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Interplay Of The Circadian Clock And Growth Hormone Signaling In The Heart

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ABSTRACT

Growth hormone (GH) signaling plays a critical role in postnatal development and the details of its release, regulation, and influence on the development of a number of organs has been well studied. GH is released from the anterior pituitary in a pulsatile fashion with pulse amplitude being greater at night than during the day in a number of species, including humans. Once in circulation, GH binds to the GH receptor (GHR) on target tissues throughout the body altering gene expression and stimulating the local production of insulin-like growth factor 1 (IGF-1). IGF-1 primarily acts in an autocrine and paracrine fashion. Interestingly, after roughly a century of GH research, comparative tissue sensitivity to a GH pulse has yet to be described. The work herein encompassed provides a comparative description of GH sensitivity and responsiveness of five key metabolic tissues in mice (heart, liver, kidney, epididymal white adipose tissue, and skeletal muscle).

We then home in on the heart and the influence of circadian rhythms. It is well known that a number of mechanisms and pathologies affecting the heart occur in a time-of-day dependent manner including normal nightly dips in blood pressure and increases in GH secretion and increased incidence of acute myocardial infarctions in the early mornings; these processes are influenced by the circadian clock. The cardiomyocyte-specific BMAL1 knockout (CBK) mice have a functional central clock in the suprachiasmatic nucleus and functional peripheral clocks everywhere but

cardiomyocytes. These animals experience altered metabolic processes, including insulin sensitivity, early age onset heart failure, and early mortality. It has also been suggested that the cardiomyocyte circadian clock influences GH related signaling processes. Here, we describe altered time-of-day dependent GH sensitivity, and other signs of increased GH signaling in CBK hearts including elevated igf-1 gene expression. We also introduce and characterize the cardiomyocyte-specific BMAL1$^{(-/-)}$ IGF-1$^{(-/+)}$ (CBKI)- Cre(+) mouse in an attempt to partially rescue the cardiac hypertrophy phenotype of the CBK mice.

Key words: GH sensitivity, circadian, CBK, cardiac, IGF-1, BMAL1

DEDICATION

To my parents Darrell R. and Katherine D. Berry, my wife Christine, my daughters, Ana and Emma, my siblings Tyler, Meagan, Trevor, and Kevin, and my in-laws, Robert M. and Pamela Norris for their unfailing support of me and my family throughout this entire process.

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INTRODUCTION

OVERVIEW

This body of work involves three distinct fields of study whose overlap forms a niche in the scientific world. Hence, an understanding of all three fields is necessary in order to fully appreciate the data herein contained. First, we will review growth hormone (GH) and insulin-like growth factor 1 (IGF-1) signaling, followed by the critical role of circadian rhythms in health and disease, and lastly heart failure (HF) and the mouse model in which these three fields overlap. Following the introduction of these fields, Chapter 1 will present cardiac GH dose responsive signaling in the context of the whole body, specifically in relation to other metabolically relevant tissues. Chapter 2 will then focus in on the overlap of GH/IGF-1 signaling, circadian biology, and heart failure found in the cardiomyocyte-specific Bmal1 knockout (CBK) mouse.

GROWTH HORMONE SIGNALING

Brief History Of Growth Hormone

The term “hormone” was first coined in the early 1900’s by Earnest H. Starling following the discovery of secretin [1]. This concept allowed for the discovery of GH in 1922 when Evans and Long reported increased growth of rats receiving intraperitoneal injections of homogenized cow pituitary glands[2]. The field of GH research was first propelled forward in 1944 by the isolation of bovine GH [3] and then again in 1969 and

1970 when the same group solved the primary structure of and subsequently synthesized human GH [4, 5].

Components of Growth Hormone Signaling

Growth Hormone

GH is a 22 kDa peptide hormone consisting of 188 amino acids including a single tryptophan residue [5]. Produced in somatotrophic cells of the anterior pituitary, it is secreted into circulation where it influences postnatal growth in tissues throughout the body. GH secretion is primarily controlled and modulated by GH releasing hormone (GHRH), somatostatin, and ghrelin.

Growth Hormone Releasing Hormone

GHRH is a short peptide hormone consisting of only 44 amino acids and is produced in neurons located in the arcuate nucleus of the hypothalamus. These neurons send axonal projections to the median eminence where they release GHRH, in a pulsatile fashion, into a capillary system fed by the superior and inferior hypophyseal arteries. GHRH travels to the anterior pituitary via the hypothalamic-pituitary portal vessels where it binds the GHRH receptor (GHRHR), a G_s-protein coupled receptor, on somatotrophic cells, stimulating the production of 3',5'-cyclic adenosine monophosphate (cAMP) and subsequent GH production and release [6]. GHRH also stimulates the expansion of the somatotroph lineage during development and retains its ability to stimulate somatotroph proliferation throughout adulthood [7].

Somatostatin

Somatostatin was discovered at the same time as GHRH when it was isolated from the hypothalamic-pituitary portal vessels[8]. There are two active forms of the peptide, somatostatin-14 and -28, consisting of 14 and 28 amino acids respectively. Somatostatin-14 is the predominant form produced in the brain. The role of somatostatin in hypothalamic-pituitary signaling is as an inhibitor of GH secretion; it does not, however, inhibit the production and accumulation of GH in somatotrophs. The effect of somatostatin is relatively short-term, with a half-life of ~2 minutes. In addition to countering the effects of GHRH on GH secretion, there is also evidence that somatostatin directly binds to GHRH containing neurons in the arcuate nucleus to inhibit GHRH release[8]. Furthermore, somatostatin has been implicated as a contributor to the pulsatile pattern of GH secretion [8].

Ghrelin

The discovery of ghrelin occurred in an atypical fashion in that pharmacological agents were in use and the receptor discovered before the endogenous ligand [9, 10]. Ghrelin is a 28- amino acid peptide hormone with one of its serine residues acylated with n-octanoic acid and is produced primarily by the X/A-like cells of the oxyntic gland located in the fundus of the stomach. Despite their location in the oxyntic gland, the X/A-like cells are closely associated with a capillary network suggesting secretion of ghrelin into the circulation[9]. The exact mechanism controlling ghrelin release has not yet been delineated; nevertheless, it has been shown that mRNA and circulating ghrelin levels are increased in fasting, starving, hypoglycemia or leptin administration and states of

malnutrition such as anorexia nervosa[11-13]. In contrast, food intake lowers ghrelin levels[14], and in conditions representing a positive energy balance, such as obesity, ghrelin mRNA expression and circulating ghrelin levels are very low [9].

Ghrelin acts on G-protein coupled receptors, GHS receptors, in the arcuate nucleus of the hypothalamus and the somatotroph cells of the anterior pituitary to stimulate GH release[9]. While ghrelin is a more potent stimulator of GH release than GHRH[15], it appears that the GHRH receptor (GHRHR) plays a permissive role and must be functional to observe the GH stimulatory effects of ghrelin [9, 16]. Despite ghrelin's signaling dependence on the GHRHR, somatostatin shows little inhibitory effect on ghrelin's ability to stimulate GH release, but is a powerful inhibitor of ghrelin secretion [9, 17].

Growth Hormone Receptor And Its Regulation

Once in circulation GH binds the GH receptor (GHR) on target tissues to activate signaling and somatogenesis. GHR was cloned in 1987 [18] and was found to contain 638 amino acids, and individual extracellular, transmembrane, and cytoplasmic domains (Figure 1 and Figure 2) [18]. A member of the cytokine/ hematopoietin receptor superfamily, GHR shares a limited sequence homology with receptors for prolactin (PRL), multiple interleukins, erythropoietin, and leptin [19]. GHR is initially synthesized as individual monomers, but exists, at least partially, as ligand independent homodimers, which are formed in the endoplasmic reticulum prior to arriving at the cell surface [20, 21]. Activation of GHR occurs when GH forms a high affinity bond to a monomer of the GH receptor followed by binding of another face of the same GH molecule to the second

monomer thereby stabilizing the GHR homodimer [19, 22]. Binding of GH to the GHR homodimer results in conformational changes, which enable the activation of two non-receptor Janus kinase 2 (JAK2) molecules associated with Box1 of the GHR intracellular domain [23, 24]. However, there is somewhat conflicting information regarding the exact nature of these conformational changes, specifically whether the intracellular domains of the homodimer pair separate or come closer together upon GH binding [21, 22, 25]. The activated JAK2 molecules phosphorylate the intracellular domain of GHR providing docking sites where downstream signaling molecules may bind and become phosphorylated by JAK2 [26, 27].

The role of JAK2 in GHR signaling cannot be downplayed. In addition to phosphorylating the intracellular domain of GHR and downstream signaling molecules, it also plays a critical role in the stabilization of the receptor by extending the half-life of the mature GHR [28, 29]. Conversely, JAK2 phosphorylation of GHR intracellular domain also increases GH-induced GHR down-regulation and degradation via lysosomal/endosomal degradation of mature GHR [26, 27, 29].

GHR and JAK2 are involved in signal activation, via phosphorylation of a number of different downstream pathways including at least 4 members of the signal transducer and activator of transcription (STAT) family (STAT 1, 3, 5a, and 5b) [19]. Other pathways include the RAS- mitogen activated protein (MAP) kinase pathway via phosphorylation of Src homology 2 domain containing (SHC) protein, and the PI3 kinase pathway via phosphorylation of insulin receptor substrates (IRS 1, 2, and 3) [19, 30]. The primary signaling pathway activated by GHR, however, is the JAK2/STAT5 pathway.

Signaling at GHR is subject to regulation via multiple mechanism including other receptors and negative feedback inhibitors. The insulin-like growth factor 1 (IGF-1) receptor (IGF-1R) is a hetero-tetromeric receptor with a tyrosine kinase located on the cytoplasmic domain. IGF-1R forms a GH dependent complex with GHR and JAK2 that is not dependent on tyrosine phosphorylation of GHR. Furthermore, activation of GH dependent gene expression is augmented synergistically in the presence of both the GH and IGF-1 ligands[31]. IGF-1R serves to physically block the interaction of protein tyrosine phosphatase 1B (PTP-1B) with the GHR intracellular domain and deletion or reduction of IGF-1R expression results in attenuation of acute STAT5 phosphorylation [31-35].

GHR also forms a complex with the PRL receptor (PRLR)[36]. However, in contrast to IGF-1R, the presence of PRLR tends to reduce GH activation of GHR, and when PRLR abundance is reduced, GHR abundance increases along with its sensitivity to GH[37]. An important caveat in this case is the ability of human GH, but not GH derived from other species, to bind to and activate signaling through both GHR and PRLR [36-40].

GHR is subject to regulation via constitutive and inducible proteolytic cleavage via the metalloproteases TNF-α converting enzyme (TACE) and α disintigrin and metalloprotease 10 (ADAM10). TACE and ADAM10 cleave off the extracellular GHR domain, creating a soluble protein fragment referred to as the GH binding protein (GHBP) and a cell-associated cytoplasmic domain GHR fragment. In this case, JAK2 also plays a protective role by preventing proteolysis of GHR [41].

The Suppressors of cytokine signaling (SOCS) protein family plays an important role in negatively regulating cytokine signaling including at GHR. The SOCS family consists of eight members, cytokine-inducible SH2 protein (CIS), and SOCS 1-7. In general, these proteins act by inhibiting the same signaling pathway that stimulated their expression in a classical negative feedback loop [42]. SOCS2 expression is stimulated by phosphorylated STAT5b and is the family member responsible for suppressing signaling at GHR. The mechanism by which SOCS2 inhibits GHR signaling may be two-fold in nature. First, it binds to two phosphorylated tyrosine residues on the intracellular GHR tail and physically blocks the association of positive regulators such as STAT5 and JAK2, and secondly it acts as a member of an ubiquitin ligase complex targeting GHR and interacting proteins for degradation [43]. Animals lacking SOCS2 display a phenotype similar to animals overexpressing GH [44-46].

Insulin-Like Growth Factors

Insulin-like growth factor 1 (Igf-1, also called Somatomedin C) is among the many genes whose expression is positively regulated by STAT5b. A peptide hormone made up of 70 amino acids, it binds to the IGF-1 receptor (IGF-1R) to promote somatogenesis and other metabolic effects [47]. Its family member, IGF-2, is a single chain polypeptide made up of 180 amino acids, which binds to the IGF-1R with similar affinity as IGF-1. IGF-2 also binds to the IGF-2R, which acts to inhibit cellular responses to IGF-2 [48]. The Igf-1 gene consists of 80 kb of genomic DNA in humans[49] and its sequence is highly conserved in mammals[49]. Within the gene there are 6 exons and multiple splice combinations resulting in different precursor peptides which are

subsequently cleaved to produce the same mature IGF-1 peptide [49]. The different precursor peptides differ in their signal peptide, of which there are two classes and the terminal peptide (E) fragment, of which there are 3 classes, A, B, and C. Class 1 mRNA transcripts result from the initiation site located on exon 1 (promoter 1) leading to the splicing of exon 1 to exon 3[49]. Class 2 transcripts result from the initiation site located on exon 2 (promoter 2), which leads to the splicing of exon 2 to exon 3. Alternative splicing of either exon 6 or exon 5 to exon 4 results in class Ea and Eb mRNA transcripts respectively[49]. Humans have a third class (Ec), which correlates to Eb in rodents and contains portions of both exon 5 and exon 6 [49]. While there are many different precursor forms of IGF-1, the specific conditions that regulate preferential expression of one form over another have not been determined [49].

IGF-1 is stabilized, post translationally, by forming a complex with one of six IGF binding proteins (BPs) [50], primarily IGFBP-3, and the acid labile subunit (ALS) [51]; less than 5% of circulating IGF-1 is in the free, unbound form [51].

Insulin-like Growth Factor 1 Receptor

IGF-1 receptor (IGF-1R) is a hetero tetromeric protein consisting of two extracellular alpha- and two membrane spanning beta- subunits held together by disulfide linkages and has intrinsic tyrosine kinase activity in the intracellular beta subunit. Each alpha- and beta- subunit pair is cleaved from a single polypeptide [52, 53]. Upon binding of IGF-1 to the alpha- subunits, the intracellular tyrosine kinase is activated and subsequently phosphorylates tyrosine moieties on the intracellular beta-subunit [54, 55]. These phosphorylated tyrosine moieties create protein tyrosine binding and SRC

homology (SH2) domains, which attract and provide binding sites for docking molecules such as members of the insulin receptor substrate (IRS) family, IRS 1-4. These attract and couple with the p85 subunit of the phosphatidyl inositol 3 kinase (PI3K) [56, 57], and downstream PDK1/2 and Akt, and the Shc docking substrate, which couples to the Ras guanine nucleotide exchange factor, Son of Sevenless (SOS) and the downstream Ras-Raf-MEK-ERK-MAPK pathway[58]. The PI3K/Akt pathway is typically responsible for preventing apoptosis and the MAPK pathway is promotes growth and proliferation[59].

Liver IGF-1 And The Somatomedin Hypothesis

For decades the Somatomedin hypothesis of GH action was the accepted explanation of GH/IGF-1 signaling [60-62]. The Somatomedin hypothesis described a linear signaling pathway which began in the hypothalamus with GHRH being released, stimulating GH secretion from the anterior pituitary which then acted on the liver to produce IGF-1 (aka Somatomedin C). IGF-1 then circulated and acted in an endocrine fashion on extrahepatic tissues to promote somatogenesis [61, 63]. In 1984 D'Ercole, *et al*. [64] began to challenge the original Somatomedin hypothesis and produced evidence that GH also stimulated the production of IGF-1 in extrahepatic tissues. Skottner, *et al*. [65] showed that administration of IGF-1 alone failed to produce increased longitudinal growth of hypophysectomized rats except at very high concentrations, whereas low levels of GH alone produced significant gains in longitudinal growth [65]. In fact, liver-specific *Igf-1* deletion, as in the liver-specific *Igf-1* deletion (LID) mouse, caused an approximately 75% reduction in circulating IGF-1 with only minimal effects on growth

of the mice [66]. This provided evidence that IGF-1 is produced in extrahepatic tissues and that this locally produced IGF-1 is primarily responsible for somatogenesis.

Nevertheless, liver derived IGF-1 plays an important role as a negative feedback mechanism on GH secretion. Indeed, when hepatic IGF-1 production is eliminated, as in the LID mouse, circulating GH levels are markedly elevated [66]. Circulating IGF-1 also appears to play an important role in splenic cellularity and myelopoesis including the survival and transition of myeloid progenitors into the spleen [67]. Additionally, circulating IGF-1 levels are related to the development of malignancies and LID mice display a reduced risk for the development of colon and breast cancers as well as metastatic lesions [68]. Interestingly, while liver produced IGF-1 has little effect on postnatal growth; circulating IGF-1 still plays an important role in bone growth and density [51]. When circulating IGF-1 was reduced even further in LID mice by deletion of the acid labile subunit (ALS), the proximal growth plates were smaller and they experienced a 10% decrease in bone mineral density and a 35% decrease in periosteal circumference and cortical thickness[51].

Pulsatility And Circadian Growth Hormone Secretion Rhythm

In nearly all mammalian species studied, GH displays an ultradian rhythm in secretion (i.e. pulsatile secretion) [69]. The ultradian rhythm is largely due to the interplay of stimulatory GHRH neurons in the arcuate nucleus and inhibitory somatostatin neurons in the periventricular nucleus. Low levels of circulating GH result in low activity of somatostatin neurons and subsequent low inhibition of GHRH neurons in the arcuate nucleus and low inhibition of GHRH signaling at the anterior pituitary.

GHRH is subsequently released to the anterior pituitary promoting the release of GH. Rising levels of circulating GH triggers the activation of the somatostatin neurons, which inhibit GHRH neurons and release somatostatin to the anterior pituitary where it directly inhibits GH secretion thereby reducing circulating GH [70].

GH pulsatility is important physiologically in that it allows for increased response to each pulse. Following an initial GH pulse, binding of GH at a target tissue from a subsequent pulse is decreased, due to a reduced number of unoccupied binding sites, and only returns to normal after two hours [71]. Indeed, it has been shown that pulsatile administration of GH resulted in increased growth of hypophysectomized rats compared to continuous administration [72, 73]. Additionally, pulsatile GH also results in increased *igf-1* mRNA in skeletal muscle compared to continuous GH controls. This, however, is not the case in the liver, where both continuous and pulsatile GH result in substantial elevations in *igf-1* mRNA [74].

Sexual dimorphism is observed in the pattern of GH pulsatility across species. Males display discrete GH peaks with very low nadirs, whereas females tend to have broader, less discrete peaks with higher GH levels between peaks [75-77]. The full ramifications of these sexually dimorphic GH secretion patterns are not completely understood. However, one major effect is in liver gene expression, particularly of the cytochrome P450 (CYP450) superfamily [78, 79]. CYPs are membrane-bound monooxygenases that are responsible for the metabolism of a broad range of compounds including fatty acids, endogenous steroids, and exogenous chemicals and drugs[80]. The sexually dimorphic patterns in GH secretion results in the expression of a different group of CYPs in males [81] than in females [82]. The same is true for humans, though the

differences between male and female GH pulsatility are less pronounced compared to rodents [83-86].

Humans display a circadian rhythm in GH secretion with greater pulse amplitudes at night, which seem to be most strongly associated with slow wave sleep [87-89]. However, chronic sleep deprivation with a two-hour delay in sleep onset results in a large GH secretion speak at the time of normal sleep onset, with an additional peak correlated with sleep onset[90]. Additionally, individuals who have an inverted sleep schedule (i.e. night shift workers) seem to lack this characteristic rhythm in GH secretion and instead have sporadic intermediate sized GH pulses throughout the entire 24 hour day [91]. Both of these studies suggest a circadian clock component of the observed GH rhythm in individuals with normal schedules. Interestingly, increased GH secretion during sleep is also present in both mouse and rat females, but not the males from either species [92, 93].

Physiologic And Metabolic Effects Of GH/IGF-1

Growth Hormone In Metabolism: An Evolutionary View

The GH/IGF-1 system evolved early on as evidenced by the presence of GH which stimulates the production of IGF-1 even in early vertebrates [94] and the conservation of the JAK-STAT5 signaling mechanism in both vertebrates and invertebrates/pre-vertebrates [95]. Additionally, there is evidence that, while IGF-1 is very similar to insulin, it actually precedes insulin and the pancreas in development and was derived from a gene that coded for an insulin-like gene that shares high sequence homology for both insulin and IGF-1 [96]. It is clear that GH and IGF-1 play important roles in development and energy metabolism. During times of abundant food and energy,

GH, IGF-1, and insulin stimulate nitrogen retention and general energy storage. During times of scarcity, levels of IGF-1 and insulin decline [97-99] and GH alone stimulates a shift in metabolism to favor the usage of fatty acids instead of carbohydrates and protein as a source of energy[97, 98]. Such an ability to convert and re-direct metabolism depending on the availability of nutrients has played a critical role in survival [100].

Fasting or food scarcity occurs on a regular basis as eating typically does not occur simultaneously with sleep. As described previously, GH release is closely associated with sleep [87-89], and by default, fasting. However, fasting alone induces increased GH secretion [97]. Fasting, or the absence of energy intake, results in reduced insulin expression and subsequent hepatic GH resistance [101, 102]. This hepatic GH resistance manifests as reduced GH induced activation of the Akt and JAK2/STAT5 pathways and increased GH induced phosphorylation of ERK1/2 and PKC [103]. The result of this shift in signaling is increased activation of GH stimulated expression and activation of hormone sensitive lipase (HSL), a major lipolytic enzyme responsible for breaking down stored lipids [104], through activation of the ERK and PKC pathways [105]. The major short-term physiologic effect of GH release is elevation of circulating free fatty acids (FFAs) and ketone bodies [106], which suggests a shift toward beta-oxidation, and therefore, an increased need for these substrates. The mobilized FFAs originate from the abdominal and femoral adipose depots [107, 108] and there is evidence to suggest that visceral adipose tissue is also involved [109]. Reintroduction of food stimulates insulin secretion, sensitization of the liver to GH, reduced ERK and PKC signaling, and reduced HSL activation and expression, and a shift back toward an anabolic state favoring glucose oxidation [97, 103].

GH has been implicated in increasing gluconeogenesis during fasting [100] because of the correlation between fasting, GH secretion, increased gluconeogenesis [110], and sustained euglycemia. However, a study in GH deficient children revealed that despite normally elevated cortisol levels, the children developed hypoglycemia after prolonged fasting and a spike in blood glucose levels was observed upon glucagon administration suggesting the GH deficient patients were unable to mobilize their glycogen stores during the fast [111]. This suggests that GH maintains euglycemia during fasting via mobilization of glycogen stores and not gluconeogenesis necessarily. There is also evidence that implicates cortisol as a major driver of gluconeogenesis [112]. It is, therefore, unclear to what degree GH influences gluconeogenesis during the fasted state, but it is abundantly clear that it shifts metabolism in favor of β-oxidation and influences the body's ability to maintain euglycemia under prolonged nutrient deprivation.

GH promotes protein or muscle sparing. GH is the only anabolic hormone to increase during fasting while insulin and IGF-1 decrease and catabolic hormones such as glucagon, and epinephrine increase [113]. Individuals receiving exogenously administered high doses of GH display nitrogen retention manifested by decreased blood urea nitrogen levels and reduced urea excretion[114-116]. These individuals also displayed an increase in nitrogen excretion upon cessation of GH treatment [114-116]. Other studies, in patients with physiological levels of endogenous GH (as produced by fasting), revealed that GH suppression using somatostatin, as also in the case of GH therapy withdrawal in growth hormone deficient (GHD) subjects, resulted in 50% increases in urea-nitrogen excretion [117, 118] and a 30-35% increase in the rate of [13C] urea production [119]. Furthermore, GHD patients with GH replacement had decreased

circulating levels of branched chain amino acids, an evidence of decreased proteolysis [118, 119]. Overall, GH causes increased circulating FFA levels, which partially mediate GH promotion of nitrogen retention and protein sparing during fasting [120].

In the case of caloric abundance and excess, GH works with IGF-1 and insulin to promote muscle building and FFA storage[100]. When this is carried to the extreme, as in obesity, the excess FFAs begin to cause insulin resistance, which can ultimately lead to the development of diabetes and cardiovascular disease. Therefore, the presence of FFAs results in protein sparing in the fasted or famine state, and in the case of over nutrition, it results in insulin resistance and cardiovascular disease [100].

GH In Longitudinal Growth (Acromegaly)

Some of the earliest evidence for the role of GH in longitudinal growth came in the form of giants [121]. History has recorded the presence of abnormally tall individuals for thousands of years, yet it wasn't until 1886 that Pierre Marie first coined the term acromegaly and provided a clinical description of the condition[122]. This early description included, principally, swelling or hypertrophy of the hands, feet, and face. At this time, physicians had described the existence of sellar enlargement (i.e. the pituitary gland housed in the sella turcica) found in autopsies of acromegalic individuals, but it was not known whether it was a cause or effect of the acromegaly [121].

