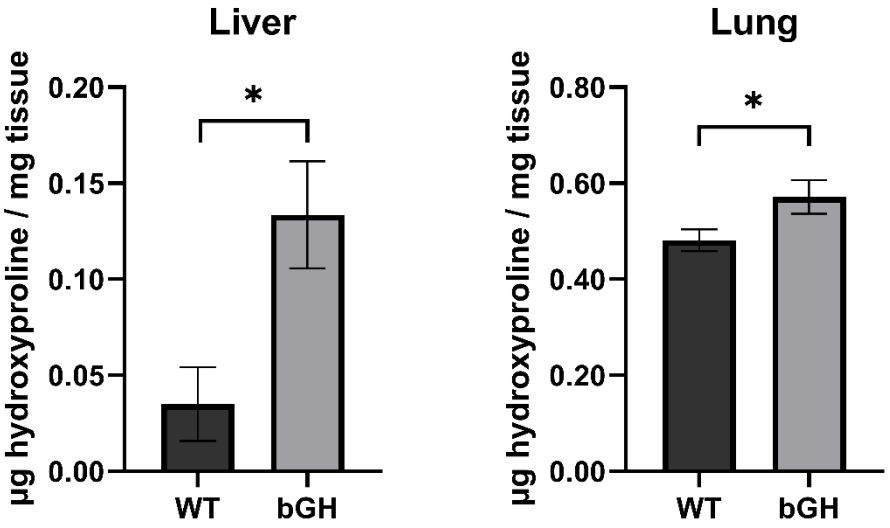


Figure 10) Hydroxyproline quantification in liver (n=4) and lung (n=10) of 11-15-month-old bGH and WT mice. Both liver and lung showed increased hydroxyproline concentration in bGH mice compared to WT mice.

## Discussion

The data collected and analyzed for this thesis project provide us with new information on the effects of GH on mouse tissues. The most striking finding was that each bGH tissue examined showed increased fibrosis compared to WT mice, which supports the conjecture that fibrosis is a common feature of the excess GH action in bGH mice. These data were supported by the higher hydroxyproline concentration in the liver and lung of 11-15-month-old bGH mice than in the same WT tissues. Despite the visualized and quantified evidence of fibrosis in old bGH tissues, the visceral WAT of younger mice demonstrated a decrease in RNA expression of certain collagen genes as well as a TIMP gene. To make this story even more complex, when single nuclear sequencing was performed on 6-month-old mice, some collagen genes were found to be expressed more in the subcutaneous WAT of bGH mice than in that of WT mice – but this differential expression was only present in a cell population that is a mixture of cell types that predominantly includes "ASPC4" cells. Ultimately, there are still many unanswered questions regarding the mechanisms through which fibrosis develops in organisms with excess GH, and our goals for future research will be addressed later in this section.

Our qPCR results did not show an increase in collagen gene expression. That is, the 3-month-old bGH mice showed decreased RNA expression of certain collagens and *Timp1* in the visceral WAT depot compared to WT mice. More specifically, *Col1a1* and *Col6a1* had decreased expression in visceral WAT of bGH mice in both types of analysis, while *Col3a1 and Col11a1* showed decreased expression in bGH mice in one of the two analyses. Note that while the two types of analyses do not agree with respect to

expression of all collagens, both types of analyses show no increase in collagen gene expression. Typically, we would expect to see an increase in collagen RNA expression in a tissue that is fibrotic, as collagen is the primary protein component of the ECM.[1] Thus, it is surprising that genes responsible for collagen production would have downregulated expression levels in bGH mouse RNA where fibrosis has repeatedly been shown to be evident.[103,113]

Our results in younger mice are similar to a study published in 2018 that reported decreases in collagen RNA expression (specifically, *Col1a1, Col3a1, Col4a1,* and *Col5a1)* in subcutaneous WAT in 6-month-old bGH mice.[103] This 2018 study had a few potential explanations for increased tissue fibrosis with simultaneous decreases in fibrosis-associated gene expression: 1) analysis of whole tissue may have masked gene expression changes in a subset of cells, 2) chronic exposure to excess GH could result in tissue dysfunction by 6 months, resulting in decreased expression by that age, and 3) finally, GH could affect post-translational modification of collagens. Collagens require posttranslational processing with at least nine specific enzymes to ensure proper folding into a triple helix and cleaving.[114] Because we isolated the SVF of WAT before performing qPCR, it is less likely that whole tissue is masking expression changes in a subset of cells than when whole tissue is analyzed. However, the SVF is comprised of many cell types, so it is still possible that there are collagen gene expression changes in various cellular subsets of the SVF that influence or mask the expression of one another. The SVF includes cell types such as endothelial cells, smooth muscle cells, fibroblasts, pericytes, immune cells, and adipose-derived stem cells.[20] However, there is no consensus on the proportions of these cell types.[20] In addition, we were unable to