We now know that acromegaly results from prolonged elevated levels of GH resulting in high IGF-1 production[123]. The most common cause of elevated GH in acromegaly is a GH-secreting pituitary adenoma derived from somatotroph cells[123]. Most acromegalic tumors are sporadic and occur as an isolated disorder [123], although

they can also be familial either as an isolated tumor or part of multiple endocrine neoplasia syndrome (MEN) 1 and 4 or Carney complex [124-126]. Approximately 43% of acromegaly cases have explainable genetic causes among which are mutations in the AIP gene (29%), X-linked acrogigantism (10%; microduplication of GPR101 gene on chromosome Xq26.3 [127, 128]), and other causes (7%) [124, 129, 130].

Acromegaly has equal occurrence in males and females and the average age of onset is 40-50 years old[131, 132]. Individuals younger than 20 years at onset of acromegaly account for approximately 5% of cases and tend to be more severe with much higher levels of GH and IGF-1 and are more likely to result in gigantic stature [131, 132]. The clinical features are varied and related to the expansion of the pituitary tumor as well as the systemic effect of excessive GH and IGF-1. Some of these features and co-morbidities include local tumor effects: hypopituitarism, headache, visual impairment, and hyperprolactinemia and systemic effects: prominence of the brow, enlargement of the nose, ears, and lips, macroglossia, acral enlargement (excessive growth of hands and feet), visceromegaly, sleep apnea, insulin resistance, and multiple forms of cardiovascular disease (CVD) including hypertension, left ventricular hypertrophy, arrhythmias, cardiomyopathy, and congestive heart failure [123, 126, 133].

There is extensive evidence showing that, when untreated, acromegaly reduces life expectancy. Causes of mortality in acromegaly are cardiovascular disease (~60%), respiratory disease (20%), and malignancies (15%) [134]. Increased age of onset and lower GH levels are generally associated with increased life expectancy while increased time to diagnosis, duration of disease, and elevated GH levels are generally associated with increased mortality [131]. As such, early diagnosis and treatment are key to

reducing the effects of acromegaly, as patients whose treatment achieved GH levels below 5 μU/L (2.5 μg/L) experienced normal mortality rates [134].

Diagnosis of acromegaly consists of a combination of clinical presentation and biochemical tests. Serum GH levels can vary widely, however, serum IGF-1 is uniformly elevated[134]. Nevertheless, the gold standard for diagnosis is failure to normally suppress serum GH following a standard 75g oral glucose tolerance test [134].

The goals of treatment in acromegaly are to provide symptomatic relief, control the tumor, preserve pituitary function, reduce or reverse the increased morbidity, and reduce circulating GH and IGF-1 levels. Treatment modalities include surgical resection of the tumor, medical therapy, and radiotherapy. Medical therapy may include a somatostatin receptor agonist (Octreotide, Lanreotide), Pegmisomant, a recombinant analog of GH which acts as a GHR inhibitor, or dopamine antagonists (Cabergonine, Bromocriptine). Radiotherapy is viewed as a last resort adjuvant therapy when surgery and medical treatments have failed. Treatment can be complicated and usually involves multiple modalities [134].

Animal models have proven valuable in studying the effects of GH excess beginning in 1982 with the first transgenic GH overexpression model of acromegaly [135]. Both male and female transgenic mice have elevated levels of GH and IGF-1 [135-138], grow to be larger than their control counterparts and have greater lean body mass and lower body fat percent and total fat mass [136]. Additionally, these animals have a dramatically reduced life expectancy (approximately 50 percent of controls) with few mice living beyond one year [139]. GH transgenic mice display significant pathology in addition to the alterations in mass and body composition including hepatomegaly,

glomerulonephritis, glomerulosclerosis, and multifocal myocardial fibrosis [139, 140]. Systolic blood pressure (SBP) is also elevated in GH transgenics beginning at 6 months of age accompanied by a decrease in circulating levels of brain natriuretic peptide (BNP) despite a curious increase in BNP mRNA [141]. Furthermore, high GH levels are associated with hepatomegaly and increased pathological lesions in the liver including increased hepatocyte size, enlarged nuclei, and most prominently, nuclear inclusions and lipid droplets within the nuclear sap [140, 142, 143]. In addition to increased body weight and bone length, the structure of bone is also altered such that cortical thickness, trabecular bone volume fraction, thickness, and number are all decreased resulting in weakened bones [144].

Mouse models overexpressing IGF-1 have also been created to determine which effects of GH overexpression are IGF-1 dependent. Liver-specific overexpression of IGF-1 resulted in a 50% increase in circulating IGF-1, body weight, lean mass, and length of long bones [145]. Muscle-specific IGF-1 overexpression results in hypertrophy dependent on the mTOR pathway [146]. The functional effects of cardiac-specific models of IGF-1 overexpression have been varied and conflicting. Some have reported increased shortening velocity and cellular compliance in primary cardiomyocytes [147] and in vivo observations revealed increased peak aortic outflow velocity (a measure of systolic function) at 10 weeks, but this benefit was eventually lost and peak aortic outflow velocity became significantly reduced by 52 weeks [148]. On the other hand IGF-1 overexpression was associated with reduced functional recovery after acute ischemia [149]. However, there is a general consensus that it increases cell viability, proliferation, and increases heart size via hyperplasia rather than hypertrophy per se [147, 150-153].

GH In Longitudinal Growth (Dwarfism)

Just as much can be learned about the effects of GH from its deficiency as from its excess. One of the best-known models of GH deficiency is Laron syndrome. Laron syndrome (LS) is described as pituitary dwarfism with high plasma GH [154] resulting from a polymorphic defect in the GHR [155-157]. The effects of a defective GHR are nearly identical to those of isolated GH deficiency (IGHD), however, in LS, levels of circulating IGF-1 are low and cannot be induced by GH treatment [157]. Individuals with LS and IGHD share the clinical characteristics of dwarfism, obesity, prominent forehead, and acromicria, specifically small hands, feet, gonads, genitalia, and face with a saddle nose. While individuals attain full sexual development and reproductive capacity, sexual development can be delayed, particularly in males [157, 158]. Perhaps one of the most interesting characteristics of LS is something that is absent, namely cancer. A study was done of 538 patients that had either LS, or another syndrome resulting in IGF-1 deficiency (i.e. congenital IGHD, congenital multiple pituitary hormone deficiency (cMPHD) including GH or GHRHR defect) and 752 first-degree family members to compare the incidence of cancer in the patients compared to their family members. None of the 230 individuals with LS had cancer and only one of the 116 individuals with IGHD had a malignancy, however, this individual also suffered from xeroderma pigmentosum. In contrast, there were 25 cases of malignancy among their 421 first-degree relatives and an additional 29 cases among 126 further relatives [154, 159, 160].

Treatment of LS consists of recombinant human IGF-1 replacement, usually via injection [161], however, the treatment produces little longitudinal growth and carries

with it the burden of multiple daily injections, intense pain at the injection site, and hypoglycemia [162]. Efforts have been made to develop a more permanent and less burdensome form of treatment using the intraperitoneal xenotransplantation of porcine Sertoli cells, but the method has only been tested in mice [162].

Mouse models of LS have reduced body weight, elevated serum GH and low serum IGF-1 [163]. Heterozygous mGHR (+/-) knockout females are only 10% smaller than normal littermates and have normal mGHR and IGF-1 levels similar to individuals who are heterozygous for LS who tended to be shorter, but still within normal height limits [163, 164]. The homozygous mGHR (-/-) (Laron Dwarf; LD) mice also accurately model the LS homozygous individuals with the notable exception of weight. LD mice are up to 55% smaller than their +/+ counterparts by 4 weeks of age, whereas LS individuals, despite short stature, have a tendency toward obesity following birth [164, 165]. Most other differences between LS individuals and the LD models stem from this tendency toward obesity or lack thereof [164]. In general the LD mouse is accepted as a meaningful model of LS.

Further findings in the LD mice are that their lifespan is significantly extended compared to normal controls [166-168] with the longest living LD mice living over a year longer than their control counterparts [169]. The LD mice have also shown resistance to age-induced memory decline [170] and an increase in the number of bone marrow stem cells [171].

Recently, Cui D, *et al*. [172] developed a miniature pig model of LS in the hopes of finding a model that is more closely related phylogenetically to humans so that treatment modalities developed therein will be more easily translatable to humans. This

pig LS model, in contrast to the LD mice, displays increased adiposity at three and a half months compared to controls [172].

GH regulation begins in the hypothalamus and pituitary of the brain and receives negative systemic feedback via elevated circulating IGF-1 and positive regulation from low circulating IGF-1 levels and ghrelin secretion. Elevated GH results in excessive growth systemically and dysregulated handling of fats and glucose including insulin resistance. Deficiency of GH results in dwarfism, increased fat storage, and protection against development of certain types of cancer. While the influence of elevated GH or IGF-1 in cardiac function may initially be beneficial it seems that it is ultimately detrimental. Overall GH/IGF-1 signaling has been extensively studied and well described, however the effect of tissue specific disruption of the circadian clock on GH/IGF-1 signaling has been less well described and our understanding of the interplay between these two systems is lacking. Therefore, further investigations in this area are needed.

THE CIRCADIAN CLOCK: OUR INTERNAL TIMEKEEPER

The Purpose Of The Circadian Clock

One fact that rules all life on this planet is the 24 hours that it takes the earth to make one full rotation around its axis. Circadian clocks allow organisms to adapt to this environment by providing the ability to anticipate regularly occurring events whether these events are the presence of light, food, arousal, foraging, fleeing, temperature changes or any number of beneficial or noxious stimuli/events and either maximize the benefit obtained or minimize the damage received. At the heart of this timing mechanism

are genetic circuits that have been conserved across all life from bacteria, to plants, to humans, although the specific components of this circuit vary between organisms [173, 174].

The Mammalian Molecular Clock

The molecular circadian clock consists of a transcription-translation negative feedback loop (Figure 4). The positive arm of this loop consists of two basic helix-loop-helix – Period-Arnt-Single-minded (bHLH-PAS)-containing transcription factors, CLOCK (circadian locomotor output cycles protein kaput) [175, 176] and BMAL1 (brain and muscle ARNT-Like 1; aka MOP3) [177, 178]. CLOCK and BMAL1 form a heterodimer, which activates gene transcription by binding to E-box enhancer elements characterized by the sequence CACGTG [176, 177]. Among the genes activated by the CLOCK-BMAL1 complex are genes coding for the negative arm of the loop including *period* genes (*Per 1-3* depending on species) and *cryptochrome* genes (*Cry1-2*). The PER and CRY proteins interact and translocate back to the nucleus and directly inhibit the CLOCK-BMAL1 complex, and therefore, their own transcription [179-182]. This process requires approximately 24 hours. CLOCK-BMAL1 also binds to the *Rev-Erbα* gene, which forms a second part of the negative regulating arm. REV-ERBα represses *Bmal1* gene expression through binding Rev-Erb/ROR response elements in its promoter [183-186].

Except for Bmal1, knockout of any single gene in the molecular circadian clock fails to completely disrupt clock oscillation, as there is redundancy in the system with Cry1 and Cry2 genes and Per1 and Per2 genes, even CLOCK has a redundant homolog

protein Npas2 [187-189]. To completely disable the molecular clock, deletion of both Per1 and Per2 genes or both Cry1 and Cry2 genes or both Clock and Npas2 genes, or simple deletion of Bmal1 is required [178, 181, 186, 190-194].

Molecular clocks are only able to approximate the 24-hour rotation of the earth and most are powerfully entrained or reset each day by the presence of light. In constant dark conditions the intrinsic clock runs free with a period of slightly greater (diurnal species) or shorter (nocturnal species) than 24 hours [195-198]. In larger organisms, where light is unable to penetrate in a significant manner to all cells, a central or master clock has evolved, which can be entrained to light and in turn synchronize the remaining cells throughout the organism; this is the suprachiasmatic nucleus (SCN) located in the anterior hypothalamus [173].

Entrainment Of The Suprachiasmatic Nucleus

The ability of a stimulus to entrain the clock or alter the period (length of one complete cycle) and phase (position w/in a cycle) classifies it as a Zeitgeber (time giver) [199]. Light is the most universal zeitgeber in circadian biology. Light cues from the environment enter through the eyes and ultimately travel down the retinohypothalamic tract to the SCN. However, upon entering they eye it penetrates the retina beyond the classical opsin-based retinal photoreceptors, the rods and cones, which are responsible for the formation of visual images from light, to the intrinsic photosensitive retinal ganglion cells (ipRGCs). ipRGCs rely on the photoreceptor melanopsin for light detection and have dendrites that project directly to the SCN where they induce changes in the molecular components of the clock [186, 200-203].

When signals from the ipRGCs reach the SCN, glutamate is released causing the stimulation of NMDA (N-methyl-D-aspartate) receptors [204, 205], which allow Ca^{2+} influx into the SCN neurons. Increased Ca^{2+} activates the calcium/calmodulin-dependent kinase II α (CaMKIIα) enabling it to phosphorylate the cyclic AMP responsive element-binding protein (CREB) [206-209]. CREB is responsible for binding to a cAMP-responsive element (CRE) in the promoters of numerous genes including *Per1* causing its robust induction [206, 210]. The increased expression of PER1 leads to its association with CRY1/2 and subsequent translocation of the complex to the nucleus where it effectively entrains the SCN molecular clock [186].

Peripheral Clocks And Their Entrainment

Entrainment of the SCN occurs primarily via signals resulting from light entering the eyes, but the peripheral clocks located in nearly every nucleated cell of the body are subject to input from many sources including the SCN, stress and associated hormones, food, arousal and locomotor activity, and melatonin (Figure 3).

The role of the SCN in the synchronization of peripheral clocks has not been explicitly outlined for each tissue and or organ. SCN efferent neuronal projections signal via GABA, VIP, and AVP, are short, and innervate regions of the hypothalamus, thalamus, preoptic area, and the basal forebrain [211]. These brain regions act as relay stations for reaching other parts of the brain and body. SCN neurons project to generally three different types of neurons, intermediate neurons, pre autonomic neurons, and endocrine neurons, although most projections are to regions containing interneurons such as the paraventriculate nucleus (PVN), the dorsomedial hypothalamus (DMH), and the

medial preoptic area (MPOA). Further connections from these interneurons directly entrain and influence circadian clocks and functions in peripheral tissues such as the liver, the pineal gland and the adrenal gland [212]. SCN projections to CRH – (corticotropin-releasing hormone), TRH – (thyroid releasing hormone), and GnRH – (gonadotropic releasing hormone) containing neurons have also been described [212, 213].

When the SCN is lesioned, rhythms in the animal are abolished [214], but when just its neuronal connections are severed, it is able to induce locomotor rhythms by one or more unknown secretory factors [211, 214, 215]. In fact, transplantation of the SCN from a donor animal into an animal in which the SCN has been lesioned, restores locomotor rhythmicity, but with the period of the donor animal [214].

Synchronization of peripheral clocks by the SCN is also achieved via rhythmic glucocorticoid (cortisol in humans) secretion, which peaks in the early morning in humans and declines to a nadir in the late evening [216]. Glucocorticoids entrain peripheral clocks via binding to its glucocorticoid receptor, causing it to interact with glucocorticoid response elements (GRE) found in the gene sequence of *Per1* [217] and the gene locus of *Per2* [218] inducing their expression [219]. Physical and psychological stress [220] as well as exercise [221] can act as zeitgebers for peripheral and central clocks through their ability to increase circulating cortisol levels. This proves evolutionarily advantageous for animals whose predators tend to hunt at certain times of day, provoking stress and necessitating fleeing.

The most powerful entrainment stimulus for most peripheral clocks is food. In nocturnal animals with free access to food, they will consume the majority of their food

during their subjective night when they are active and the peripheral clocks in organs regulating metabolism and the digestion, absorption, utilization and storage of nutrients synchronize to this rhythm. However, when food is restricted to the subjective day, when the animals would normally be resting, the feeding pattern shifts and the internal organs align themselves with the feeding rhythm in opposition to the light entrainment occurring at the SCN [199, 222]. While the feeding rhythm, in general, entrains the peripheral clocks, individual nutrients found in foods can cause a range of effects on the peripheral clocks including phase shifting (e.g. ketogenic diet, high salt and caffeine) lengthening (e.g caffeine and high fat diet) and shortening (e.g. cinaminic acid) the period of the rhythm [223].

Other external entraining stimuli are arousal during the rest period and physical activity. Arousal in the middle or latter stages of an animal's rest period (subjective day/light phase for nocturnal animals) will entrain the clock by advancing the phase of the activity rhythm. Conversely, arousal early in the rest period causes a phase delay. Arousal can be stimulated in a number of different ways including by cage changes, wheel running, or even gentle handling in the case of hamsters [199, 224-227].

Melatonin's role in synchronization of the circadian clock is limited. Melatonin is produced by the pineal gland and is controlled by outputs from the SCN [212, 228-230]. Melatonin production and release in mammals are highest at night and low throughout the day regardless of whether the animal is nocturnal or diurnal [231]. Melatonin production is highly sensitive to light input at the retina and will quickly cease upon nighttime light exposure [232]. Melatonin, however, is not required for rhythmic animal behavior as C57BL/6 (naturally melatonin deficient [233]) mice exhibit normal rhythmic

behavior compared to the melatonin-proficient C3H-strain [234]. One difference between the two strains is the effect of a light pulse late in the subjective night. C3H mice experience a smaller phase shift as a result of a light pulse administered during the second half of the subjective night compared to C57BL/6 mice [234]. This provides evidence that melatonin can influence the circadian clock. Additionally, melatonin regularly administered to rodents free-running (in the absence of light entrainment, i.e. constant dark conditions) shifts the phase of their circadian rhythms [235]. Early on, melatonin was believed to be the main SCN output whereby the rest of the body was entrained to the light-dark cycle and melatonin is still used to treat desynchronization of circadian rhythms caused by shift work or jetlag [236]. Therefore, melatonin does indeed influence circadian rhythms, but remains one of the less potent entrainment stimuli.

Circadian Clock Disturbances And Their Effects

Physiologic Modes Of Clock Disruption

Modes of physiologic circadian disruption principally involve alteration of behavior relative to the external environment (shift work, night eating, social jetlag) or altering the environment and our behavior relative to the internal clock (jetlag).

Jetlag is the classical example of circadian disruption. When an individual crosses time zones they are entering a new light dark cycle, yet their circadian clock continues to send them cues as if they were in the original light-dark and sleep-wake cycle. The individual's circadian clock will eventually shift or entrain itself to the new light-dark cycle, but it requires between one and two days for each hour that it must shift depending on whether it is undergoing a phase advance or delay [237]. During the time required for

entrainment, the circadian timing system is out of sync with the external environment. Upon return to the original time zone, the clock must again shift back, requiring one day for each hour. Individuals making frequent trips crossing time zones are frequently in a state of dysynchrony with their environment.

Another example of physiologic circadian clock disruption is shift work. Similar to jet lag, the individual working three night shifts each week shifts their sleep wake cycle by approximately 12 hours two times each week. The circadian clock, unable to keep up with the constantly changing conditions goes into disarray and biological processes lose their rhythmicity[238, 239], or even worse, occur out of sync with the rest of the body and the environment[240, 241]. Nearly 6% of Americans participate in shift work of some kind, including doctors, nurses, police officers, fire fighters, truck drivers, and individuals serving in the military [242].

Night eating simply consists of eating at night when the circadian clock/body is primed for sleeping. The regular introduction of food to the digestive system at night provokes shifting or entrainment of many peripheral circadian clocks (i.e. biological rhythms) relative to the central clock [243].

Perhaps the most ubiquitous example of physiologic circadian clock disruption is social jetlag. Social jetlag occurs when an individual's median sleep time differs significantly on the weekend compared to weekdays [244, 245]. Median sleep time refers to the time halfway between falling asleep and waking up. A stereotypical example is the individual that sleeps from 10:00PM – 6:00AM Monday through Friday, but who then stays awake till 2:00 AM Friday night and sleeps until noon and repeats this behavior Saturday night before returning to their regular weekly schedule on Monday. The median

sleep time during the week is three hours earlier on weekdays (2:00AM) compared to weekends (7:00AM).

Disruptions of the circadian clock, such as those described above, place an individual at increased risk of developing depression [246], cancer [247], metabolic syndrome [248-250], diabetes [251], obesity [248, 250, 252], and cardiovascular disease [253-256]. Due to the complexity and breadth of health problems influenced by circadian dysregulation, it is difficult to assess the actual health burden of circadian misalignment and disruption on the population. Treatment can be equally as complex.

Treatment Options For Circadian Misalignment And Sleep Disorders

The best and most effective treatment modality for physiologic circadian dysregulation is, intuitively, behavioral; consistently eating only during the day, sleeping at night and going to bed and waking up at the same time each day. However, for many individuals, shift work and circadian disruption are inevitable, such as for military personnel, health care professionals, and those in public services. For these individuals, treatment strategies include light therapy, melatonin, and in certain cases, pharmacotherapy.

Light therapy consists of timing light exposure to certain times of day. In the case of delayed sleep phase disorder (DSPD) (difficulty falling asleep before 2:00-6:00 am accompanied by difficulty waking on time in the morning and daytime sleepiness), bright light is administered in the early mornings to induce a phase advance and exposure to light is limited in the afternoon/evening before sleep to avoid inducing a phase delay. In advanced sleep phase disorder (ASPD) (falling asleep between 6:00-8:00 pm and early

arousal around 1:00-3:00 am), bright light is administered in the evenings to induce a phase delay and light is limited in the early morning. The principles for treating DSPD and ASPD can also be applied to individuals doing shift work; exposure to bright light during the beginning of the desired wake time and reducing light exposure in the hours preceding sleep [257, 258].

Melatonin can also be used to entrain the circadian clock. Melatonin is typically ingested 2-5 hours before the desired onset of sleep depending on the specific situation [258].

Pharmacotherapy consists of hypnotics (benzodiazepine receptor agonists) to promote sleep, wake promoting agents (modafinil and armodafinil), and stimulants such as caffeine [258].

Typically, a combination of all of these therapeutic modalities is most effective at treating circadian rhythm sleep disorders [258].

Genetic Circadian Clock Disruption: Lessons From Animal Models

Genetically disrupting the molecular clock has provided a better understanding of the role of the clock itself as well as individual players in the negative feedback loop mechanism, including the idea that not all genes influenced by core clock components are expressed rhythmically [259]. Specific animals models will be discussed below.

The *Bmal1* knockout (KO) model is the simplest way to disrupt the circadian clock due to the fact that Bmal1 is the only core clock component for which there is no redundancy [178]. Global knockout of *Bmal1* results in complete loss of rhythmic behavior and an overall reduction in activity levels in mice as well as loss of rhythmic

expression of core clock (*Per1/2*) genes in the SCN and output genes (*Dbp*) in the liver [178]. Effects of *Bmal1* KO include pathologies associated with early aging, such as sarcopenia, cataracts [260, 261], increased mortality and systemic organ failure [262] with decreased organ weight [263, 264]. *Bmal1* KO also increases adipogenesis [265], reduces glucose stimulated insulin secretion in beta cells [266], and reduces insulin sensitivity [267], glycolysis, and glucose uptake [268]. Reproduction is also affected with whole body *Bmal1* KO resulting in infertility with altered steroidogenesis in both male and female mice [269]. Even hemizygocity in *Bmal1* expression in female *Bmal1*(-/+) mice results in reduced reproductive capacity compared to controls [270]. Female mice further display disrupted luteinizing hormone (LH) and follicle stimulating hormone surges in whole body *Bmal1* KO but not in the gonad-specific model [271], and while ovulation persists in both cases, ovulation in theca cell-specific *Bmal1* KO lacks the usual rhythm and LH surges do not consistently result in ovulation [272]. Loss of *Bmal1* also reduces oocyte fertilization [273], increases implantation failure (steroidogenic cell-specific) [274] and alters normal time-of-day dependent parturition (myometrial specific) [275]. Loss of *Bmal1* in males results in failure to mate with receptive females and reduced aggression and avoidance response to a predator's scent, indicating neurological impairment [276]. In fact, *Bmal1* KO results in reduced habituation [277], learning and memory functions [277, 278], possibly through reduced neurogenesis [279], dysfunctional redox homeostasis [280], neurodegeneration, reduced cortical functional connectivity [281], and altered cell cycle control [280, 282, 283]. The effect of whole body *Bmal1* KO in muscle is reduced myogenesis, muscle mass, and strength, with an additional effect of reducing mitochondrial volume [284, 285]. Loss of *Bmal1*, either

whole body or endothelial-specific, also increases risk of cardiovascular events by creating a hypercoagulable prothrombotic state by increasing a number of prothrombotic factors [286-288]. Significant effects on vasculature include pathologic remodeling and increased wall thickness in arteries [289], impaired endothelial function [287], nitric oxide production [289, 290], and response to vasoconstriction agonists [291]. Loss of *Bmal1* has severe consequences in the heart whether the model is whole body *Bmal1* KO or cardiomyocyte specific *Bmal1* KO (CBK). These mice experience dilated cardiomyopathy, increased fibrosis and expression of collagen genes, altered metabolism that resembles the fasted state with a shift away from glycolysis toward fatty acid oxidation, impaired resolution of inflammation, and contractile dysfunction that decompensates to heart failure and early mortality [259, 292, 293].

Other strategies for disabling the circadian clock include double KO of *Clock* and *Npas2* or transgenic expression of a dominant negative form of *Clock* [285, 289, 294-297], *Cry1/2* double KO [277, 294, 298], and *Per1/2* double KO [299-304].

Therefore, the circadian clock provides the mechanism whereby regularly occurring events are anticipated and biological processes are aligned and optimized. When these rhythms are disrupted this optimization is lost to varying degrees and disease risk increases. Los of circadian rhythms by *Bmal1* deletion in cardiomyocytes leads to development of cardiomyopathy and eventually heart failure.

CARDIOVASCULAR DISEASE

General Cardiovascular Disease Background

Cardiovascular disease (CVD) presents a major disease burden in the United States with an estimated 85.6 million Americans (>1 in 3) having more than one form of CVD. This problem, however, is not new. In the United States, CVD has claimed more lives every year except one since 1900 than any other major cause of death. While efforts to reduce the prevalence of some risk factors have been successful in reducing projected CVD deaths, the increased prevalence of obesity has offset progress substantially [305].

Risk factors for CVD can be classified as either behavioral or clinical. Behavioral risk factors include smoking and tobacco use, physical inactivity, poor nutrition, and being overweight/obese. The clinical risk factors are family history and genetics, hypercholesterolemia, hyperlipidemia, hypertension, diabetes mellitus, metabolic syndrome, and chronic kidney disease [305].

CVD is a broad term, which encompasses a number of related, but distinct diagnoses including stroke (cerebrovascular disease), congenital cardiovascular defects, cardiac arrhythmias, sudden cardiac arrest, subclinical atherosclerosis, coronary heart disease, acute coronary syndrome, angina pectoris, peripheral artery disease, valvular, venous, and aortic diseases, and finally cardiomyopathy and heart failure (HF) [305]. Here, we will focus on HF.

Heart Failure

Heart Failure Background

HF accounts for a substantial disease burden. In 2012, there were 5.7 million Americans with HF and that is projected to increase by 46% to greater than 8 million by the year 2030 and there are an additional 915,000 newly diagnosed HF cases each year [305]. Risk factors for HF are similar to those for CVD in general, but also include, coronary heart disease, dietary sodium intake, and valvular heart disease [305].

Heart failure is a clinical diagnosis and is made based upon a combination of a number of different signs and symptoms. Symptoms of HF include, dyspnea on exertion (DOE), orthopnea, paroxysmal nocturnal dyspnea (PND), dyspnea at rest, fatigue, dizziness, and syncope[306]. Clinical signs of heart failure are elevated jugular venous pressure (JVP; almost always present at diagnosis), pulmonary venous congestion, pulmonary edema, cardiomegaly on chest x-ray, and lower extremity swelling, and/or pitting edema [306].

Heart Failure Definition of General Terms

To understand heart failure, we must first understand the terms that describe the parameters of cardiac function. Stroke volume (SV) is obtained by subtracting the end systolic volume (ESV) from the end diastolic volume (EDV). Cardiac output (CO) is the volume of blood per minute that is ejected from the heart. Ejection fraction (EF) is defined as SV/EDV.

Systolic Versus Diastolic Heart Failure

There are two broad classifications of HF, systolic HF (SHF; HF with reduced ejection fraction (HFrEF)) and diastolic HF (DHF; HF with preserved ejection fraction (HFpEF)). SHF refers to the state at which there is a failure in systole; the heart's ability to contract and maintain sufficient cardiac output. In SHF, the left ventricle experiences eccentric hypertrophy described as normal wall thickness, but reduced ratio of wall thickness/ventricle diameter, and reduced EF (Figure 5). Systolic dysfunction and eventual failure occurs when the heart is exposed to abnormally large internal pressures during diastole [307]. Abnormally large internal left ventricle (LV) pressures result from conditions such as myocardial infarction, valvular stenosis and regurgitation of the aorta or the mitral valve, patent ductus arteriosus, or ventricular septal defects [307, 308]. In these conditions, the left ventricle must accommodate a larger than normal amount of blood during diastole while continuing to maintain a sufficient net CO. The adaptive mechanism is eccentric hypertrophy, which consist of sarcomeres (the functional contractile unit of muscle) adding in series thereby lengthening the cardiomyocyte and effectively resulting in dilation of the LV [307, 309]. In SHF, EF is reduced by definition, though early on the heart may be able to maintain CO (compensated HF). However, by the law of Laplace, increased LV diameter increases wall stress resulting in further sarcomere elongation leading to further increases in wall stress [307], and the heart is no longer able to compensate for the increased volume and both EF and CO decline.

DHF is characterized by a failure of the heart to relax during diastole and is typically caused by increased afterload (i.e. the pressure against which the LV must contract to expel blood; correlated to systemic vascular resistance), commonly caused by

hypertension (HTN) [308]. In diastolic dysfunction, the LV experiences concentric hypertrophy (Figure 5) described as an increased wall thickness/ventricle diameter [309], while preserving EF [308]. The LV undergoes remodeling to compensate for the increased afterload by adding new sarcomeres in parallel to the existing sarcomeres, thereby thickening the LV wall and increasing the force of contraction [307]. If the pressure overloaded state continues, the remodeled LV eventually becomes fibrosed [310], and resistant to distension (i.e. unable to receive much blood during diastole), has a limited EDV, and reduced SV, resulting in reduced CO despite a normal EF [307-309]. Other less common causes of DHF may include amyloid deposition, scleroderma, and sarcoidosis [307]. Of important note, heterogeneity exists between SHF and DHF, as comorbidities (e.g. HTN, myocardial infarction) do not exclusively follow one or the other.

Treatment Strategies For Heart Failure

Treatment of SHF and DHF targets similar parameters (though there is significantly less data on effective treatment of DHF), namely heart rate, blood pressure (BP) and fluid volume. To this end, a combination of different drug classes is used. The recommended therapy includes an angiotensin converting enzyme inhibitor (ACEI) and a β-blocker (BB) with the use of a mineralocorticoid receptor antagonist (MRA) as a second line agent. If the patient does not tolerate an ACEI, this may be substituted by an angiotensin II receptor blocker (ARB). Recently, a new class of drug, angiotensin receptor-neprilysin inhibitor (ARNI), was recommended as a viable substitute for an ACEI and or ARB if they are not tolerated [311-314]. The regimen described above

primarily targets BP and heart rate. Diuretics are also indicated if the patient has signs of volume overload such as PND, pulmonary edema, lower extremity edema and ascites [314].

Metabolic Changes In Heart Failure

In the normal state, the heart relies heavily on oxidative metabolism for the energy production, deriving 95% of its ATP from mitochondrial oxidative metabolism compared to only 5% from glycolysis. Fatty acid (FA) oxidation accounts for 70-90% of ATP production resulting from oxidative metabolism[315]. The heart can also metabolize ketone bodies (β-hydroxybutyrate; βOHB) and branched chain amino acids (BCAA), though to a lesser extent than FAs and glucose[315]. In HF, the heart loses some of its oxidative capacity due to mitochondrial dysfunction. A barrage of different processes contribute to this declining mitochondrial function including a reduction in expression of key transcriptional regulators for both mitochondrial biogenesis (PGC1α) and FA oxidation (PPARα), increases in production of reactive oxygen species (ROS), decreases in mitochondrial fission (isolation of dysfunctional mitochondrial components for mitophaghy) leading to loss of functional mitochondria [315]. However, while both SHF and DHF show reductions in oxidative metabolism, their handling of specific metabolites is not the same. In SHF, oxidation of FAs [316-319], and glucose [320-323], and coupling between glucose oxidation and glycolysis [324, 325] are reduced, while glycolysis [323, 324] and ketone body oxidation [316, 326, 327] are increased. However, in DHF, FA oxidation [328, 329] and glycolysis [330, 331] are either unchanged or increased, and coupling between glucose oxidation and glycolysis is unchanged [331,

332]. Glucose oxidation is reduced [332, 333] as in SHF, but there is insufficient information in DHF models to conclude whether or not ketone body oxidation is altered.

Various animal models have aided in the study of HF and include both rodent and large animals. For the study of SHF [334], rodent models include coronary artery ligation (CAL) [335, 336], ischemia/reperfusion (I/R) [337, 338], or Cryo injury, which includes damaging a section of the heart tissue by exposing it to extremely cold temperatures [339]. The benefit of Cryo injury is that it gives a more reproducible area of injury compared to CAL and I/R. Other rodent models include toxicity-induced models employing the administration of doxorubicin [340, 341] or isoproterenol [342, 343]. Large animals models of SHF are dogs, pigs, and sheep employing the following techniques to induce HF: CAL [344-346], coronary microembolization [347, 348], pacing-induced tachycardia [349-351], and toxic doxorubicin administration [352, 353]. There are also fly [354] and fish [355] models of SHF, and while they are advantageous for studying/genetically modifying genes, they are far removed from humans phylogenetically and findings are not easily translatable to humans [334].

DHF models [356] include the Dahl salt-sensitive (Dahl/SS) rat that develops HF upon administration of a high salt diet from 7 weeks of age through 12-19 weeks [357]. The deoxycorticosterone acetate (DOCA) salt-induced rat receives DOCA 1 week post hemi-nephrectomy followed by chronic DOCA treatment for 4-5 weeks [358]. The DOCA-TAC model received 2 weeks of TAC prior to DOCA treatment [359]. DHF models can also be induced using chronic stimulation with pro-hypertrophic agents such as angiotensin II [360] and isoprenaline [361]. Moderate TAC, administered at an early age, can also induce DHF [362]. Models imitating diabetes include the leptin-deficient

ob/ob and the leptin receptor-deficient db/db mice [363]. A mouse model of age-associated DHF is the spontaneous senescence-prone (SAMP8) mouse whose control is the SAMR1 mouse [364]. Rat models include the Zucker rat, which was originally bread to model obesity and HTN [365], the Otsuka Long-Evans Tokushima Fatty (OLETF) rat [366], which was created by selecting for spontaneously diabetic rats from a colony of Long-Evans rats. Dahl/SS/obese rats are a model of metabolic syndrome and were created from crossing Dahl/SS and Zucker rats [367]. Large animal models of DHF include dogs, pigs, and non-human primates by use of renal wrapping [368, 369], aortic banding [370], gradual aortic cuff inflation, and spontaneous development of diabetes mellitus type 2 [356, 371].

Heart Failure and Growth Hormone / Insulin-like Growth Factor 1

There is a clear association between GH/IGF-1 and the development of heart failure [372]. Individuals with acromegaly nearly always show signs of cardiac hypertrophy, at the time of diagnosis and at autopsy[373, 374]. In the early stages of acromegaly, the heart phenotype is relatively similar to DHF. The GH/IGF-1 stimulates cardiomyocytes to undergo concentric hypertrophy, adding sarcomeres in parallel to the existing sarcomeres, increasing ventricular wall thickness, thereby reducing wall strain. Fibrosis also increases, followed by diastolic dysfunction due to impaired ventricular relaxation [373]. In later stages of acromegaly, the HF becomes decompensated, contractility is impaired, followed by EF declines [373].

There is a great deal of conflicting information relating to GH/IGF-1 and cardiac function with reports describing positive effects of GH or IGF-1 treatment in HF [372] in

opposition to others reporting on cases of acromegaly in which hypertrophy and HF symptoms and cardiac function improved substantially after resection of the GH secreting adenoma or treatment with a somatostatin analog [375]. There are even reports that the heart becomes GH resistant in HF [376]. As described elsewhere, it seems that the relationship with GH and the heart is a delicate balance that results in deleterious effects of having either a shortage or excess of GH/IGF-1 [377]. However, what is clear is that chronic GH/IGF-1 excess, either systemic or local, leads to cardiac hypertrophy and eventual dysfunction.

THE INTERSECTION OF GH/IGF-1 SIGNALING, CIRCADIAN BIOLOGY AND HEART FAILURE

The Cardiomyocyte-Specific Bmal1 Knockout Mouse

The cardiomyocyte-specific Bmal1 knockout (CBK-Cre(+)) mouse lies at the juncture of circadian biology, HF and GH/IGF-1 signaling. The mouse was created on the C57BL/6J mouse background with *Bmal1*$^{(flox/flox)}$ and an α-myosin heavy chain promoter driven CRE recombinase. CBK-Cre(+) mice display age-onset dilated HF (SHF) with increased ventricle weight by 12 weeks of age, slightly reduced EF at 20 weeks, which remains compensated until 28 weeks at which point they decompensate and EF plummets, leading to early mortality. Additional changes that occur include increased fibrosis, expression of collagen and pro-fibrotic genes, a shift in metabolism away from glucose oxidation toward FA oxidation, and a reduction in glycogen synthesis. Markers of inflammation are increased in CBK-Cre(+) mice compared to controls [259, 293, 297].

Processes related to insulin signaling are also affected in the CBK-Cre(+) hearts. These changes include altered expression of key insulin signaling components namely

reduced expression of *Insr*, *Pik3r1*, *Akt1*, and *Akt2* and increased expression of *Irs1*. At the protein level expression of p85α, PDPK1, and Akt was significantly reduced while IRS1 was increased[378]. CBK-Cre(+) hearts presented increased cardiomyocyte insulin sensitivity in both in vivo and ex-vivo perfused hearts manifested by increased levels insulin stimulated levels of phosphorylation, additionally, pAkt was chronically increased throughout the day[378]. Interestingly, while CBK-Cre(+) hearts display increased basal and insulin stimulated pAkt, both oxidative and non-oxidative glucose utilization are reduced. Congruent with this observation abundance and phosphorylation of Akt targets AS160 (GLUT4 trafficking and glucose utilization) and GSK3β (regulation of glycogen synthesis) are reduced[378]. It was further observed that inhibitor 1 (I-1; endogenous inhibitor of protein phosphatase 1 (PP1; Responsible for targeting GSK3β and AS160)), is reduced in CBK-Cre(+) hearts.

Autophagy also appears to be impacted by abblation of the cardiomyocyte clock with chronically activated mTOR resulting in a repressive effect on autophagy via the Akt/mTOR/ ULK1 pathway. Reduction in autophagy was accompanied by augmented protein synthesis via the Akt/mTOR/S6/4EBP1 pathway[378].

Overall, the effect of cardiomyocyte-specific *Bmal1* knockout is to reduce autophagy, increase protein synthesis, via increased insulin stimulated Akt phosphorylation as well as increased basal pAkt. These effects are accompanied by cardiac hypertrophy and dilated cardiomyopathy eventually ending in heart failure and early mortality.

Although the CBK-Cre(+) mice present with dilated cardiomyopathy or systolic heart failure, in contrast to the diastolic heart failure classically associated with

acromegaly, some of the effects of GH/IGF-1 are to cause a state of insulin resistance reduce glucose oxidation[100], and hypertrophy dependent on the mTOR pathway[146], all of which are observed to some degree in the CBK-Cre(+) hearts. Therefore, Hypothesis 1 (H1) states that cardiomyocyte-specific deletion of *Bmal1* resulted in altered GH/IGF-1 signaling in the heart, thereby contributing to the development of hypertrophy and the metabolic effects observed in the CBK-Cre(+) hearts.

To address H1 we devised two study aims. H1 Aim 1, to describe the effect of cardiomyocyte-specific *Bmal1* deletion on GH/IGF-1 signaling. H1 Aim 2 determine the contribution of GH/IGF-1 signaling in development of HF in CBK-Cre(+) hearts by hemizygous deletion of the igf-1 gene in CBK-Cre(+) hearts.

H1 Aim 1 was addressed by analyzing gene expression of major components of the GH/IGF-1 signaling pathway in CBK-Cre(+) animals compared to CBK-Cre(-) controls throughout the day. Positive gene expression findings, namely increased expression of *ghr* and *igf-1* mRNA, prompted the assessment of GH sensitivity in the heart.

H1 Aim 2 was addressed by creating the CBKI-Cre(+) mouse, which attempts to ameliorate or rescue some of the cardiac hypertrophy phenotype in the CBK-Cre(+) mouse by reducing or normalizing *igf-1* gene expression in the CBK-Cre(+) hearts by hemizygous deletion of the *igf-1 gene*. We were able to confirm the homozygous deletion of *Bmal1* and hemizygous deletion of *igf-1* in the CBKI-Cre(+) mouse model as well as reduction in *igf-1* gene expression. We then initiated a longitudinal echocardiography study assessing cardiac form and function in CBK-Cre(+) and CBKI-Cre(+) mice as well

as in their Cre(-) controls, CBK-Cre(-) and CBKI-Cre(-) respectively, beginning at 16 weeks of age, prior to the development of cardiac dysfunction in CBK-Cre(+) mice.

This work establishes a basic understanding of the influence of cardiomyocyte-specific circadian clock ablation via *Bmal1* deletion on GH/IGF-1 signaling. Furthermore, it describes time of day differences in cardiac response to GH in control mice, which are lost upon cardiomyocyte-specific clock ablation. We also describe the partial rescue effect of cardiomyocyte-specific *igf-1* hemi-deletion with simultaneous cardiomyocyte-specific Bmal1 deletion (CBKI-Cre(+) mouse). These findings establish aberrant GH signaling upon circadian clock disruption as a possible contributing factor to increased cardiovascular disease risk in such individuals.

The assessment of cardiac GH sensitivity in CBK-Cre(+) and CBK-Cre(-) mice and paucity of published data on tissue-specific GH sensitivity and responsiveness prompted another study designed to address Hypothesis 2 (H2) that GH sensitivity and responsiveness differ between key metabolic tissues, namely heart, liver, kidney, white adipose tissue (WAT), and skeletal muscle (gastrocnemius). From this hypothesis we developed two study aims. H2 Aim 1: obtain detailed GH dose response information in major metabolic tissues in wild-type male mice and compare the relative tissue sensitivity and responsiveness between tissues. H2 Aim 2: describe possible correlations between protein abundance of key GH signaling components in major metabolic tissues and their relative GH sensitivity and responsiveness.

H2 Aim 1 was addressed in the following manner. Sixteen-week old wild-type C57B/6J mice were treated with an intravenous injection of either saline or one of nine GH doses for 5 minutes. The acute GH response of heart, liver, kidney, WAT, and

gastrocnemius was assessed using immunoblot comparison of STAT5 phosphorylation between doses within each tissue and also between tissues. Significant differences were found in tissue-specific GH sensitivity with liver and kidney both significantly more sensitive than heart, gastrocnemius, and WAT. Differences were also noted in tissue-specific responsiveness.

H2 Aim 2 was addressed by assessing protein abundance of key GH signaling components (GHR, JAK2, and STAT5) followed by plotting the relative tissue-specific abundance for each protein against the relative tissue-specific sensitivity and responsiveness. Correlation analysis was performed to determine possible correlation between protein abundance and relative sensitivity or responsiveness. This produced the key finding that STAT5 protein abundance has a trend toward being significantly correlated to maximum responsiveness.

This work is important as it addresses an arena of knowledge that has received little attention. We describe tissue-specific sensitivity and responsiveness in heart, liver, kidney, WAT, and gastrocnemius. We also describe the trend for correlation between STAT5 abundance and inter-tissue differences in maximum responsiveness. This work provides an invaluable reference for anyone attempting to treat any of these tissues with a submaximal dose of GH. It also provides the field of GH research with a basic understanding of how these tissues differ one from another in their response and sensitivity to GH. This understanding could provide clues as to the role of GH signaling in these tissues.

The following chapter entitled “DIFFERENTIAL TISSUE RESPONSE TO GROWTH HORMONE IN MICE” will address Hypothesis 2 and will be followed by the

chapter entitled “THE INFLUENCE OF THE CIRCADIAN CLOCK ON THE GROWTH HORMONE SIGNALING PATHWAY IN THE HEART”, which will address Hypothesis 1.

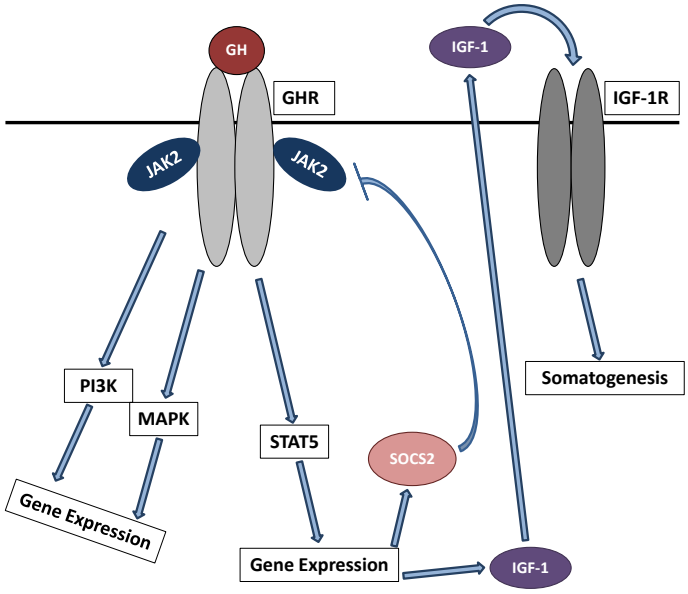

Figure 1 GH signaling pathways.

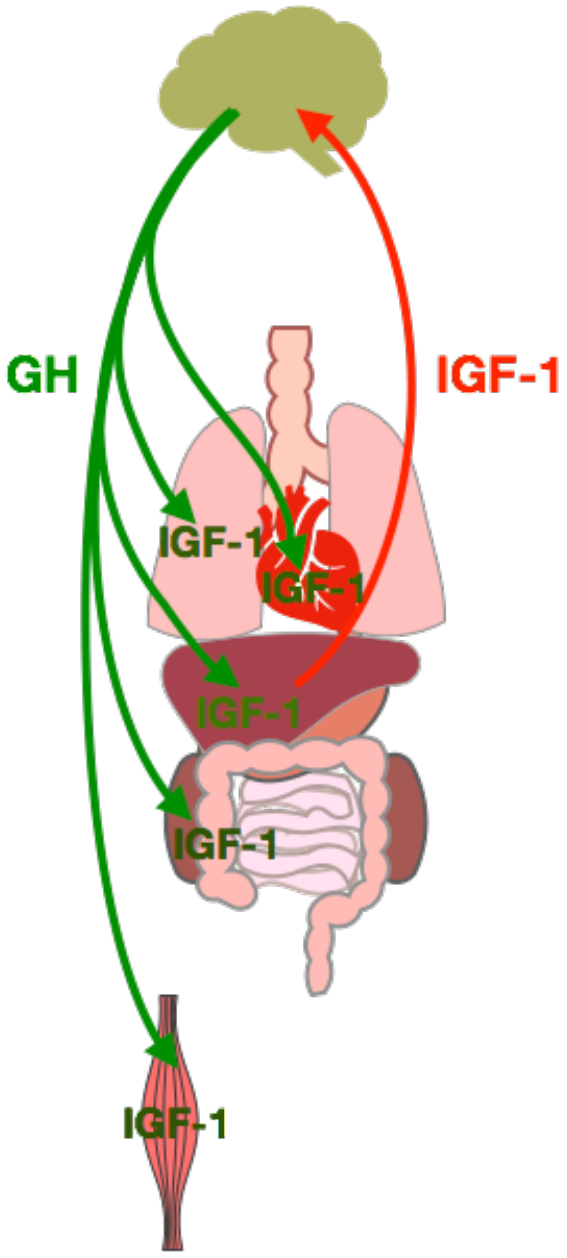

Figure 2 Schematic of basic systemic GH/IGF-1 signaling.

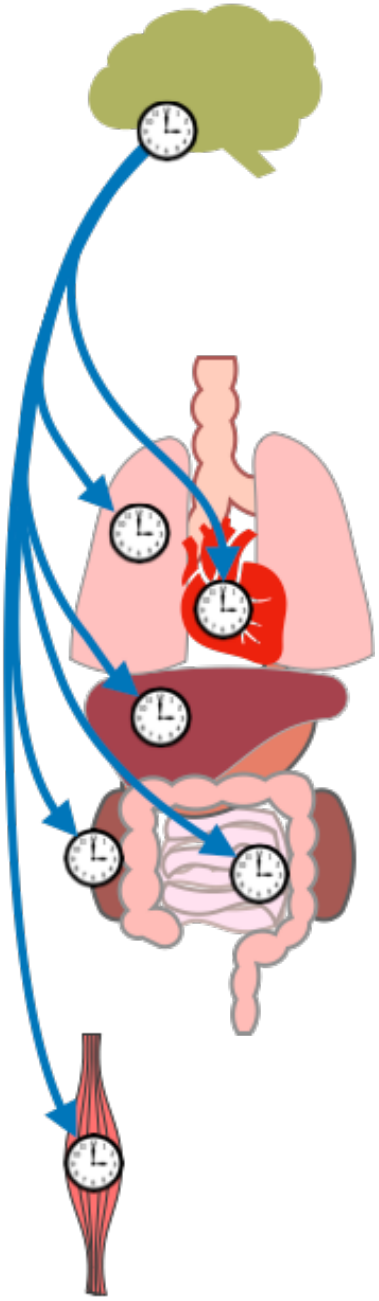

Figure 3 The central circadian clock synchronizes peripheral clocks located in nearly every nucleated cell in the body.