successfully analyze adipocyte RNA expression, so there could have been important gene expression changes in that cell population. Potential cell types involved in fibrosis-associated gene expression will be explored more when single nuclear sequencing results are discussed. Additionally, we used 3-month-old mice in contrast to the previous paper's 6-month-old mice, and still observed decreases in collagen expression in bGH WAT. These findings indicate that either 1) the fibrotic cascade and increased collagen gene expression is initiated prior to 3 months of age or 2) that genes/proteins involved in collagen turnover may be more important than collagen gene expression. However, it is important to note that the MMPs tested do not show a decrease in expression as might be expected if collagen turnover is decreased. This leaves the hypothesis that GH affects post-transcriptional modification of fibrosis-associated genes (specifically collagens, MMPs, and TIMPs), resulting in discrepancies between the amount of fibrosis in bGH WAT and fibrosis-associated RNA expression. The possibility that post-translational modifications are responsible for increases in collagens in bGH WAT is likely. Supporting this is the finding that the overexpression of endotrophin, which is a C-terminal cleavage product of collagen, results in increased adipose tissue fibrosis in mice fed with a high fat diet.[48] Of course, there is also the possibility that gene expression does not correlate with protein levels; however, other reports have shown that collagens are controlled at the transcriptional level.[115]

According to the qPCR analysis data, visceral WAT was impacted more by GH in terms of collagen gene expression than subcutaneous WAT. More specifically, the visceral WAT depot bGH RNA expression of specific collagens decreased significantly, while there were no significant changes in gene expression in the subcutaneous depot.

This is surprising, considering that the subcutaneous depot of bGH mice has more significant fibrosis than visceral WAT in both male and female bGH mice.[103,113] Further, GH has been shown to alter many characteristics of subcutaneous WAT more dramatically than visceral WAT, such as immune cell infiltration, adipocyte cell size, adipokine secretion, progenitor cell differentiation/proliferation, gene transcription, and senescence.[116] As for fibrosis, the 2018 Householder study found a decrease in RNA expression of select collagens in only the subcutaneous depot of bGH mice, with no significant changes in visceral WAT.[103] Also, adipocyte-specific GH receptor knockout mice[80] and global GH receptor knockout mice[81] show more significant depot changes in subcutaneous WAT. Specifically, the adipocyte-specific GH receptor knockouts have a more profound increase in subcutaneous depot mass than visceral depot mass and display decreases in picrosirius red stained collagen in only the subcutaneous depot.[80] GH gene-disrupted (GH -/-) mice have enlarged subcutaneous WAT depots, but decreased perigonadal depot mass compared to controls (this decrease is not significant when perigonadal mass is normalized to body mass).[81] In addition, GH -/- mice, like the adipocyte-specific GH receptor knockout mice, have decreased picrosirius red stained collagen only in the subcutaneous depot.[81] However, most of this prior research has only observed these depot-specific effects in 6-month-old mice or older mice. Bell et al (2023) does report qPCR data from 3-month-old mice in whole WAT tissue. This study reports an increase in *Col3a1* RNA expression in bGH visceral perigonadal WAT, but not in subcutaneous WAT.[113] The same paper also demonstrates that cultured visceral perigonadal WAT treated with bGH from 3-month-old female mice – so an acute bGH treatment – exhibits the most prominent increases in RNA levels of collagen genes.

However, the next most affected ex vivo depot is subcutaneous WAT from male mice. Overall, data on the effects of excess GH on 3-month-old mice is inconclusive and warrants additional investigation.

Two methods were used to analyze qPCR data: delta-delta Ct analysis[108] and a modified Pfaffl analysis.[109] The modified Pfaffl analysis method uses gene-specific amplification efficiencies and accounts for uncertainty on the estimated amplification efficiencies,[109] while the delta-delta Ct analysis method assumes 100% amplification efficiencies.[108] In addition, delta-delta Ct analysis relies on the assumption that the amplification efficiencies of the target and reference genes are approximately equal.[108] For these reasons, the modified Pfaffl analysis method probably provides more accurate determinations of relative RNA expression for this research.