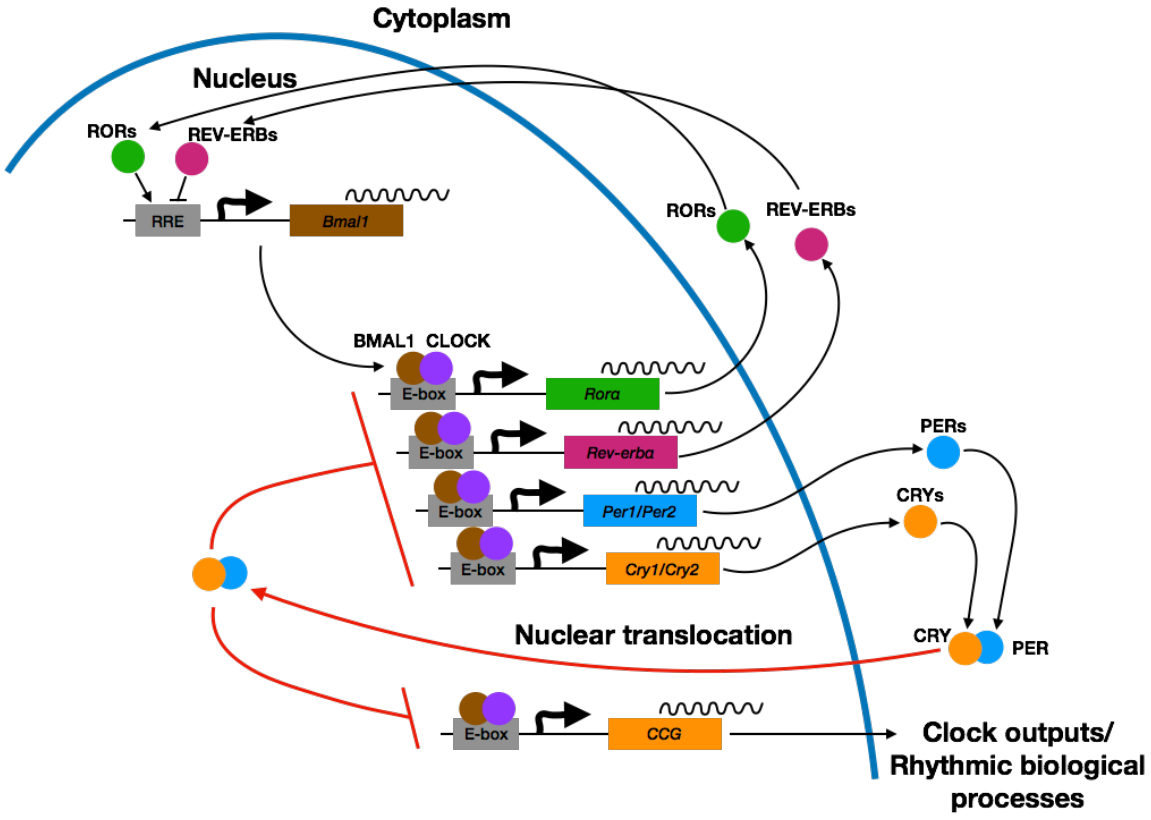

Figure 4 Schematic of the circadian clock molecular mechanism.

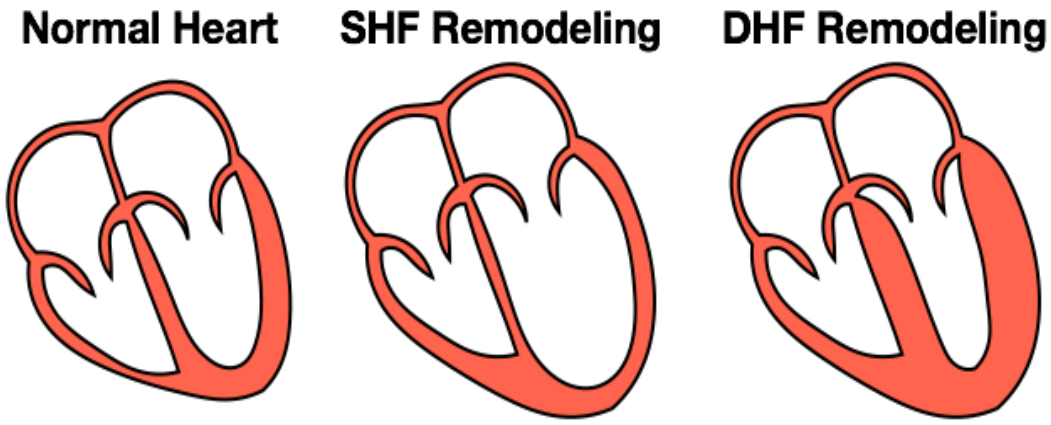

Figure 5 Remodeling observed in systolic HF (SHF) and diastolic HF (DHF).

DIFFERENTIAL TISSUE RESPONSE TO GROWTH HORMONE IN MICE

by

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ABSTRACT

Growth hormone (GH) released from the anterior pituitary acts on multiple tissues throughout the body. To date, a comparative analysis of acute GH signaling in key metabolic tissues has not been performed. Herein we address this knowledge gap. Acute tissue response to GH challenge was assessed by measuring signal transduction via immunoblotting. Our results revealed differential tissue sensitivities with liver and kidney being equally GH sensitive and more sensitive than white adipose tissue, heart, and gastrocnemius. Analysis of maximal (MAX) responsiveness revealed gastrocnemius to have the greatest MAX response (615A.U.) compared to heart (296A.U.), liver (104A.U.), white adipose tissue (54A.U.), and whole kidney (1.8A.U.). Differences in MAX responsiveness were positively correlated with tissue STAT5 abundance, while differences in sensitivity were not explained readily by differences in GH receptor protein abundance. In summary, GH sensitivity and responsiveness of distinct metabolic tissues differ substantially. Such differences may play a physiologic role in healthy animals. These data will serve as a template for analyzing changes in disease states.

Keywords: GH sensitivity, GH responsiveness, tissues

Introduction

Growth hormone (GH), produced in the anterior pituitary, plays a major role in both longitudinal growth and metabolism [1, 2]. Dysregulation in GH signaling, either increased in acromegaly and gigantism, [3] or decreased in short stature or dwarfism, has profound consequences on growth and development [4, 5]. GH also impacts lifespan; GH excess is associated with increased morbidity [6] while GH deficiency promotes longevity [7]. GH binds cell surface receptors (GH receptor; GHR) on target cells, resulting in GHR-associated Janus kinase 2 (JAK2) autophosphorylation and subsequent phosphorylation of GHR intracellular domain tyrosine residues [8-13]. Signal transducer and activator of transcription 5 (STAT5) docks at the phosphorylated GHR and is phosphorylated by JAK2. pSTAT5 dimers translocate to the nucleus to influence transcription of genes including insulin-like growth factor (IGF)-1 [14-16]; GH's metabolic and somatogenic effects are related to its influence on target cell gene expression.

Assessing acute GH effects in different key metabolic tissues may have once been considered an irrelevant question. Classically, GH was thought to exclusively target the liver, which would then produce IGF-1, (aka somatomedin C) [17]. IGF-1 would subsequently act in an endocrine manner, modulating growth/metabolism in extra-hepatic tissues. This is the somatomedin hypothesis of GH action [18]. Later, D'Ercole et al. [19] showed that IGF-1 is also produced locally by extra-hepatic tissues in response to GH, and that the level of IGF-1 produced after GH administration differs between tissues.

Further, Skottner et al [20] demonstrated that administration of IGF-1 did not affect longitudinal growth in hypophysectomized rats, except at very high concentrations, whereas GH administration induced significant growth. These pioneering studies suggested that IGF-1 might be produced and act locally within target tissues, in contrast to the somatomedin hypothesis. Consistent with these observations, liver-specific IGF-1 knockout mice grow and develop normally, despite diminished circulating IGF-1 [21-23]. As such, a revised hypothesis suggests that circulating (hepatic-derived) IGF-1 is responsible for negatively regulating GH secretion, whereas local (extrahepatic) IGF-1 plays a primary role in longitudinal growth [24].

Despite interest in extra-hepatic actions of GH and IGF-1, little information is available that compares GH signaling among organs in intact animals. Because of the distinct roles of GH signaling in the liver compared to other metabolic tissues, we hypothesized that GH sensitivity and responsiveness would differ in hepatic versus extra-hepatic tissues. Herein, we compare acute *in vivo* sensitivity and MAX responsiveness to exogenously administered GH in mice among liver, heart, kidney, skeletal muscle, and adipose tissue. Our results indicate substantial differences between tissues that may be important for understanding tissue-specific metabolic and growth promoting effects of GH.

Materials and Methods

Unless otherwise stated, reagents were obtained from Sigma (St. Louis, MO).

Animals

All animal husbandry and experimental protocols were carried out according to the Guide for the care and use of Laboratory animals [1996 (7th ed.) Washington, DC: National Research Council, National Academies Press] and in compliance with the local IACUC standards. At 15 weeks of age (+/- 3 days), male C57B6J mice (Jackson Laboratories; cat # 000664) were individually housed in standard conditions under a 12hr:12hr light:dark cycle and had ad libitum access to standard rodent chow and water. After acclimatization to single housing, mice were placed in wire bottom cages without food at the beginning of the light cycle. Following a 6-hr fast, mice were anesthetized with 400 mg/kg chloral hydrate intraperitoneally; once completely anesthetized, either saline (control) or human recombinant GH (2, 4, 8, 12.5, 20, 50, 80, 120, 200 ng/g_{bw}; gift from Eli Lilly Co, Indianapolis, IN) was injected into the inferior vena cava. Five minutes after saline or GH administration, heart, liver, kidney, white adipose tissue (epididymal fat; WAT), and skeletal muscle (gastrocnemius: gastroc) were rapidly excised in that order and flash frozen in liquid nitrogen prior to biochemical analysis. Total time of tissue extraction for each animal was 3-4 minutes. Five-minute treatment was selected because unpublished data from a collaborator suggested that acute STAT5 phosphorylation stimulated by intravenous administered GH peaked around 5 minutes, and to eliminate possible secondary effects so we could more directly assess the acute effect of GH treatment on STAT5 phosphorylation.

Female samples for PRLR mRNA control were harvested from female C56Bl6/J that were ad libitum fed and aged matched, age 2-3 months. Pregnant samples were harvested at gestational day 16.5.

Immunoblotting

Protein lysates were prepared from tissues crushed to powder under liquid nigrogen (~20mg) using 300ml of tissue lysis buffer (50 mM Tris 7.3, 150 mM NaCl, 1mM EDTA pH 8.1, 1.5mM $MgCl_2$, 10% Glycerol, 1% Triton X-100, 10mM $Na_4P_2O_7$, 100mM NaF, 1mM Na_3VO_4, 1mM phenylmethanesulfonyl fluoride, 5 mg/ml aprotinin, and 5mg/ml leupeptin). Lysates were resolved under reducing conditions by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences), followed by blocking with 2% BSA. Membranes were immunoblotted with anti-phospho-STAT5 antibody (Y694; Cell Signaling; 9351L) (1:1000), anti-STAT5 antibody (Santa Cruz Biotechnology; sc-835) (1:1000), anti-GHR (polyclonal anti-$GHR_{cytAL-47}$; against the intracellular domain of GHR) [25] (1:1000), anti-PRLR (anti-$PRLR_{cytAL-84}$; against the human PRLR ICD) [26] (1:1000), PRL-R (H-300) (Santa Cruz Biotechnology; sc-20992), and anti-JAK2 (anti-$JAK2_{AL-33}$) [27] (1:1000). Densitometry was performed using UVP Software 8.0. Ponceau staining as a loading control was done using ponceau stain (0.5g Ponceau S, 1mL Glacial acetic acid, 99mL distilled and deionized water).

Table 1 Antibody Table

Antibody	RRID	References
Phospho-Stat5 (Tyr694) Antibody	AB_331594	
Stat5 (C-17) antibody	AB_632446	
anti-GHR (polyclonal anti-$GHR_{cytAL-47}$; against the intracellular domain of GHR)		25
anti-PRLR (anti-$PRLR_{cytAL-84}$; against the human PRLR ICD)		26
PRL-R (H-300) antibody	AB_2237692	
anti-JAK2 antibody		27

Curve fitting and Statistical Analysis

Dose response curve data were fit to the Sigmoidal Dose Response (with variable slope) curve: [Y=BOTTOM + (TOP-BOTTOM)/(1+10^((LogEC_{50}-X)*HillSlope))]; Y=response; X=log[dose]; HillSlope=slope of linear section of the dose response curve; TOP=point in the dose response curve at which an increase in "X" yields little to no increase in "Y"; EC_{50}=the effective concentration (or dose) at which 50% of the MAX response is achieved [28]. This analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Sensitivity was defined by the value EC_{50}. Responsiveness was defined by the TOP value, herein referred to as the MAX response. During the constrained fit, the TOP and BOTTOM parameters were fixed at 100 and 0 respectively. Tissue-specific differences in protein abundances, were assessed via 1-way ANOVA using SPSS followed by post hoc analysis via Tukey test. Regression analysis to assess the correlation between STAT5 abundance and MAX response was performed using Excel.

Gene analysis

mRNA was isolated from male mouse tissues using a QIAGEN RNeasy Mini Kit (Cat. No. 74104). Reverse transcription was done using the HIGH CAPACITY CDNA

RT KIT (Cat. No. 4368814) from ThermoFisher. qPCR measurements were done using the mPRLR TaqMan Gene Exp. Assay (Assay ID Mm04336676_m1, Cat. No. 4351372) from ThermoFisher.

Data display

Due to differences in normalizing proteins across tissues, densitometry data are normalized to total protein loaded on the gel except in Figure 2 where pSTAT5 is normalized to STAT5 as a loading control; tissue differences in STAT5 abundance do not influence sensitivity. To reduce positional bias during the immunoblot transfer procedure, samples were loaded on gels in randomized order; where possible, n=1 for each GH dose was included on each gel. Densitometry was performed on non-manipulated blots. For clarity, representative blots presented were constructed as follows: a single gel was chosen for each tissue, after which lanes were rearranged such that GH doses were displayed in ascending order.

Results

Murine peripheral tissues display differential sensitivity and MAX response to GH

Although they varied greatly in responsiveness, kidney being the least maximally responsive, all tissues examined displayed dose-dependent GH effects on STAT5 phosphorylation (Figures 1A, and 2; SI 1-10). Calculation of EC_{50} values (see Methods section for details) revealed tissue-specific differences in GH sensitivity (Figure 1B, Table 1; SI 1,3,5,7,9,11; SI File 1). EC_{50} values for liver and kidney did not differ significantly, although both were substantially lower (i.e. greater sensitivity) than WAT,

heart, and gastrocnemius. Differences were likewise observed in tissue responsiveness (Figure 1B, Table 1; SI 1-12; SI File 1). Gastrocnemius had the greatest (extrapolated) MAX GH response, followed by heart, liver, WAT, and kidney (Figure 1B, Table 1; SI 1-12; SI File 1). There was large variability (i.e. confidence intervals) in the EC_{50} and MAX values for gastrocnemius, heart, and WAT because the predicted MAX value was not defined by experimental data points (as predicted GH doses required for MAX response were too high). Therefore, as a secondary analysis we normalized the data for each curve such that the highest experimental data point was 100 while the lowest was 0, and fit the data to the sigmoidal dose response curve using the constraints TOP=100 and BOTTOM=0 (Figure 2). This analysis yielded similar EC_{50} calculations for liver and kidney, as well as similar R^2 values for all curve fits. Furthermore, it confirmed, statistically, that liver and kidney have the same EC_{50} and that they are significantly more sensitive than WAT, heart, and gastrocnemius (Figure 2, Table 1).

Table 2

	Liver	Kidney	WAT	Heart	Gastrocnemius
Free Fit					
EC_{50} (ng/g_{bw})	10	14	1248	4901	1642
MAX Response (A.U.)	104.3	1.8	53.6	296.1	615.2
R^2	0.8622	0.6500	0.6343	0.6928	0.7379
Constrained Fit					
EC50 (ng/g_{bw})	11	14	46	63	82
EC50 (ng/g_{bw}) 95% CI	7.7 - 16.9	9.2 – 20.9	30.9 – 67.1	47.0 – 83.9	65.0 – 103.5
R^2	0.7344	0.6744	0.6288	0.7220	0.7241

Table 2. Curve fit parameters: from fitting dose response data from Figure 1B to the Hill equation without constraints (Free Fit), and from Figure 2 using fit constraints (Constrained Fit).

Differential abundance of GH signaling proteins among tissues

The factors that influence tissue sensitivity to a hormone often reside at the level of the receptor. Accordingly we assessed GHR abundance by immunoblotting with an antibody against the GHR intracellular domain, which revealed highest abundance in WAT (2.05 A.U.), followed by liver (1.00 A.U.), heart (0.61 A.U.), kidney (0.29 A.U.), and gastrocnemius (0.28 A.U.) (Figure 3A,E; SI 13-14, 21; SI File 2). The current study utilized human GH, which can also induce STAT5 phosphorylation via the prolactin receptor (PRLR) [29-31]. PRLR was not detected by immunoblotting within any of the five tissues investigated by use of the anti-$PRLR_{cytAL\text{-}84}$ (SI 15A); this was validated using a second anti-PRLR antibody, the PRL-R (H-300) antibody (SI 15B); although analysis of prlr mRNA levels revealed higher expression in the liver (compared to the other tissues), absolute expression levels were very low (SI 16). Thus, analyses of GHR and GHR-associated receptor abundance did not readily explain observed tissue-specific differences in GH sensitivity (although relatively high GHR expression in the liver may contribute to elevated GH sensitivity in this tissue).

In contrast to hormone sensitivity, the responsiveness of a tissue to a hormone is influenced by factors downstream of the receptor, including abundance of downstream signaling molecules. To this end, we assessed JAK2 and STAT5 abundance by immunoblotting. Relatively modest differences were observed in JAK2 abundance, with lowest levels in gastrocnemius, and highest levels in kidney (Figure 3B,F; SI 17-18, 21; SI File 2). STAT5 abundance did not differ between liver, gastrocnemius and heart, but was significantly lower in WAT and kidney (Figure 3C,G; SI 19-21; SI File 2).

Regression analysis revealed a correlation (correlation coefficient: +0.8296) between the STAT5 abundance in a tissue and its MAX response ($p = 0.082$) (Figure 3D).

Discussion

The purpose of the current study was to define tissue-specific differences in GH sensitivity and MAX responsiveness to GH. Here, we report that the order of GH sensitivity was liver=kidney>WAT=heart=gastrocnemius, while the order of GH MAX responsiveness was gastrocnemius>heart>liver>WAT>kidney and roughly correlated with STAT5 protein abundance. Such observations lead to questions with regards to physiologic significance. While the MAX response predicted from the free curve fitting in gastrocnemius and heart were much greater than in the other tissues, the levels of GH required to attain that MAX stimulation are far beyond physiologic levels. However, despite the error in these predicted values being large, the fact that there was a near-significant correlation between STAT5 and MAX response supports the idea that these values are good estimates. The liver and kidney exhibit the highest level of GH sensitivity (relative to other tissues investigated) and the other three tissues were indistinguishable statistically. That this general relationship holds true regardless of whether constraints were used supports the idea that the liver and kidney respond to GH at much lower concentrations than WAT, heart, and gastrocnemius. GH plays a number of important roles in the liver, including generation of circulating IGF-1 (which acts in a feedback manner on GH secretion) and hepatic metabolism. In the latter case, GH effects generally oppose those of insulin specifically they suppress glycolysis in favor of fatty acid oxidation and promote glycogenolysis and in prolonged fasting conditions promote

gluconeogenesis [1, 32-35]. Thus, increased GH secretion during sleep likely plays an important role in maintenance of blood glucose levels. Interestingly, the kidney is also a gluconeogenic tissue, contributing up to 50% of endogenous glucose production in the starved state [36]. GH signaling in the kidney is also important for normal sodium and water retention; GH deficiency leads to renal insufficiency, while excess leads to hypertension, renal hypertrophy and failure [36]. Thus, our observation that the kidney is relatively GH sensitive (similar to the liver) is consistent with essential GH actions in this tissue. GH signaling is also important in WAT, as this endocrine factor shifts metabolism from glucose utilization toward lipolysis and fatty acid oxidation, thereby minimizing reliance on muscle protein catabolism during periods of fasting (such as the sleep period) [1, 37-39]. In contrast, GH signaling in the adult heart must be closely regulated, thus preventing excessive growth (e.g., in acromegaly) and subsequent contractile dysfunction [40]. Similar to the heart, GH signaling in skeletal muscle mainly influences muscle size, but not contractile force [37, 39, 41]. The low GH sensitivity of gastrocnemius muscle may suggest that skeletal muscle growth in an adult mouse, in response to exercise for example, may be through GH-independent mechanisms.

Subsequent interrogation of known GH signaling components provided potential mechanistic insights with regards to tissue-specific differences in GH responsiveness/sensitivity. For example, STAT5 levels were correlated with MAX response in a given tissue. Additionally, GHR, levels were relatively high in liver, consistent with high GH sensitivity. Our findings are consistent with those of Walker et al [42], who reported that GHR mRNA in the rat kidney was roughly 33% that of liver.

Additional studies are required to elucidate fully the mechanisms mediating tissue-specific differences in GH sensitivity/responsiveness.

The current study focused on a particular acute signaling response of various tissues to exogenously administered GH (namely STAT5 phosphorylation). This approach has benefits and drawbacks. Although we did not assess the long-term response to endogenous GH pulses, this approach allowed us to directly compare acute responses to GH in multiple tissues simultaneously. As STAT5 is a critical mediator of acute GH action, we were able to observe direct GH effects, rather than compensatory effects over longer periods.

As noted above, GH stimulates glycogenolysis in liver and kidney during fasting[34, 35]. The mice in this study were fasted for 6 hours prior to GH treatment. Therefore, it is possible that we would have observed a different relationship among tissues of GH sensitivity in mice if food had not been withdrawn in the 6 hours leading up to GH treatment. However, the period of fasting corresponded to the first 6 hours of the rest phase, during which food consumption is generally reduced (relative fasting), compared to the active period [43]. Thus, the relative physiological effects of the strict fast are likely limited. We are mindful, however, that GH sensitivity and MAX response were only assessed at one time of day in our study. Because the circadian clock may control both secretion and sensitivity to hormones [44], it is possible that relative tissue sensitivity to GH may vary depending on the time of day.

In summary, the current study reveals a correlation between STAT5 abundance and the MAX GH response in these tissues, while GH sensitivity is not correlated to GHR. Thus, an important determinant of MAX GH response appears to be STAT5 abundance,

while the determinants of in vivo GH sensitivity are more complex. We speculate that in pathological states GH action may be influenced by alterations in GH sensitivity and/or responsiveness, not solely by changes in circulating GH levels. Our data from wild-type mice will serve as a template for analyzing such changes in disease states.

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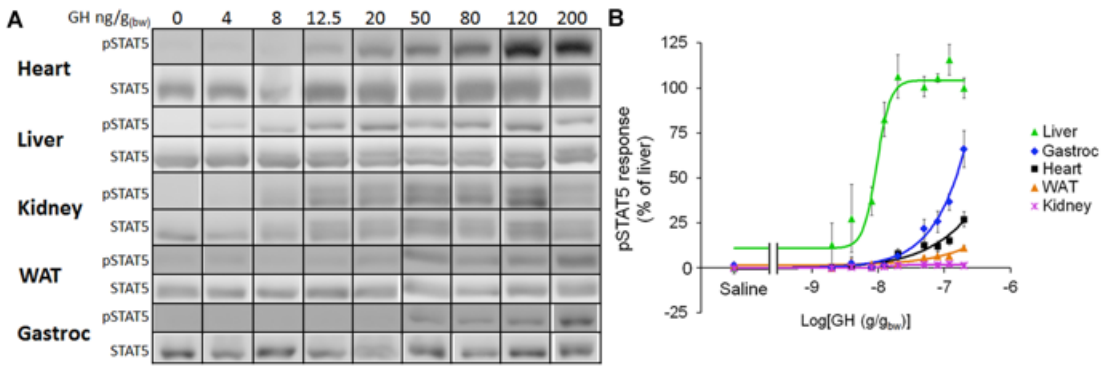


Figure 1. Tissue GH Sensitivity and Responsiveness (Free Fit). (A) Representative blots for dose-dependent GH-induced STAT5 phosphorylation in liver, kidney, WAT, heart and gastrocnemius muscle. Exposure time was adjusted to allow for visual comparison of dose dependencies between the tissues and as such the intensities of bands may not be compared between tissues. (B) Dose response data from liver, kidney, WAT, heart and gastrocnemius fit to the Hill Equation without fit constraints (Mean +/- SEM; n = 3-10).

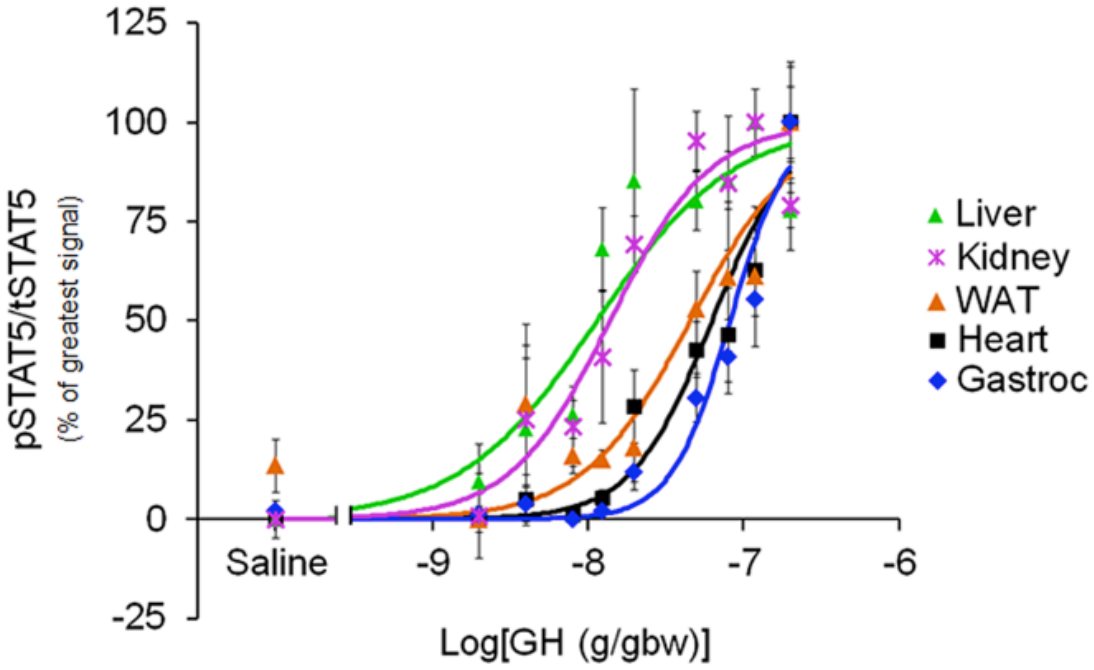


Figure 2 Tissue GH Sensitivity (Constrained Fit). Dose response data normalized such that the highest value within each tissue is 100 and lowest is 0, and fit to the Hill Equation with the constraints: TOP=100 and BOTTOM=0.

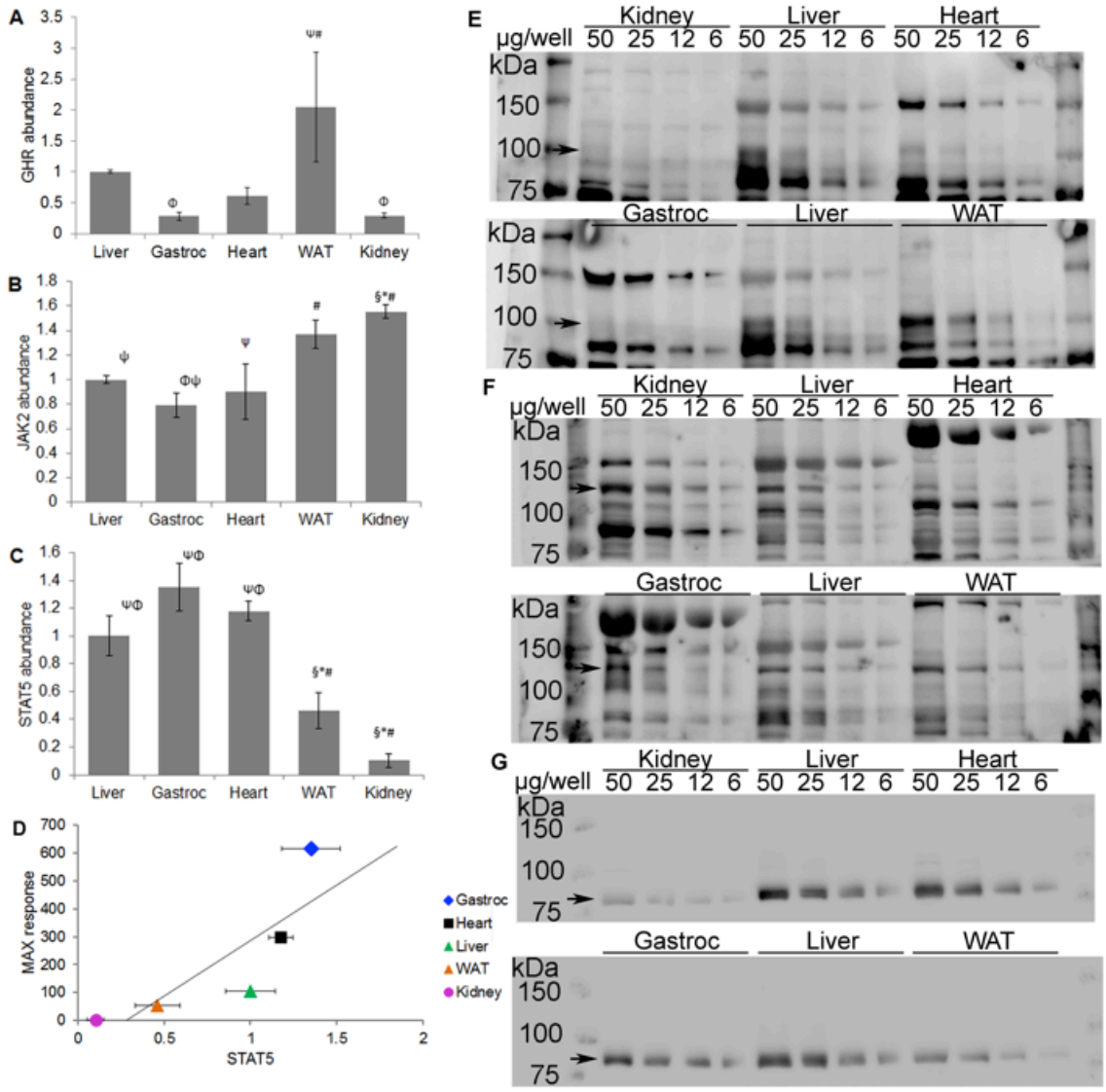
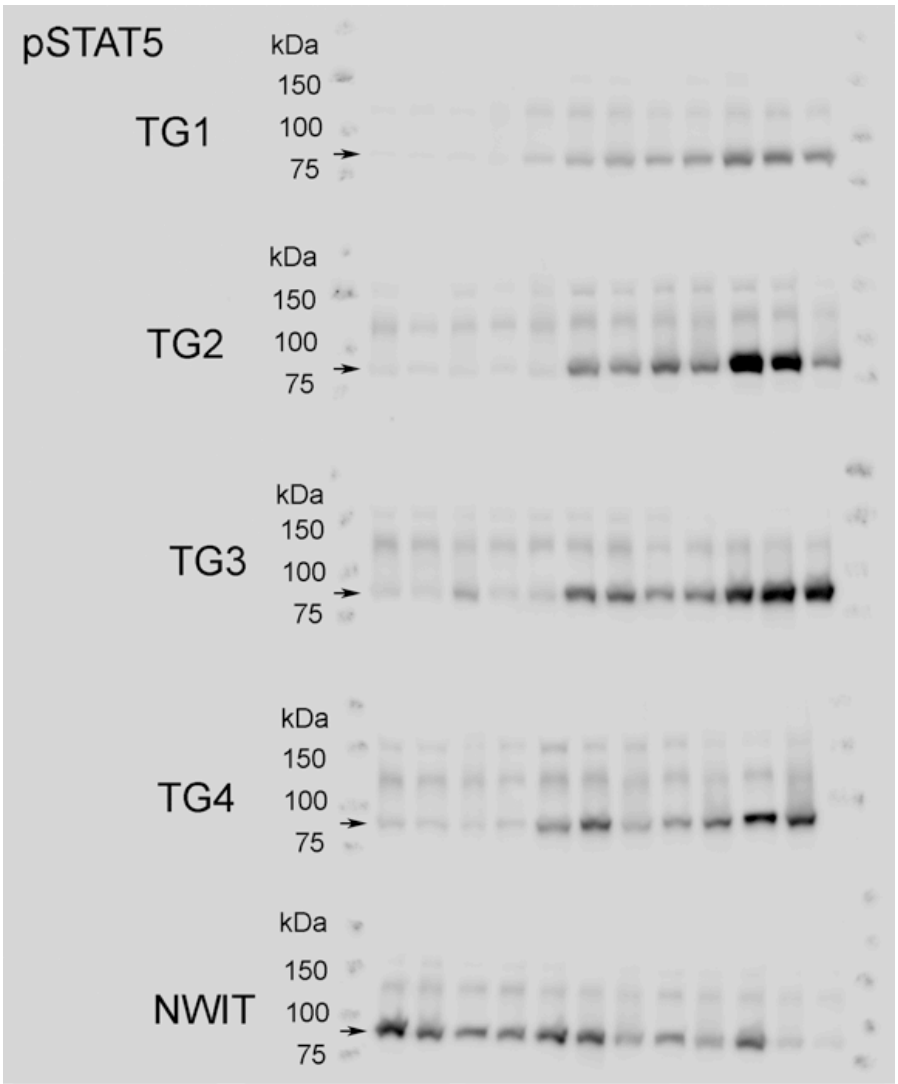


Figure 3 GH Signaling Components. Densitometry analysis and representative blots of tissue-specific comparison of protein abundance for (A,E) GHR, (B,F) JAK2, and (C,G) STAT5 (relative to liver). (Mean +/- SEM; n = 5). L = liver; K = kidney; H = heart; G = gastrocnemius; W = WAT. Significance: all symbols represent $p < 0.05$ compared to: *, liver; #, gastrocnemius; §, heart; Φ, WAT; Ψ, kidney. (D) Linear regression analysis displaying the correlation between tissue-specific MAX response and STAT5 (Mean; n=5) abundance (correlation coefficient: +0.83; p=0.082).

SI 1-12 Raw Immunoblot Images: raw immunoblots created from heart (SI 1-2), liver (SI 3-4), kidney (SI 5-6), WAT (SI 7-8), and Gastrocnemius (SI 9-10) samples, used to compile the representative figure 1A (SI 1-10 TG1) and on which densitometric analysis was performed to obtain the data used in Figure 1B (SI 1,3,5,7,9,11) and Figure 2 (SI 1-12). The blots were first probed for pSTAT5 followed by stripping and re-probing for STAT5. TG 1-4 denotes Tissue Gels or the blots that contain all of the samples from a given tissue. NWIT denotes Normalize Within Tissue or the gel that was used to normalize the 4 TGs to one another. NBT denotes Normalize Between Tissues or the gels that were used to normalize the data between the five tissues so that relative comparisons of dose response between tissues might be made. Note: 4 liver samples are found on both NBT1 and NBT2 and were used to compare relative band intensities between the two gels.



Tissue Gel (TG1)

Lane #	Animal #	Dose (ng/g)
1	1	0
2	2	0
3	3	4
4	4	8
5	5	12.5
6	6	20
7	7	50
8	8	50
9	9	80
10	10	120
11	11	200
12	12	200

Tissue Gel (TG2)

Lane #	Animal #	Dose (ng/g)
1	13	0
2	14	2
3	15	4
4	16	8
5	17	12.5
6	18	20
7	19	50
8	20	80
9	21	120
10	22	200
11	23	200
12	24	200

Tissue Gel (TG3)

Lane #	Animal #	Dose (ng/g)
1	25	0
2	26	2
3	27	4
4	28	8
5	29	12.5
6	30	50
7	31	50
8	32	80
9	33	120
10	34	200
11	35	200
12	36	200

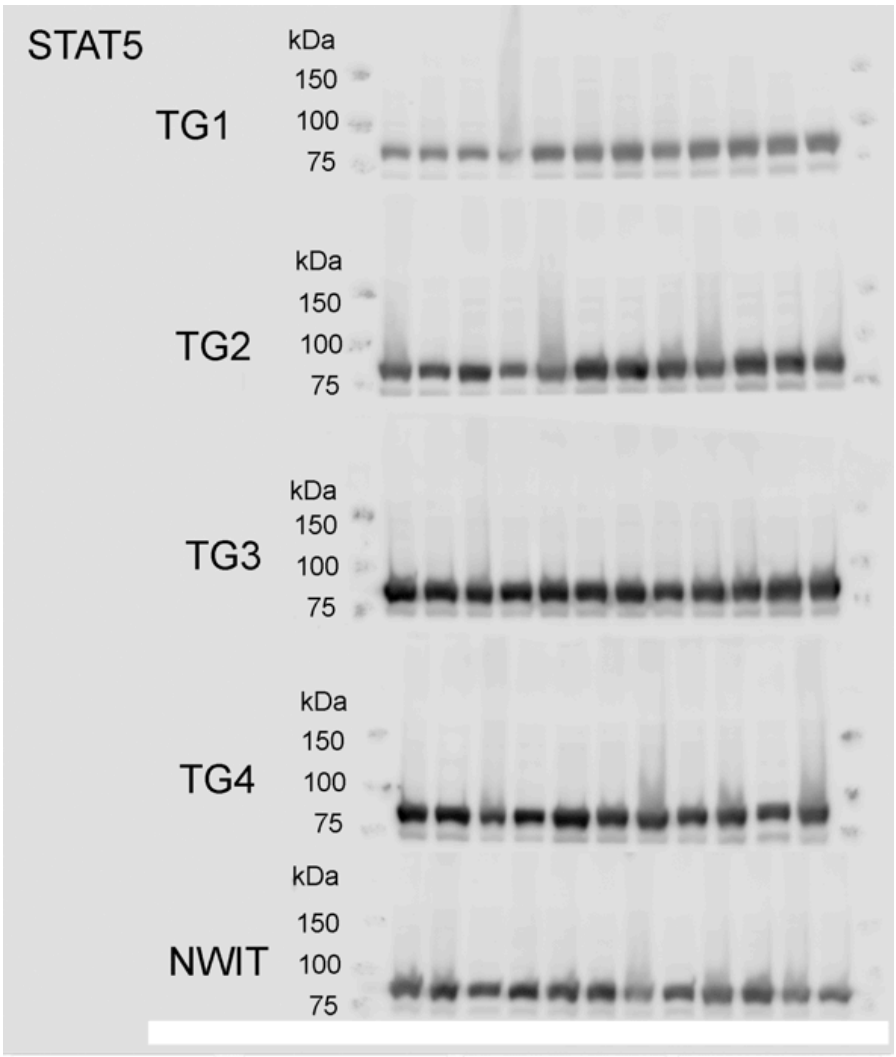
Tissue Gel (TG4)

Lane #	Animal #	Dose (ng/g)
1	37	0
2	38	2
3	39	4
4	40	8
5	41	20
6	42	50
7	43	50
8	44	80
9	45	120
10	46	200
11	47	200

Normalizer Gel w/in tissue Gel (NWIT)

Lane #	Animal #	Dose (ng/g)	Originating (TG)
1	22	200	2
2	34	200	3
3	46	200	4
4	11	200	1
5	23	200	2
6	35	200	3
7	47	200	4
8	12	200	1
9	24	200	2
10	36	200	3
11	7	50	1
12	43	50	4

SI Figure 1



Tissue Gel (TG1)

Lane #	Animal #	Dose (ng/g)
1	1	0
2	2	0
3	3	4
4	4	8
5	5	12.5
6	6	20
7	7	50
8	8	50
9	9	80
10	10	120
11	11	200
12	12	200

Tissue Gel (TG2)

Lane #	Animal #	Dose (ng/g)
1	13	0
2	14	2
3	15	4
4	16	8
5	17	12.5
6	18	20
7	19	50
8	20	80
9	21	120
10	22	200
11	23	200
12	24	200

Tissue Gel (TG3)

Lane #	Animal #	Dose (ng/g)
1	25	0
2	26	2
3	27	4
4	28	8
5	29	12.5
6	30	50
7	31	50
8	32	80
9	33	120
10	34	200
11	35	200
12	36	200

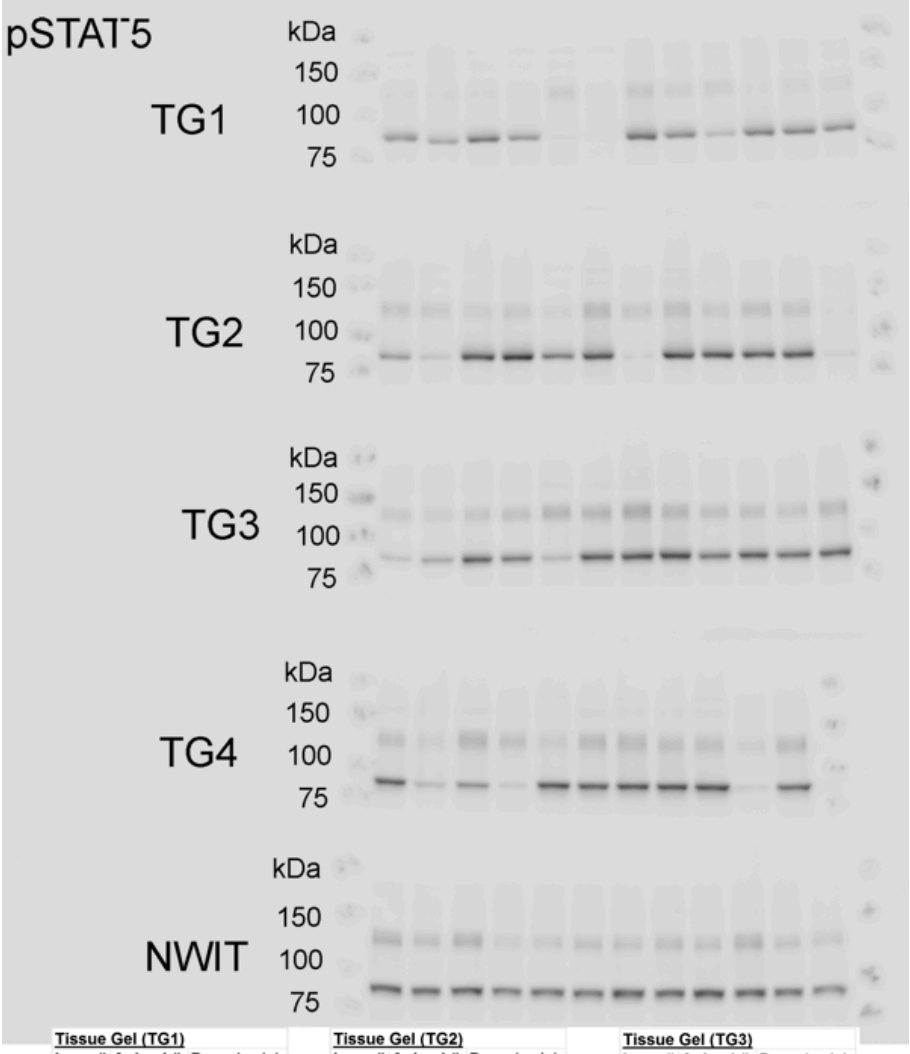
Tissue Gel (TG4)

Lane #	Animal #	Dose (ng/g)
1	37	0
2	38	2
3	39	4
4	40	8
5	41	20
6	42	50
7	43	50
8	44	80
9	45	120
10	46	200
11	47	200

Normalizer Gel w/in tissue Gel (NWIT)

Lane #	Animal #	Dose (ng/g)	Originating (TG)
1	22	200	2
2	34	200	3
3	46	200	4
4	11	200	1
5	23	200	2
6	35	200	3
7	47	200	4
8	12	200	1
9	24	200	2
10	36	200	3
11	7	50	1
12	43	50	4

SI Figure 2



Tissue Gel (TG1)

Lane #	Animal #	Dose (ng/g)
1	8	50
2	4	8
3	6	20
4	11	200
5	1	0
6	2	0
7	10	120
8	5	12.5
9	3	4
10	9	8
11	7	50
12	12	200

Tissue Gel (TG2)

Lane #	Animal #	Dose (ng/g)
1	16	8
2	14	2
3	23	200
4	24	200
5	17	12.5
6	19	50
7	15	4
8	20	8
9	18	2
10	22	200
11	21	120
12	13	0

Tissue Gel (TG3)

Lane #	Animal #	Dose (ng/g)
1	25	0
2	26	2
3	35	200
4	27	4
5	28	8
6	32	80
7	33	120
8	31	50
9	29	12.5
10	34	200
11	30	50
12	36	200

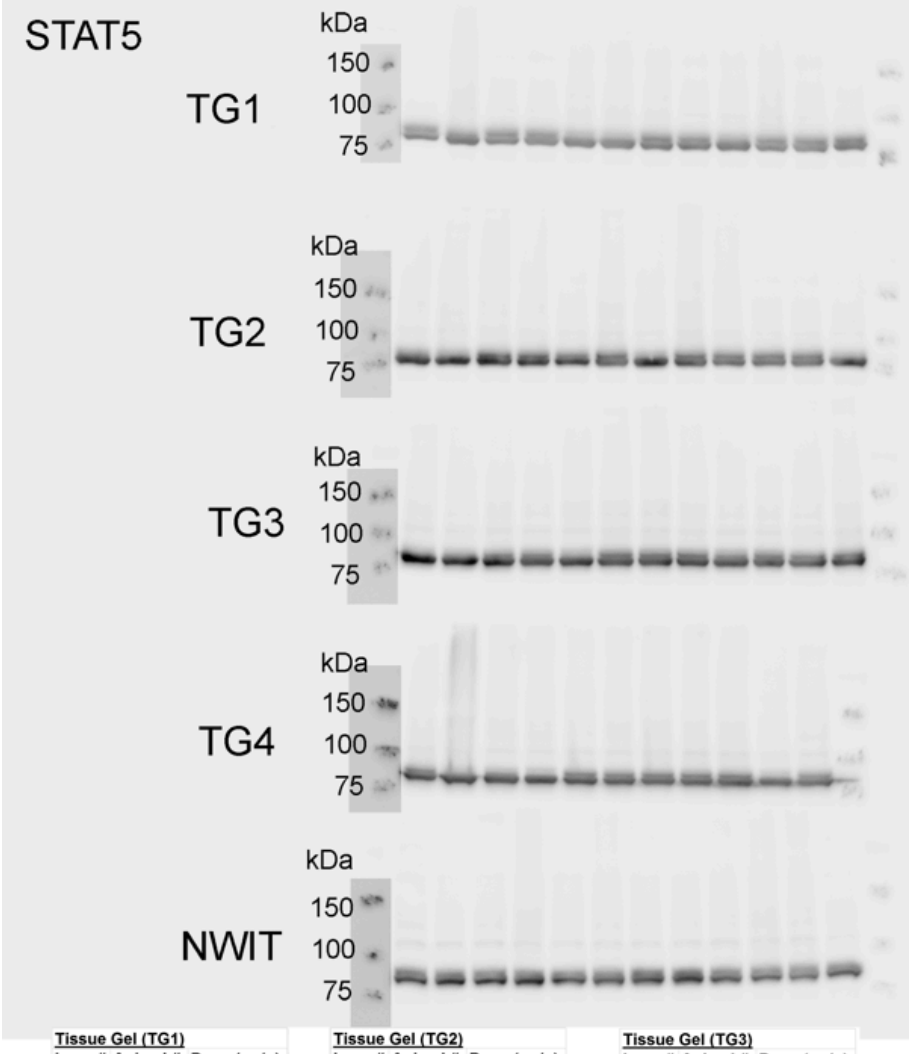
Tissue Gel (TG4)

Lane #	Animal #	Dose (ng/g)
1	41	20
2	39	4
3	40	8
4	38	2
5	42	50
6	43	50
7	44	80
8	45	120
9	47	200
10	37	0
11	46	200

Normalizer Gel w/in tissue Gel (NWIT)

Lane #	Animal #	Dose (ng/g)	Originating (TG)
1	22	200	2
2	34	200	3
3	46	200	4
4	11	200	1
5	23	200	2
6	35	200	3
7	47	200	4
8	12	200	1
9	24	200	2
10	36	200	3
11	7	50	1
12	43	50	4

SI Figure 3



Tissue Gel (TG1)