An increase in collagen gene expression is only seen in a specific cell type in bGH WAT via single nucleus sequencing as shown in this thesis. Unfortunately, this cell type was not identified using the method for cell identification outlined in the methods.[110] Upon closer inspection of the data within this cell cluster, it appears that this group of cells has a gene expression profile similar to adipose stem and progenitor cells (ASPCs). Most of the unidentified cells in this cell cluster are mASPC4 cells, which may be CD142+ adipogenesis regulatory cells that inhibit adipogenesis.[110,117] ASPC4 cells in humans are more prevalent in subcutaneous adipose tissue than in visceral adipose tissue; however, they are also found in a higher proportion in people with BMIs > 40, which is inconsistent with the lean bGH phenotype.[110] However, high BMI is also associated with increased fibrosis in WAT;[34,35] thus, this cell type may be a critical player in initiating the

fibrotic cascade in both obese and lipodystrophic states. Further investigation will be done into this cell type.

One of the most exciting findings from this study was that the bGH mice have increased fibrosis in all tissues assessed. This suggests a common feature of excess GH that likely contributes to the early death of bGH mice. It is well-established that GH-transgenic mice have drastically shortened lifespans compared to controls from the same mouse line.[118,119] bGH transgenic mice have a lifespan of 12-18 months, and typically die due to heart, liver, and kidney problems.[120]

bGH mice, but not mice that overexpress human IGF-1, develop glomeruli with mesangial (mesangial cells form the vascular pole of the renal corpuscle) hypercellularity and eventually experience progressive glomerulosclerosis, which is a result of increased ECM.[121] We are able to qualitatively observe these enlarged glomeruli in the images of picrosirius red and fast green stained bGH kidney. GH transgenic mice also have sclerotic kidney lesions, contributing to the renal abnormalities that are thought to be the primary cause of shortened bGH lifespan.[122] Because these changes do not appear in the presence of excess IGF-1 alone, kidney fibrosis is likely a result of direct GH action on the tissue. In addition, excess GH induces Notch1 signaling in podocytes, and the inhibition of Notch1 prevents GH-induced glomerular fibrosis.[123] Also, there is a strong body of evidence that supports the role of GH in epithelial-mesenchymal transition (EMT) (for example, bGH transgenic mice have a twofold increase in integrin-linked kinase (ILK) mRNA in their podocytes,[124] and ILK negatively regulates P-cadherin and activates $\beta$-catenin,[125] which are changes consistent with EMT).[126] Although, EMT has a debated

role in the development of kidney fibrosis.[127,128] Overall, GH appears to have direct actions on the kidneys, which may result in the fibrosis that we observe in bGH mice.

Another tissue that is significantly altered in bGH mice, potentially leading to their short lifespans, is liver. Chronic exposure to excess GH in mice is associated with hepatomegaly (due to both hypertrophy and hyperplasia) and liver inflammation, and GH-transgenic mice (but not IGF-1 transgenic mice) develop hepatocarcinogenesis.[129] A 1989 study observed that bGH mice have sclerotic hepatic lesions that progress with age, and transgenic mice expressing high levels of IGF-1 do not have similar lesions, suggesting the possibility that GH action alone results in these fibrotic lesions.[122] Interestingly, non-alcoholic fatty liver disease (NAFLD), a fibrotic disease, may be associated with low levels of GH.[130] Hepatic steatosis is more frequently observed in patients with GH deficiency than in patients without GH deficiency, but it is theorized that fibrosis in NAFLD is due to the metabolic syndrome associated with low levels of IGF-1, which would be consistent with low serum GH.[131] This implies that the direct actions of GH on the liver could affect fibrosis differently.

Increased collagen deposition was also present in other bGH tissues that have been less well studied with respect to fibrosis. For lung, no studies appear to assess the direct role of GH in pulmonary fibrosis. However, a growth hormone-releasing hormone receptor antagonist, which would decrease GH secretion, reduces lung fibrosis in mice.[132] While these mice present an opposite condition to bGH mice, the relationship between GH and tissue fibrosis is consistent with our findings. Both acute and chronic treatment of GH has been shown to increase collagen expression in skeletal muscle;[98] however, the ability of this increased collagen expression to promote fibrosis has not been assessed.