Lane #	Animal #	Dose (ng/g)
1	8	50
2	4	8
3	6	20
4	11	200
5	1	0
6	2	0
7	10	120
8	5	12.5
9	3	4
10	9	8
11	7	50
12	12	200

Tissue Gel (TG2)

Lane #	Animal #	Dose (ng/g)
1	16	8
2	14	2
3	23	200
4	24	200
5	17	12.5
6	19	50
7	15	4
8	20	8
9	18	2
10	22	200
11	21	120
12	13	0

Tissue Gel (TG3)

Lane #	Animal #	Dose (ng/g)
1	25	0
2	26	2
3	35	200
4	27	4
5	28	8
6	32	80
7	33	120
8	31	50
9	29	12.5
10	34	200
11	30	50
12	36	200

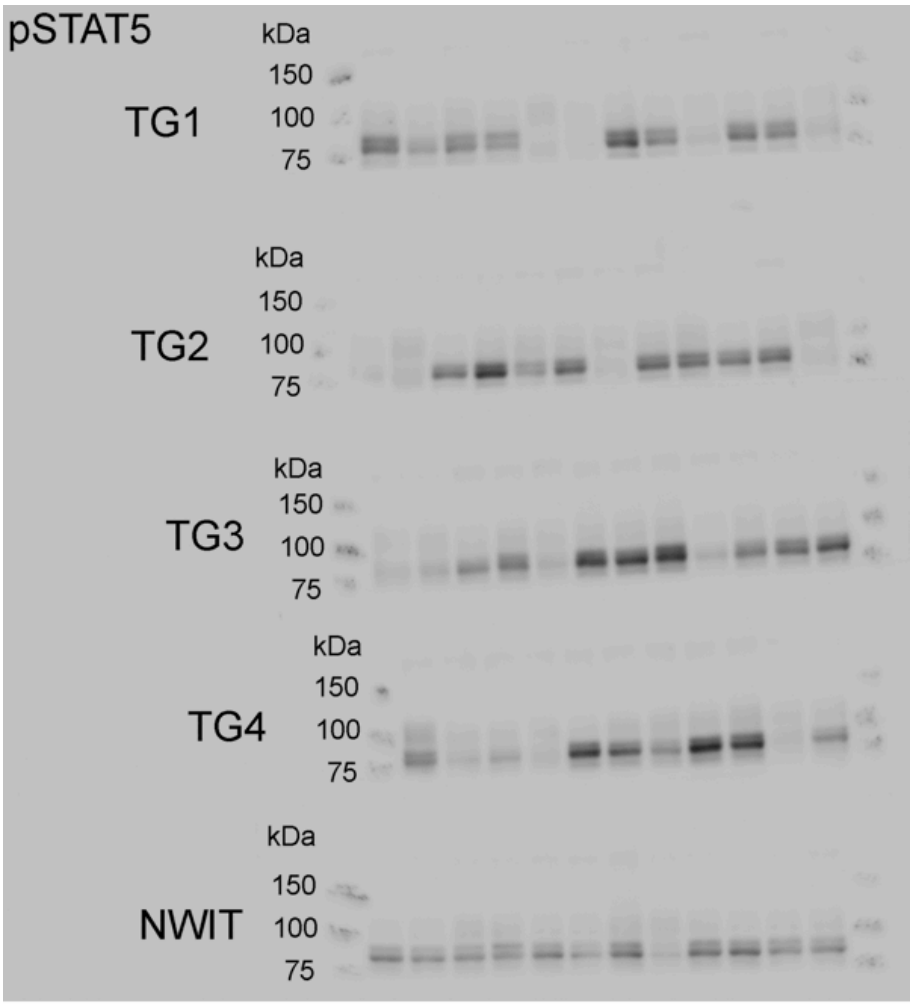
Tissue Gel (TG4)

Lane #	Animal #	Dose (ng/g)
1	41	20
2	39	4
3	40	8
4	38	2
5	42	50
6	43	50
7	44	80
8	45	120
9	47	200
10	37	0
11	46	200

Normalizer Gel w/in tissue Gel (NWIT)

Lane #	Animal #	Dose (ng/g)	Originating (TG)
1	22	200	2
2	34	200	3
3	46	200	4
4	11	200	1
5	23	200	2
6	35	200	3
7	47	200	4
8	12	200	1
9	24	200	2
10	36	200	3
11	7	50	1
12	43	50	4

SI Figure 4



Tissue Gel (TG1)

Lane #	Animal #	Dose (ng/g)
1	8	50
2	4	8
3	6	20
4	11	200
5	1	0
6	2	0
7	10	120
8	5	12.5
9	3	4
10	9	8
11	7	50
12	12	200

Tissue Gel (TG2)

Lane #	Animal #	Dose (ng/g)
1	16	8
2	14	2
3	23	200
4	24	200
5	17	12.5
6	19	50
7	15	4
8	20	8
9	18	2
10	22	200
11	21	120
12	13	0

Tissue Gel (TG3)

Lane #	Animal #	Dose (ng/g)
1	25	0
2	26	2
3	35	200
4	27	4
5	28	8
6	32	80
7	33	120
8	31	50
9	29	12.5
10	34	200
11	30	50
12	36	200

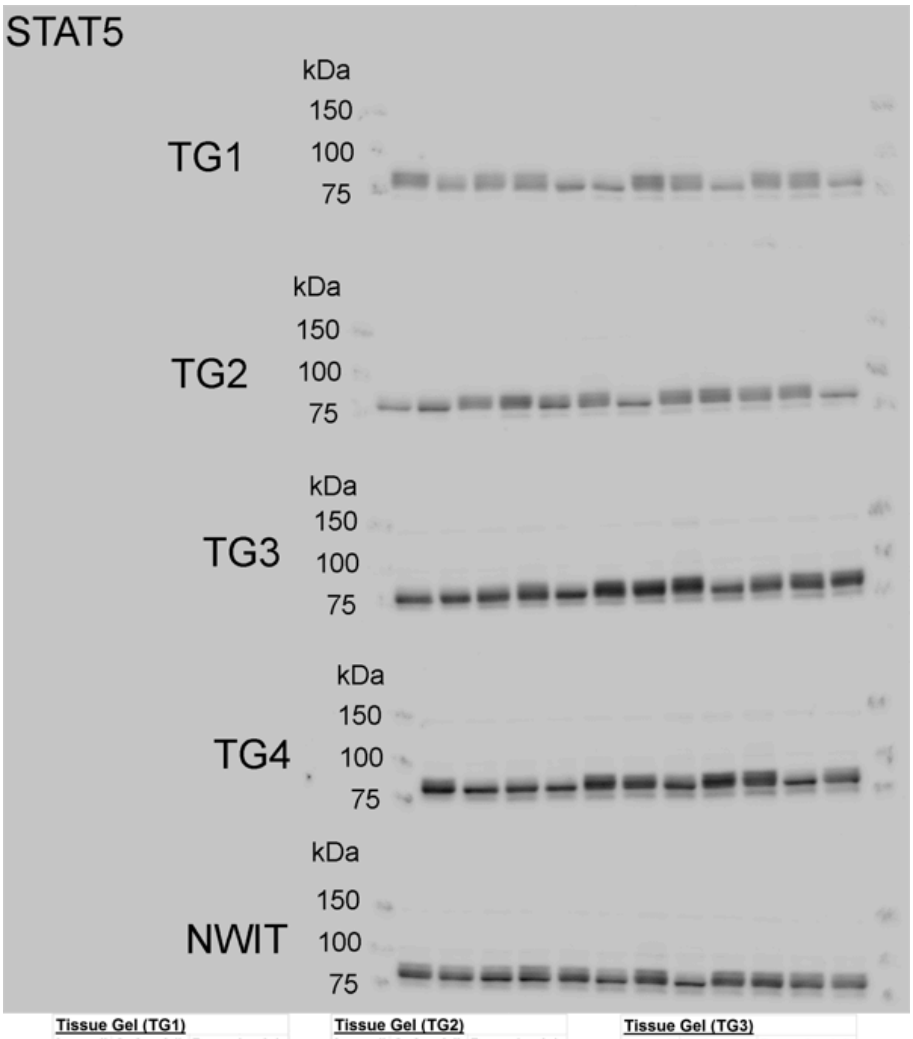
Tissue Gel (TG4)

Lane #	Animal #	Dose (ng/g)
1	41	20
2	39	4
3	40	8
4	38	2
5	42	50
6	43	50
7	44	80
8	45	120
9	47	200
10	37	0
11	46	200

Normalizer Gel w/in tissue Gel (NWIT)

Lane #	Animal #	Dose (ng/g)	Originating (TG)
1	22	200	2
2	34	200	3
3	46	200	4
4	11	200	1
5	23	200	2
6	35	200	3
7	47	200	4
8	12	200	1
9	24	200	2
10	36	200	3
11	7	50	1
12	43	50	4

SI Figure 5



Tissue Gel (TG1)

Lane #	Animal #	Dose (ng/g)
1	8	50
2	4	8
3	6	20
4	11	200
5	1	0
6	2	0
7	10	120
8	5	12.5
9	3	4
10	9	8
11	7	50
12	12	200

Tissue Gel (TG2)

Lane #	Animal #	Dose (ng/g)
1	16	8
2	14	2
3	23	200
4	24	200
5	17	12.5
6	19	50
7	15	4
8	20	8
9	18	2
10	22	200
11	21	120
12	13	0

Tissue Gel (TG3)

Lane #	Animal #	Dose (ng/g)
1	25	0
2	26	2
3	35	200
4	27	4
5	28	8
6	32	80
7	33	120
8	31	50
9	29	12.5
10	34	200
11	30	50
12	36	200

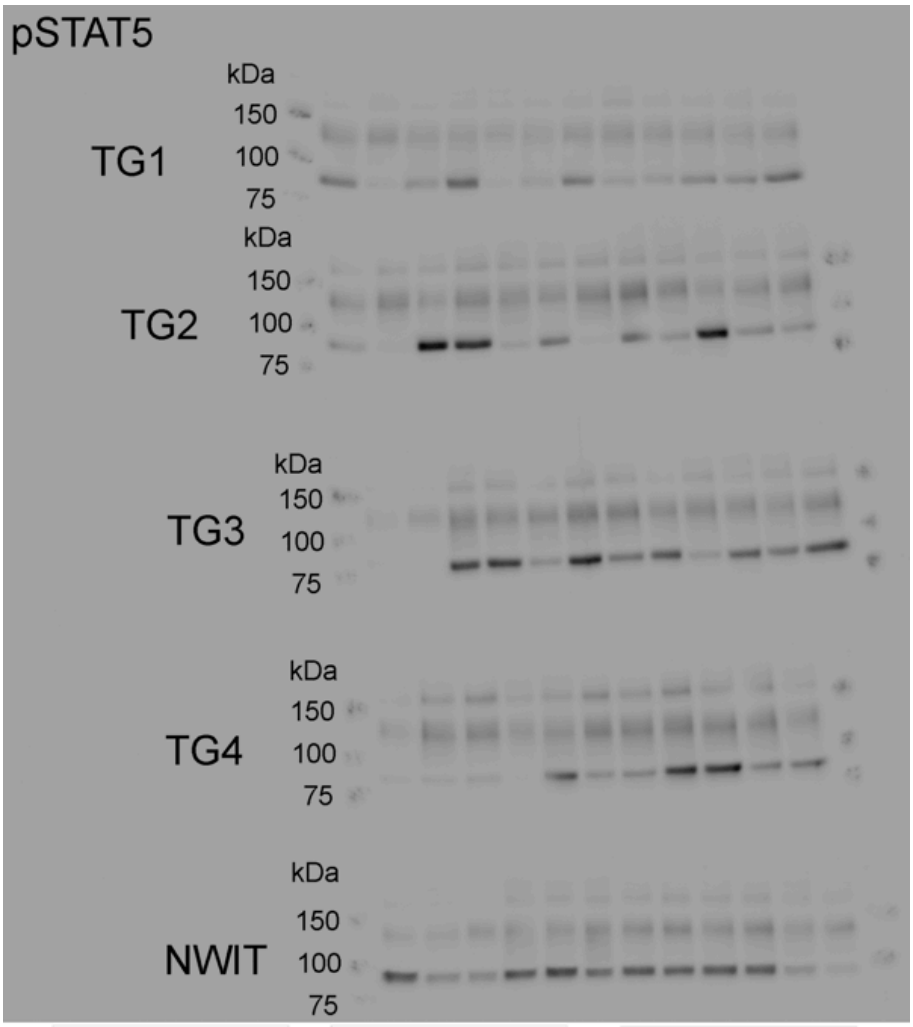
Tissue Gel (TG4)

Lane #	Animal #	Dose (ng/g)
1	41	20
2	39	4
3	40	8
4	38	2
5	42	50
6	43	50
7	44	80
8	45	120
9	47	200
10	37	0
11	46	200

Normalizer Gel w/in tissue Gel (NWIT)

Lane #	Animal #	Dose (ng/g)	Originating (TG)
1	22	200	2
2	34	200	3
3	46	200	4
4	11	200	1
5	23	200	2
6	35	200	3
7	47	200	4
8	12	200	1
9	24	200	2
10	36	200	3
11	7	50	1
12	43	50	4

SI Figure 6



Tissue Gel (TG1)

Lane #	Animal #	Dose (ng/g)
1	8	50
2	4	8
3	6	20
4	11	200
5	1	0
6	2	0
7	10	120
8	5	12.5
9	3	4
10	9	8
11	7	50
12	12	200

Tissue Gel (TG2)

Lane #	Animal #	Dose (ng/g)
1	16	8
2	14	2
3	23	200
4	24	200
5	17	12.5
6	19	50
7	15	4
8	20	8
9	18	2
10	22	200
11	21	120
12	13	0

Tissue Gel (TG3)

Lane #	Animal #	Dose (ng/g)
1	25	0
2	26	2
3	35	200
4	27	4
5	28	8
6	32	80
7	33	120
8	31	50
9	29	12.5
10	34	200
11	30	50
12	36	200

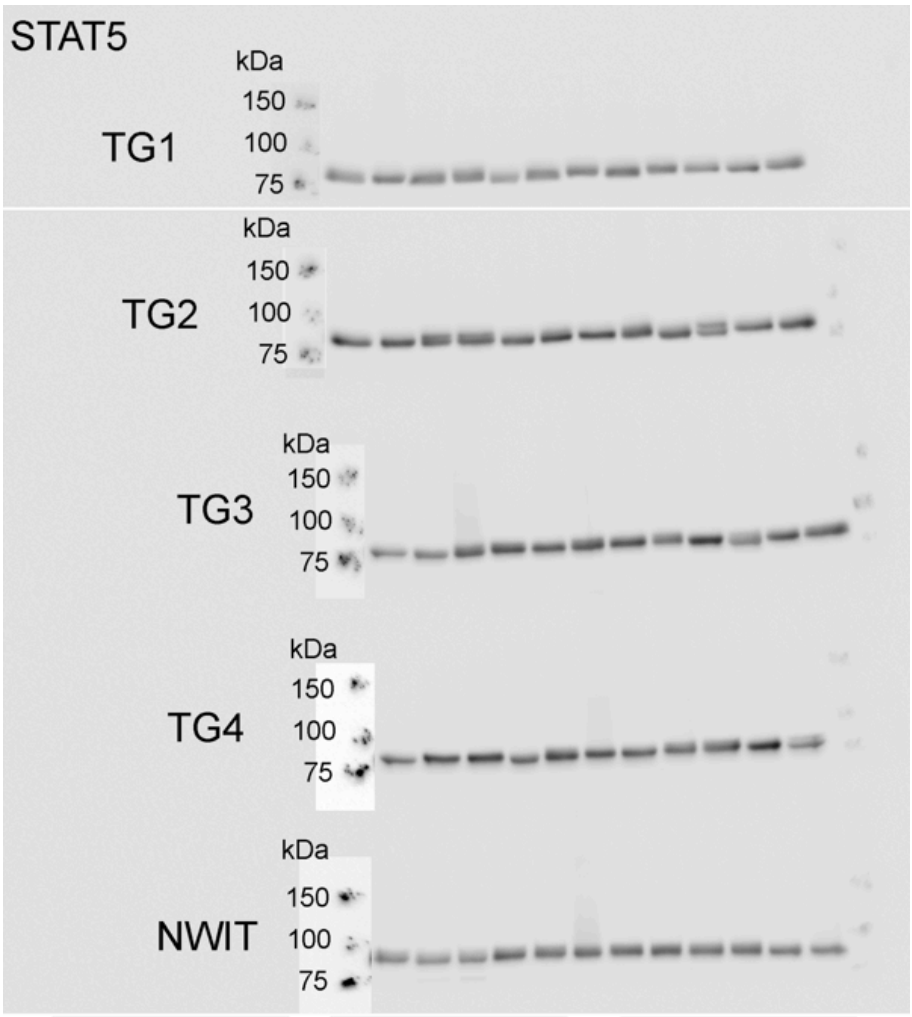
Tissue Gel (TG4)

Lane #	Animal #	Dose (ng/g)
1	41	20
2	39	4
3	40	8
4	38	2
5	42	50
6	43	50
7	44	80
8	45	120
9	47	200
10	37	0
11	46	200

Normalizer Gel w/in tissue Gel (NWIT)

Lane #	Animal #	Dose (ng/g)	Originating (TG)
1	22	200	2
2	34	200	3
3	46	200	4
4	11	200	1
5	23	200	2
6	35	200	3
7	47	200	4
8	12	200	1
9	24	200	2
10	36	200	3
11	7	50	1
12	43	50	4

SI Figure 7



Tissue Gel (TG1)

Lane #	Animal #	Dose (ng/g)
1	8	50
2	4	8
3	6	20
4	11	200
5	1	0
6	2	0
7	10	120
8	5	12.5
9	3	4
10	9	8
11	7	50
12	12	200

Tissue Gel (TG2)

Lane #	Animal #	Dose (ng/g)
1	16	8
2	14	2
3	23	200
4	24	200
5	17	12.5
6	19	50
7	15	4
8	20	8
9	18	2
10	22	200
11	21	120
12	13	0

Tissue Gel (TG3)

Lane #	Animal #	Dose (ng/g)
1	25	0
2	26	2
3	35	200
4	27	4
5	28	8
6	32	80
7	33	120
8	31	50
9	29	12.5
10	34	200
11	30	50
12	36	200

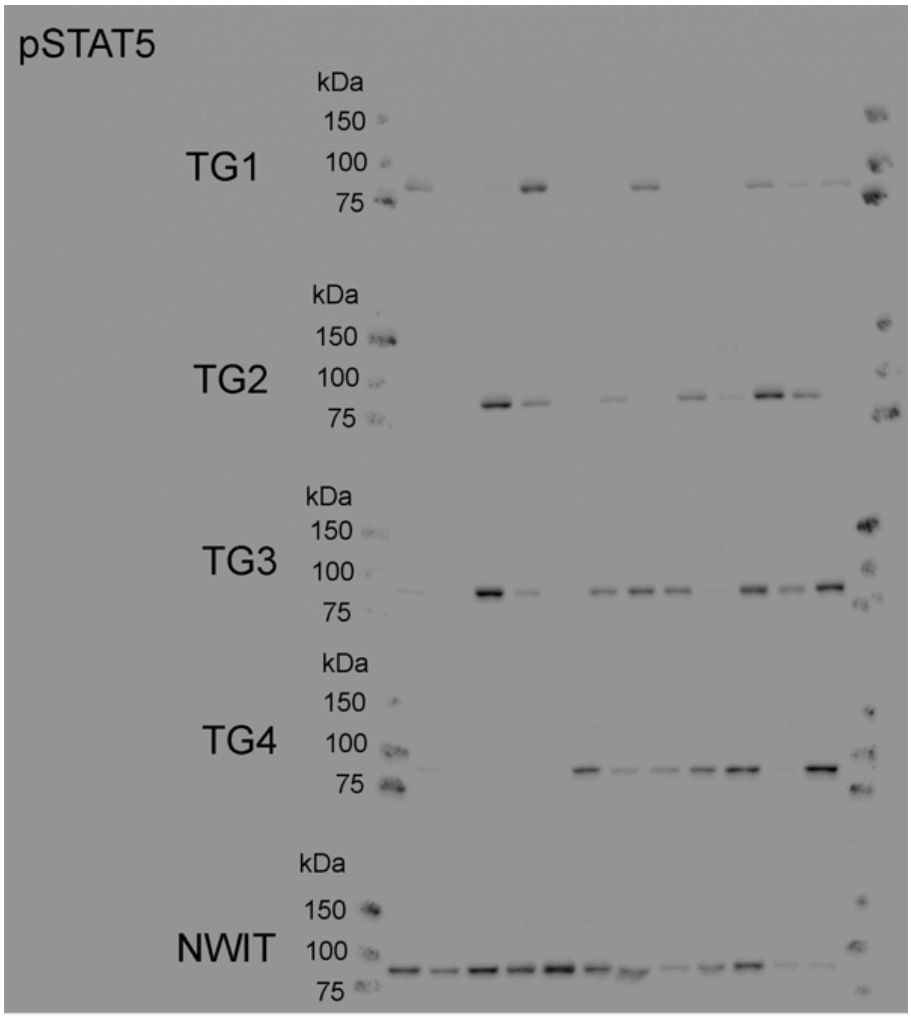
Tissue Gel (TG4)

Lane #	Animal #	Dose (ng/g)
1	41	20
2	39	4
3	40	8
4	38	2
5	42	50
6	43	50
7	44	80
8	45	120
9	47	200
10	37	0
11	46	200

Normalizer Gel w/in tissue Gel (NWIT)

Lane #	Animal #	Dose (ng/g)	Originating (TG)
1	22	200	2
2	34	200	3
3	46	200	4
4	11	200	1
5	23	200	2
6	35	200	3
7	47	200	4
8	12	200	1
9	24	200	2
10	36	200	3
11	7	50	1
12	43	50	4

SI Figure 8



Tissue Gel (TG1)

Lane #	Animal #	Dose (ng/g)
1	8	50
2	4	8
3	6	20
4	11	200
5	1	0
6	2	0
7	10	120
8	5	12.5
9	3	4
10	9	8
11	7	50
12	12	200

Tissue Gel (TG2)

Lane #	Animal #	Dose (ng/g)
1	16	8
2	14	2
3	23	200
4	24	200
5	17	12.5
6	19	50
7	15	4
8	20	8
9	18	2
10	22	200
11	21	120
12	13	0

Tissue Gel (TG3)

Lane #	Animal #	Dose (ng/g)
1	25	0
2	26	2
3	35	200
4	27	4
5	28	8
6	32	80
7	33	120
8	31	50
9	29	12.5
10	34	200
11	30	50
12	36	200

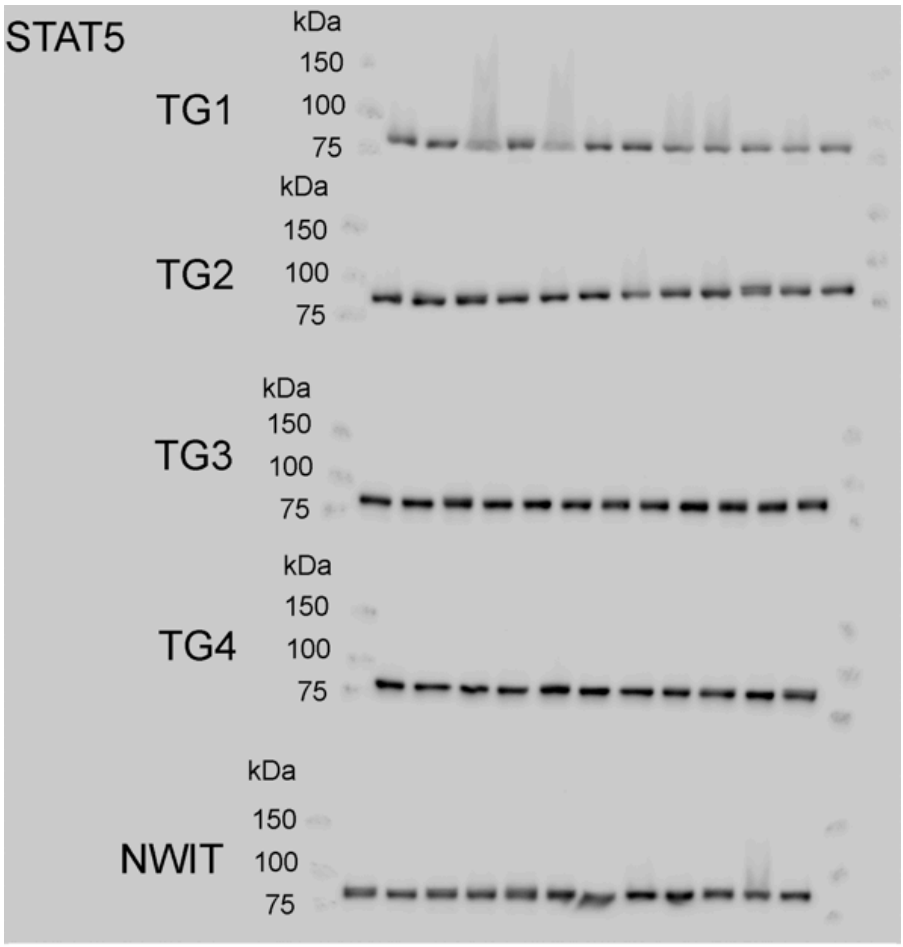
Tissue Gel (TG4)

Lane #	Animal #	Dose (ng/g)
1	41	20
2	39	4
3	40	8
4	38	2
5	42	50
6	43	50
7	44	80
8	45	120
9	47	200
10	37	0
11	46	200

Normalizer Gel w/in tissue Gel (NWIT)

Lane #	Animal #	Dose (ng/g)	Originating (TG)
1	22	200	2
2	34	200	3
3	46	200	4
4	11	200	1
5	23	200	2
6	35	200	3
7	47	200	4
8	12	200	1
9	24	200	2
10	36	200	3
11	7	50	1
12	43	50	4

SI Figure 9



Tissue Gel (TG1)

Lane #	Animal #	Dose (ng/g)
1	8	50
2	4	8
3	6	20
4	11	200
5	1	0
6	2	0
7	10	120
8	5	12.5
9	3	4
10	9	8
11	7	50
12	12	200

Tissue Gel (TG2)

Lane #	Animal #	Dose (ng/g)
1	16	8
2	14	2
3	23	200
4	24	200
5	17	12.5
6	19	50
7	15	4
8	20	8
9	18	2
10	22	200
11	21	120
12	13	0

Tissue Gel (TG3)

Lane #	Animal #	Dose (ng/g)
1	25	0
2	26	2
3	35	200
4	27	4
5	28	8
6	32	80
7	33	120
8	31	50
9	29	12.5
10	34	200
11	30	50
12	36	200

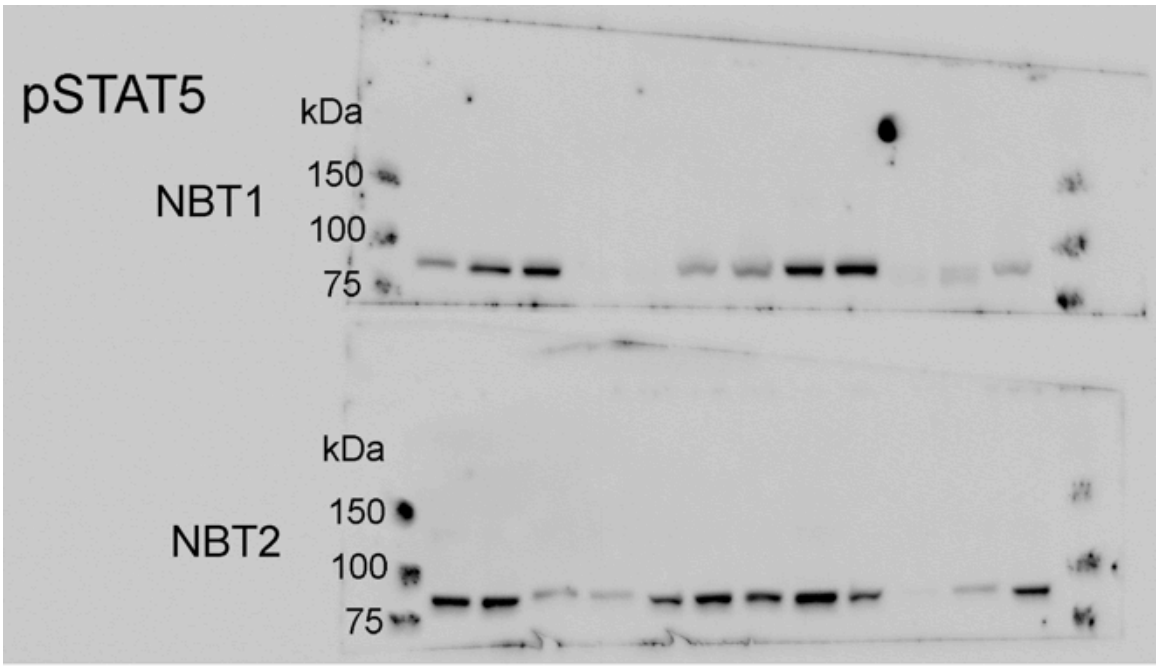
Tissue Gel (TG4)

Lane #	Animal #	Dose (ng/g)
1	41	20
2	39	4
3	40	8
4	38	2
5	42	50
6	43	50
7	44	80
8	45	120
9	47	200
10	37	0
11	46	200

Normalizer Gel w/in tissue Gel (NWIT)

Lane #	Animal #	Dose (ng/g)	Originating (TG)
1	22	200	2
2	34	200	3
3	46	200	4
4	11	200	1
5	23	200	2
6	35	200	3
7	47	200	4
8	12	200	1
9	24	200	2
10	36	200	3
11	7	50	1
12	43	50	4

SI Figure 10



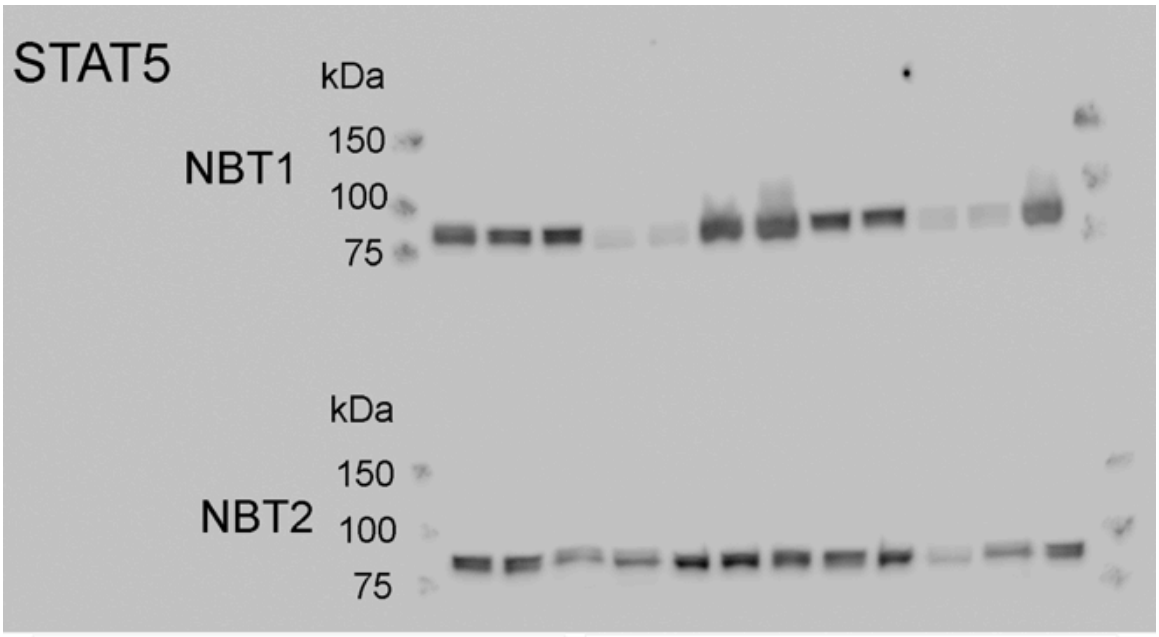
Normalizer Gel b/w tissues (NBT1)

Lane #	Animal #	Tissue	Dose (ng/g)	Originating (TG)
1	46	Heart	200	3
2	35	Liver	200	2
3	23	Liver	200	1
4	35	Kidney	200	2
5	46	Kidney	200	3
6	35	Heart	200	2
7	23	Heart	200	1
8	46	Liver	200	3
9	47	Liver	200	4
10	23	Kidney	200	1
11	47	Kidney	200	4
12	47	Heart	200	4

Normalizer Gel b/w tissues (NBT2)

Lane #	Animal #	Tissue	Dose (ng/g)	Originating (TG)
1	46	Liver	200	3
2	23	Liver	200	1
3	23	WAT	200	1
4	35	WAT	200	2
5	47	Gastroc	200	4
6	23	Gastroc	200	1
7	46	Gastroc	200	3
8	47	Liver	200	4
9	35	Gastroc	200	2
10	46	WAT	200	3
11	47	WAT	200	4
12	35	Liver	200	2

SI Figure 11



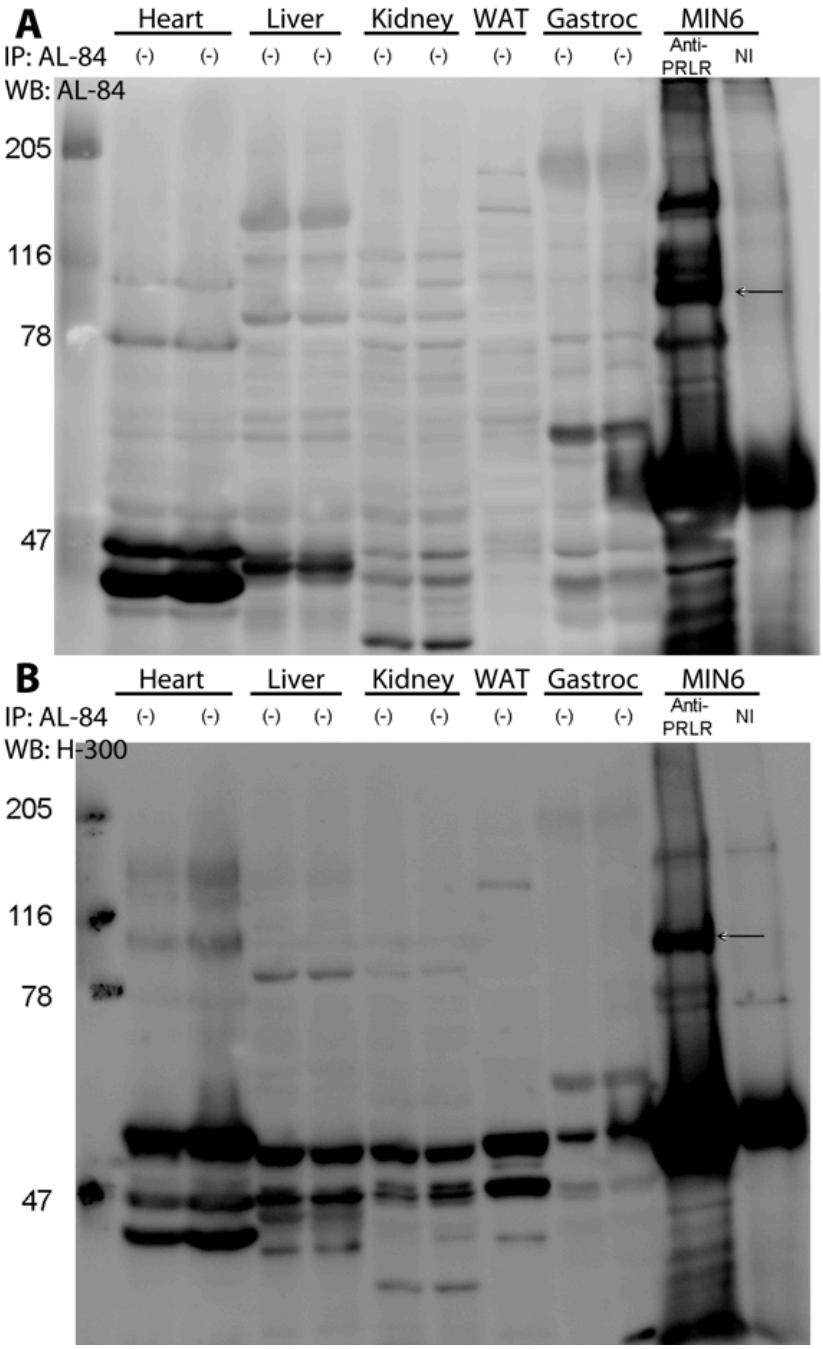
Normalizer Gel b/w tissues (NBT1)

Lane #	Animal #	Tissue	Dose (ng/g)	Originating (TG)
1	46	Heart	200	3
2	35	Liver	200	2
3	23	Liver	200	1
4	35	Kidney	200	2
5	46	Kidney	200	3
6	35	Heart	200	2
7	23	Heart	200	1
8	46	Liver	200	3
9	47	Liver	200	4
10	23	Kidney	200	1
11	47	Kidney	200	4
12	47	Heart	200	4

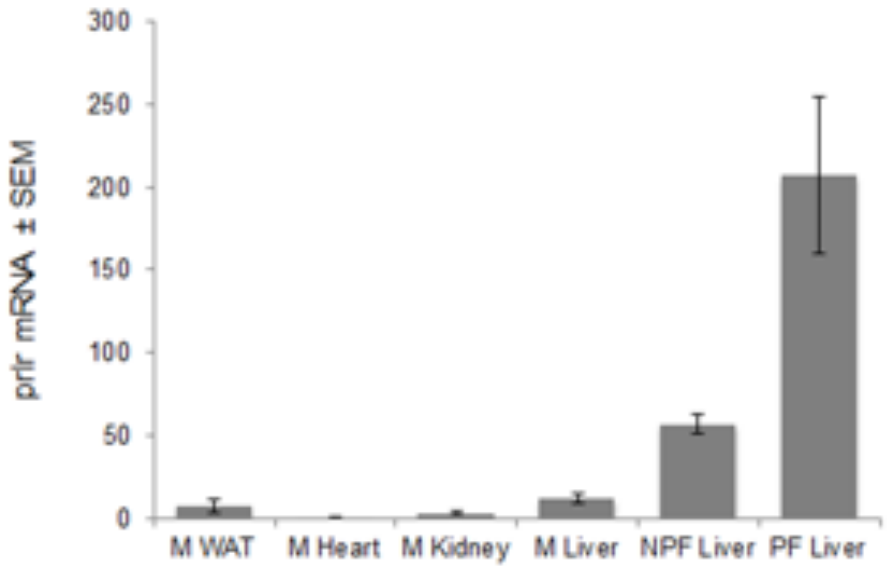
Normalizer Gel b/w tissues (NBT2)

Lane #	Animal #	Tissue	Dose (ng/g)	Originating (TG)
1	46	Liver	200	3
2	23	Liver	200	1
3	23	WAT	200	1
4	35	WAT	200	2
5	47	Gastroc	200	4
6	23	Gastroc	200	1
7	46	Gastroc	200	3
8	47	Liver	200	4
9	35	Gastroc	200	2
10	46	WAT	200	3
11	47	WAT	200	4
12	35	Liver	200	2

SI Figure 12

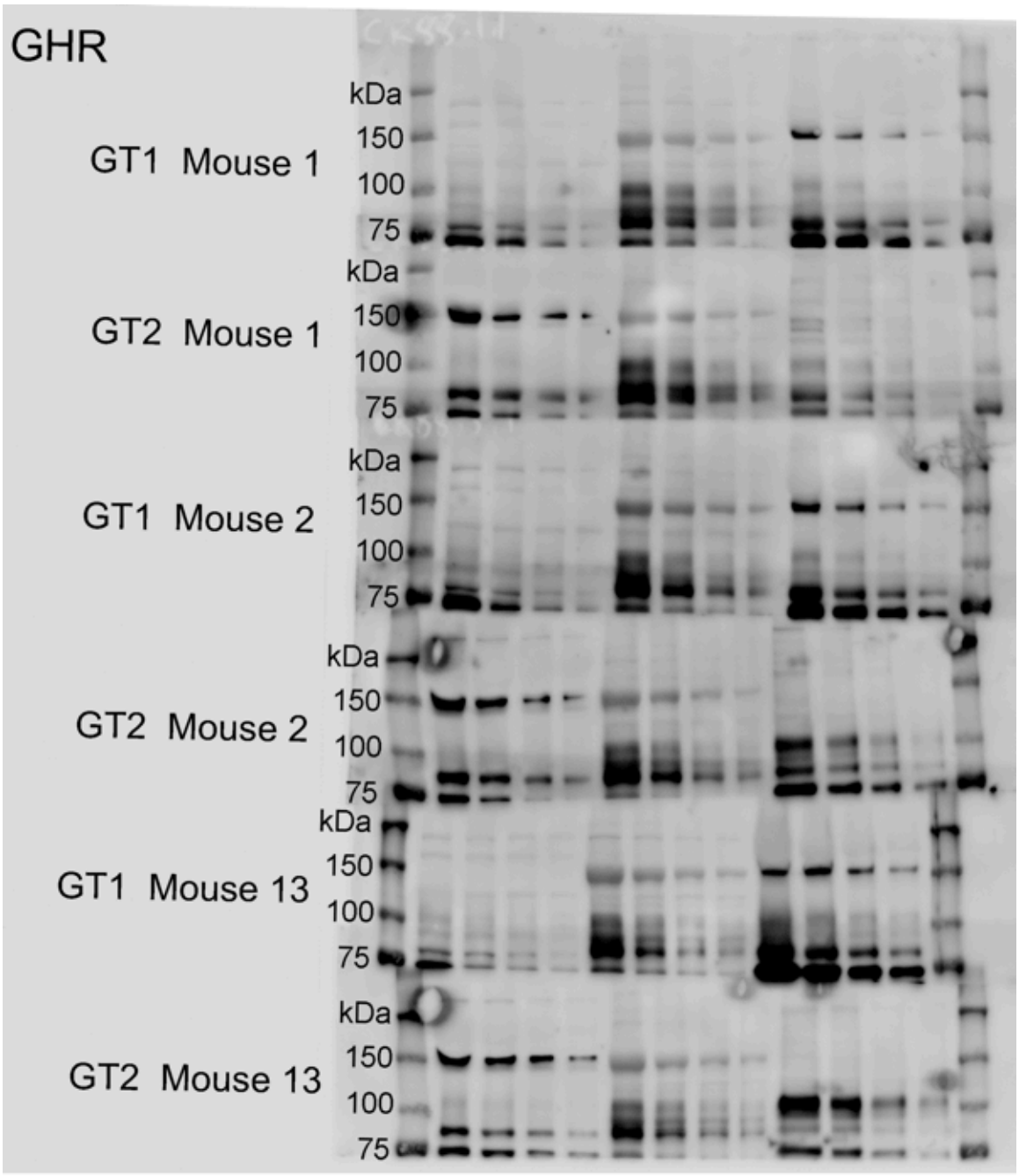


SI 15 PRLR immunoblot of tissue lysates from various mouse tissues and in MIN6 mouse insulinoma cells (positive control). The black arrows denote the PRLR in the IP control. (-) denotes that no immunoprecipitation was done. NI denotes non-immune serum. Immunoprecipitation was done with anti-$PRLR_{cytAL\text{-}84}$, and blotted with (A) anti-$PRLR_{cytAL\text{-}84}$ and (B) PRL-R (H-300).



SI 16 prlr mRNA expression in various mouse tissues. M, male; NPF, non-pregnant virgin female; PF, pregnant female.

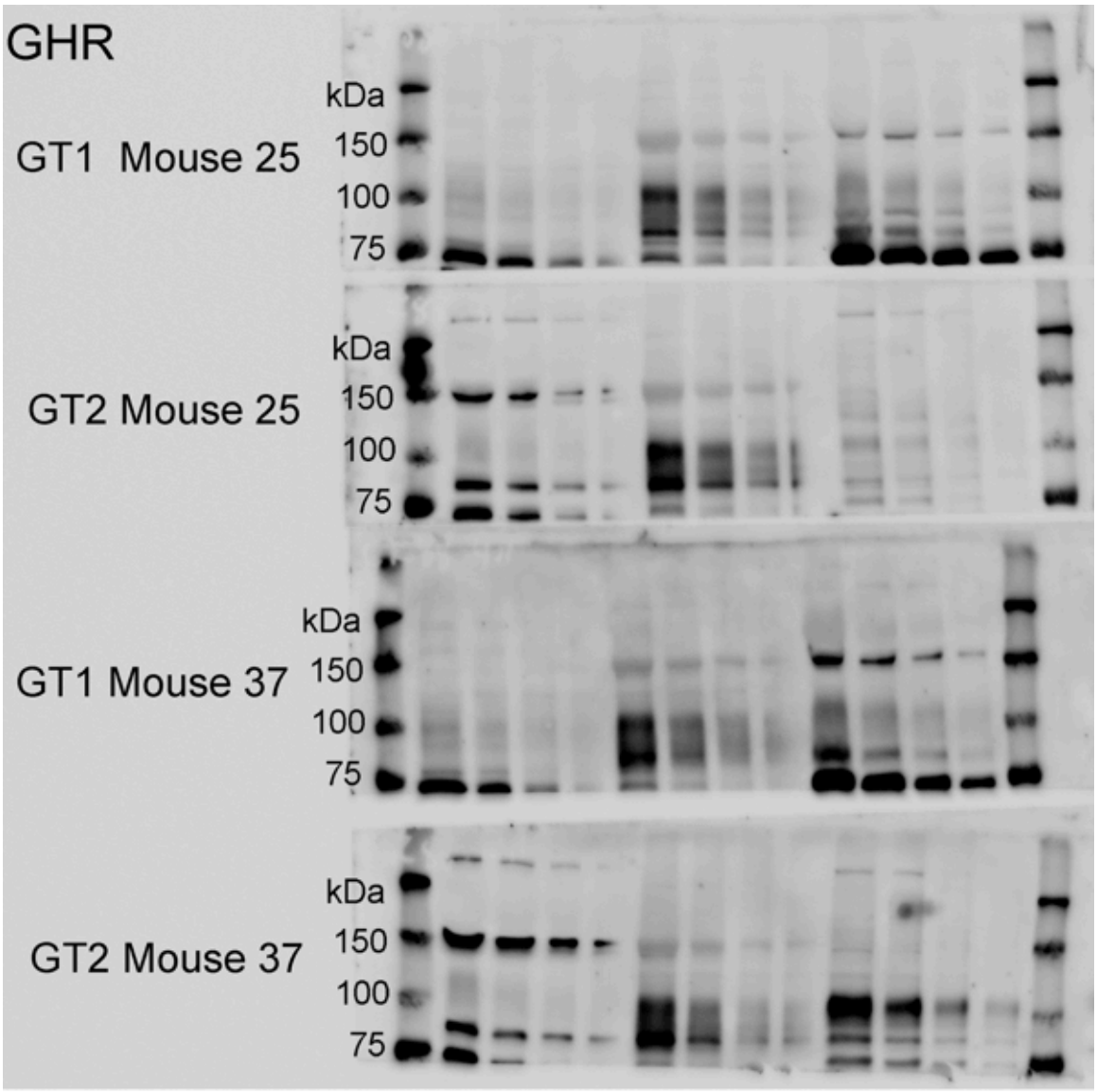
SI 13-14, 17-20 raw immunoblot images: immunoblots used to compare the relative tissue-specific abundance of GHR, JAK2, and STAT5 protein in Figure 3A-C and the representative blots (GT1-2) for Mouse 1 were used as representative blots (Figure 3 E-G). Tissue homogenates from only one mouse were included on any given gel. On each gel tissue homogenates were loaded into 4 sequential wells 50, 25, 12.5, and 6.25 μg of total protein respectively. Tissue homogenates from each mouse were loaded onto two gels: Gel Type 1 (GT1), containing kidney, liver and heart or GT2 containing gastrocnemius, liver, and WAT. Liver was included on both GT1 and GT2 such that relative protein abundance could be compared between GT1 and GT2 relative to the well containing 50 μg of total liver protein. Tissue-specific protein abundance was first compared within each of the five mice relative to the liver after which the data from all five mice was combined by assigning a value of 1 to the signal in the well containing 50 μg of total liver protein. We then analyzed the combined data for each protein – GHR, JAK2, and STAT5 – to ensure that the total protein amount used was in the linear range for immunoblot. For example, if the intensity of the STAT5 band did not decrease by half between the 50 μg well to the 25 μg well, but did decrease by half between the 25 μg well and the 12.5 μg well, only the data originating from the 12.5 μg well would be used to create Figure 3 A-C.