We could find no papers that examined the relationship between GH and fibrosis in spleen or BAT. Overall, little research exists that investigates the relationship between GH and tissue fibrosis, but the research that is available comes to similar conclusions to this thesis: GH has a significant, positive impact on fibrosis across various tissues.

### *Future Directions*

There are many possible paths that future research can take towards understanding the cell types, genes, and pathways involved in GH-induced fibrosis. First, we will further investigate the "other cells 3" cell population that we now believe to be comprised of ASPCs. Also, we could look at mice that are even younger than 3 months of age to determine if there are changes in the RNA expression of fibrosis-associated genes that may explain the discordance between WAT fibrosis and gene expression that is apparent in 3- and 6-month-old bGH mice. Tissues with increased fibrosis in bGH mice (BAT, liver, quad, kidney, lung, and spleen) could be analyzed for fibrosis-associated gene expression, as this may provide us with hints as to why we see decreases in these genes in WAT.

To determine whether specific fibrosis-associated genes undergo post-translational modifications, western blots could be performed. More precise methods for measuring post-translational modifications are still being developed, but strategies to detect target proteins and their post-translational modification isoforms exist. For example, there is an approach to detect proteins and their post-transcriptional modification isoforms in single cells.[133]

The picrosirius red and fast green staining of mouse heart was unsuccessful, but the heart is known to be significantly altered in bGH mice. For example, bGH mice have myocardial hypertrophy, impaired cardiac insulin signaling, increased heart mass, impairment of systolic function, and increased interstitial fibrosis.[134,135] Because GH appears to have such an important impact on the heart, as seen in people with acromegaly (who are more likely to experience cardiomyopathy and heart failure),[136] it is important that we accurately visualize fibrosis in bGH heart tissue. Sometimes, yellow myocyte staining occurs when cardiac tissue is stained with picrosirius red.[137] The stained myocytes can obscure the collagen fibers, and there is a method to eliminate cytoplasmic staining that we could use to more clearly demonstrate fibrosis presence in heart tissue.[137] There are also cardiac tissue-specific picrosirius red staining kits that would assist in the visualization of collagen fibers.

Picrosirius red and fast green staining of mouse brain was also unsuccessful. Reasons for this are unclear, as collagens are important and present in the vertebrate nervous system.[138,139] There also does not seem to be discussion of brain-specific problems with picrosirius red and fast green staining in the literature. We may attempt to stain brain tissue again in the future.

In the future, we will continue to examine the impacts of GH on fibrosis through further analysis of cell types, gene expression, and tissue histology. This future work aims to further our understanding of the mechanisms through which fibrosis develops due to GH action.

***Potential Impacts***

Studies estimate that 45% of deaths in the United States can be attributed to fibrotic disorders such as pulmonary fibrosis, liver cirrhosis, and cardiovascular disease.[12] Thus, there is an urgent need to discern the contributing factors to fibrosis in individual tissues, to develop effective antifibrotic agents, and to identify suitable biomarkers to monitor fibrosis. Through improving our understanding of the ways in which fibrosis arises and progresses, we will get closer to preventing and potentially reversing its development.

Acromegaly is associated with fibrosis, with most studies focusing on the fibrosis in the myocardium.[140] Although the role of fibrosis in mortality of patients with acromegaly is still unclear, it is likely that GH is involved in its development. Myocardial fibrosis is associated with cardiomyopathy, a heart muscle disease that is often seen in people with acromegaly.[87] Because of the relationship between excess GH and fibrosis in the heart, it is crucial to better understand the role that GH may play in promoting fibrosis in other tissues to ensure improved health outcomes for patients with acromegaly. As sampling multiple tissues in patients with acromegaly is not desirable or feasible, our bGH mice will be used instead to assess fibrosis across multiple tissues.

Finally, GH is used therapeutically to treat growth hormone deficiency, poor growth due to renal failure, and other conditions resulting in insufficient growth.[141] It is also abused voluntarily for multiple reasons, including as a means to reverse effects of ageing and to improve athletic performance. Currently, there is not sufficient evidence to suggest that excess GH has beneficial effects on the body. Supplementing GH has not been shown to improve athletic performance (though it has become a target of abuse in

athletes), and muscles in people with acromegaly are larger but weaker than in people with typical GH levels.[142] In fact, prolonged exposure to excess GH is associated with increased mortality.[143] To minimize unnecessary harm in people who choose to supplement GH, it is critical that we continue to pursue knowledge surrounding the effects of this hormone on the body, such as its role in promoting tissue fibrosis.

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