Gel type 1 (GT1)		Kidney				Liver				Heart			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

Gel type 2 (GT2)		Gastrocnemius				Liver				WAT			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

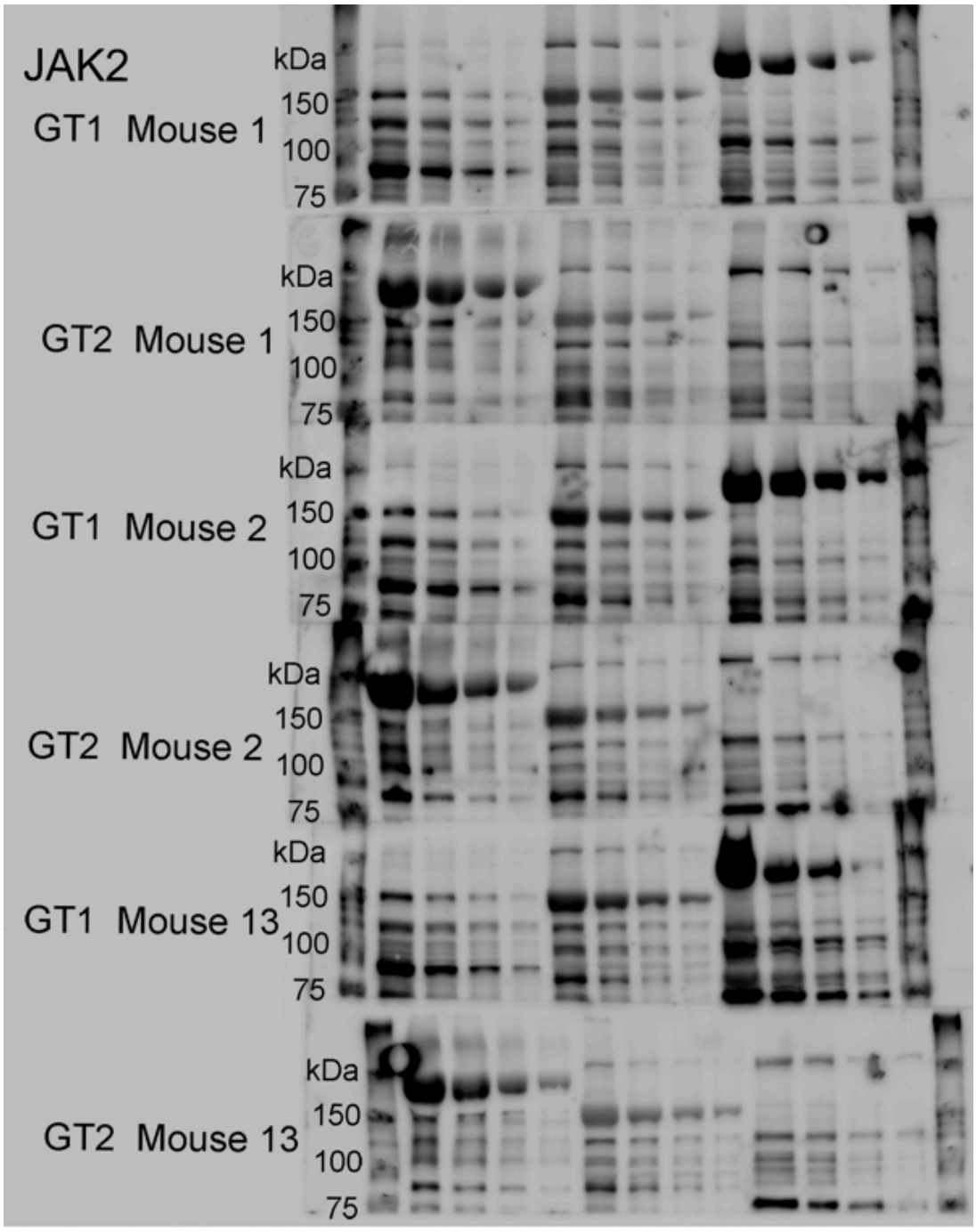
SI Figure 13



Gel type 1 (GT1)		Kidney				Liver				Heart			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

Gel type 2 (GT2)		Gastrocnemius				Liver				WAT			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

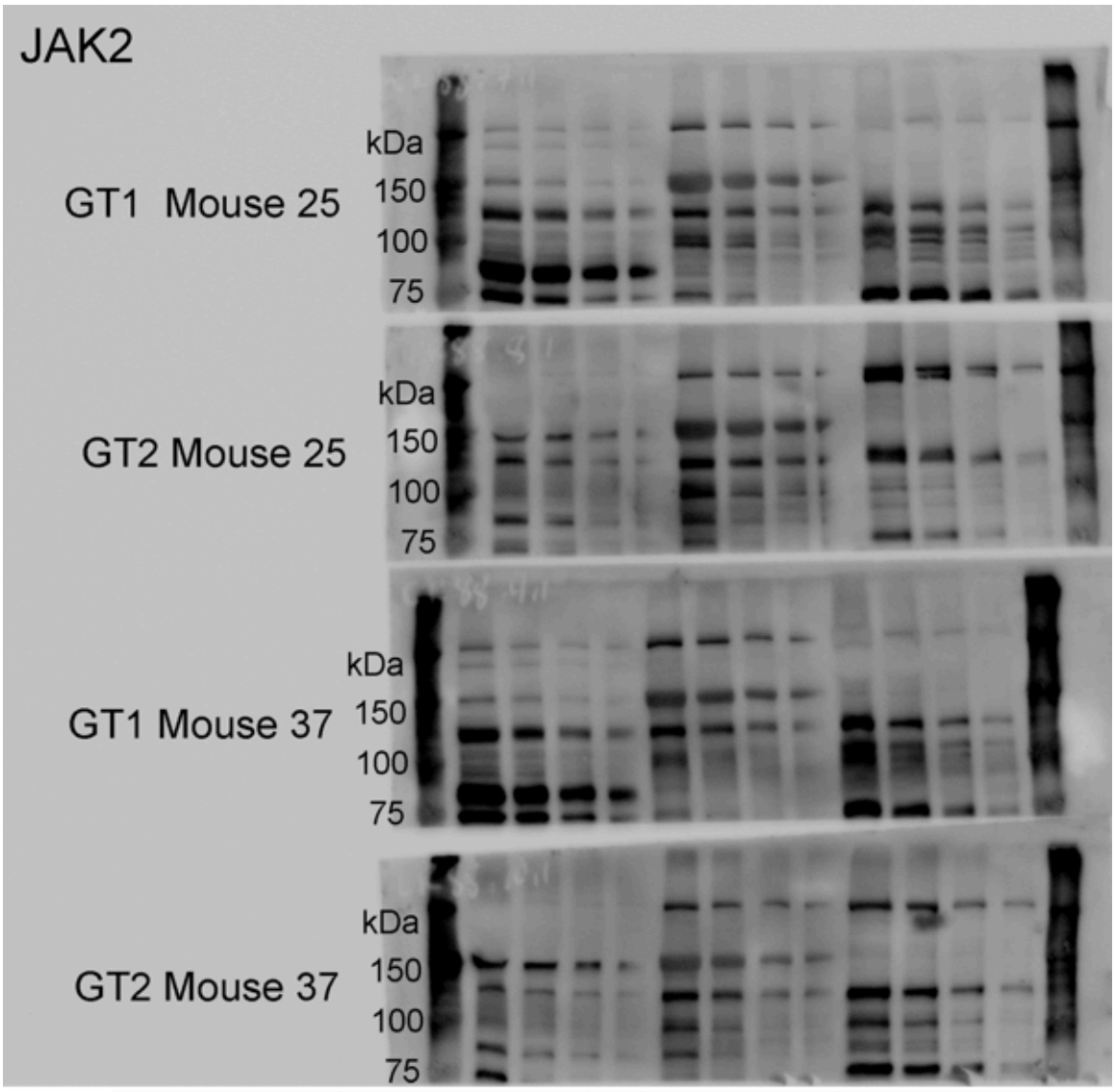
SI Figure 14



Gel type 1 (GT1)		Kidney				Liver				Heart			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

Gel type 2 (GT2)		Gastrocnemius				Liver				WAT			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

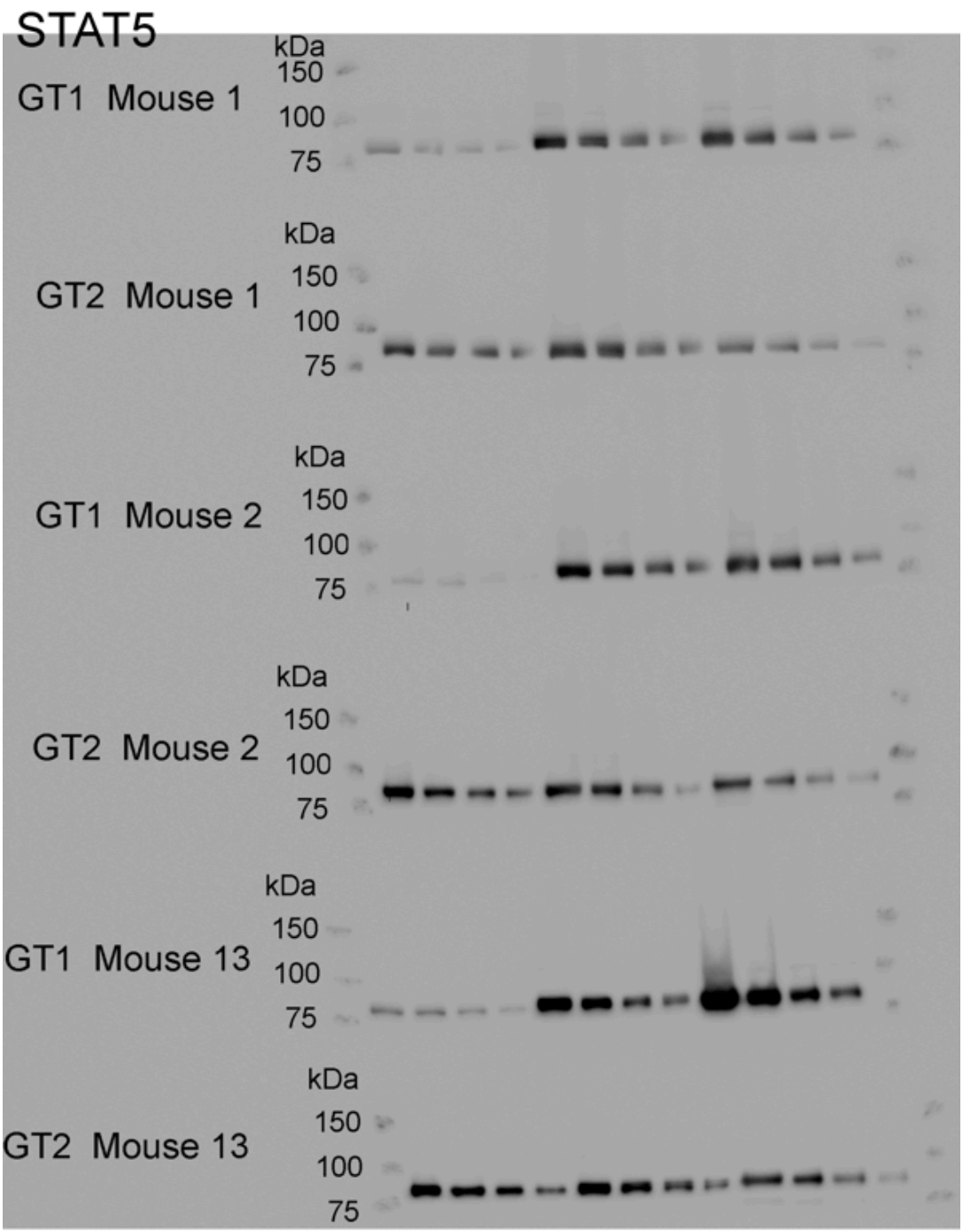
SI Figure 17



Gel type 1 (GT1)		Kidney				Liver				Heart			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

Gel type 2 (GT2)		Gastrocnemius				Liver				WAT			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

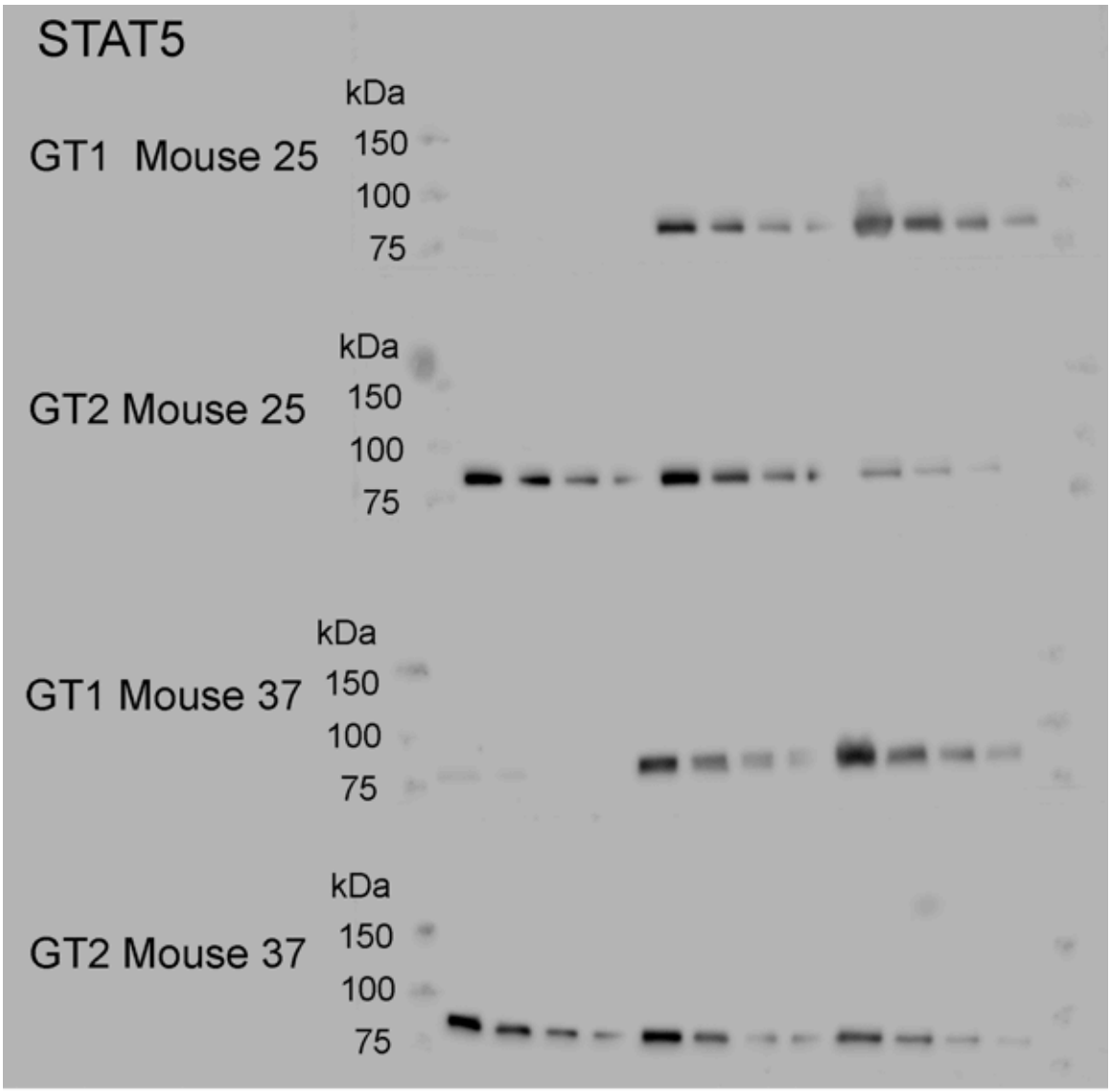
SI Figure 18



Gel type 1 (GT1)		Kidney				Liver				Heart			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

Gel type 2 (GT2)		Gastrocnemius				Liver				WAT			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

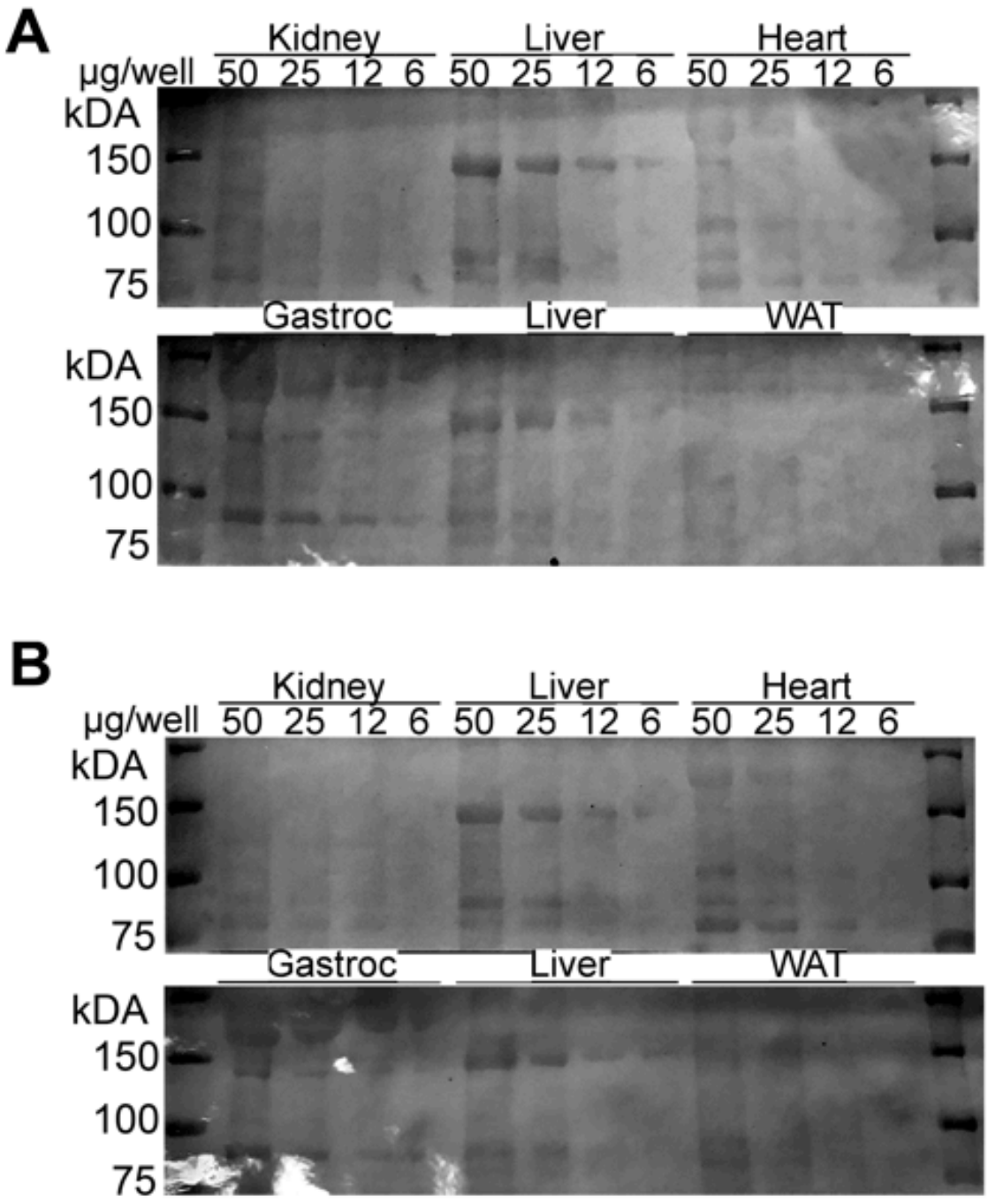
SI Figure 19



Gel type 1 (GT1)		Kidney				Liver				Heart			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

Gel type 2 (GT2)		Gastrocnemius				Liver				WAT			
Animal #s:	Amt.	50	25	12.5	6.25	50	25	12.5	6.25	50	25	12.5	6.25
1,2,13,25,37													

SI Figure 20


A
Kidney
Liver
Heart
µg/well 50 25 12 6 50 25 12 6 50 25 12 6
kDA
150
100
75
Gastroc
Liver
WAT
kDA
150
100
75
B
Kidney
Liver
Heart
µg/well 50 25 12 6 50 25 12 6 50 25 12 6
kDA
150
100
75
Gastroc
Liver
WAT
kDA
150
100
75

SI Figure 21

SI 21 Ponceau Stain Loading Control: This file contains the ponceau stain loading controls for the blots in Figure 3E-G. A) The top and bottom blots represent the ponceau stain of the top and bottom blots respectively in Figure 3E. B) The top blot represents the ponceau stain of the top blots in Figures 3F and 3G. The bottom blot represents the ponceau stain of the bottom blots in Figures 3F and 3G. Note: The top and bottom membranes in Figure 3G have been stripped and re-probed with anti-JAK2 antibody to produce Figure 3F.

SI File 1 Raw densitometry data: this file contains the raw densitometry data for the blots found in SI 1-12. These data were used to create Figure 1B and Figure 2.

SI File 2 Raw densitometry data: this file contains the raw densitometry data for the blots found in SI 13-14 and SI 17-20. This data was used to create Figure 3A-C.

THE INFLUENCE OF THE CARDIOMYOCYTE CIRCADIAN CLOCK ON THE GROWTH HORMONE SIGNALING PATHWAY IN THE HEART

by

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In preparation for *Endocrinology*

Format adapted for dissertation

Abstract

Heart failure (HF) is a major cause of morbidity and mortality in the United States with 915,000 new cases each year. Heart failure (HF) is heavily impacted by risk factors influenced by circadian clocks, including prior myocardial infarctions and hypertension. Chronic circadian dysregulation, as with shift work, results in an increased risk of cardiovascular disease. Genetic disruption of the cardiac circadian clock in the cardiomyocyte-specific BMAL1 knockout (CBK)-Cre(+) mouse result in cardiac hypertrophy, age onset heart failure and early mortality. Excessive growth hormone (GH), as in acromegaly, results in cardiac hypertrophy, and if untreated, HF. The aim of this study was to determine whether genetic disruption of the cardiomyocyte circadian clock alters GH signaling thereby influencing the hypertrophic phenotype observed in CBK-Cre(+) mice. A candidate approach revealed increased expression of a number of growth hormone (GH) signaling related genes in CBK-Cre(+) hearts, throughout the day, including growth hormone receptor (GHR), insulin-like growth factor 1 (IGF-1), and IGF-1 receptor (IGF-1R). When CBK-Cre(+) mice and controls received intravenous injection of GH at ZT0 and ZT12, the control mice displayed reduced sensitivity to GH at ZT 12 compared to ZT0; this time-of-day dependent response is lost in CBK-Cre(+) mice and sensitivity remains elevated at ZT 12. Driven by the hypothesis that increased cardiac GH signaling response in CBK-Cre(+) mice contributes to the elevated igf-1 gene expression, which in turn results in the development of hypertrophy, we attempted to

ameliorate the CBK-Cre(+) cardiac hypertrophy phenotype by creating the CBK IGF-1(-/+) (CBKI-Cre(+)) mouse thereby normalizing cardiac igf-1 gene expression. Echocardiography revealed that CBKI-Cre(+) mice displayed partial protection against the development of cardiomyopathy as evidenced by slower development of LV dilation. In conclusion, CBK-Cre(+) mice display increased gene expression of a number of GH/IGF-1 signaling pathway components especially that of IGF-1, loss of time of day variations in cardiac GH sensitivity resulting in overall elevated GH signaling, and IGF-1 haplodeficiency in CBKI-Cre(+) mice partially protects against ventricular enlargement observed in CBK-Cre(+) mice.

Keywords: CBK, circadian, growth hormone, heart failure

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in the United States, being the attributable cause of one out of every three deaths[1]. Advances in treatment of CVD risk factors including metabolic syndrome and diabetes has reduced the incidence of acute myocardial infarction (AMI) and improved AMI interventions have increased AMI survival. However, ischemic heart disease is the single greatest risk factor for the development of heart failure, so despite improved treatments of HF treatment, increased AMI survival keeps the rates of HF high [1]. HF itself is the end product of a conglomerate of contributing factors primarily, coronary heart disease, but also including cigarette smoking, antecedent hypertension, obesity, and diabetes[1].

Aberrant secretion of GH is another factor that potently contributes to HF. In acromegaly, hypersecretion of GH and subsequent increases in insulin-like growth factor 1 (IGF-1), results in organomegaly, including cardiac hypertrophy[2]. When left untreated, the prevalence of left ventricular hypertrophy in acromegaly patients ranges between 60-90% depending on disease duration[3]. This can be recapitulated in mouse models through overexpression of IGF-1 the heart, resulting in cardiac hypertrophy and even dysfunction[4, 5].

The circadian clock allows the body to anticipate regularly occurring events (i.e. waking, eating, sun exposure, and sleeping/resting), thereby maximizing the benefit or minimizing the negative impact of these factors. The central circadian clock, responsible for entrainment to the light:dark cycle, is located in the suprachiasmatic nucleus and

consists of a cell autonomous transcriptional-translational negative feedback loop. Interestingly, this molecular clock is found in nearly every cell of the body. The circadian clock controls the secretion of major metabolic hormones[6]. For example, cortisol secretion from the adrenal glands peaks in the early morning aiding in preparing the body for arousal and commencement of activity[6, 7]. Growth hormone (GH) displays pulsatile release from the anterior pituitary with greater pulse amplitude at night when it influences growth and metabolism[6]. Physiologically, many neurohumoral factors oscillate throughout the day [6] and dysregulation of the circadian clock has been shown to disrupt hormone secretion of various HF-contributing factors [6-8]. The circadian clock also influences diurnal oscillations in hormone sensitivity. Insulin sensitivity, for example, is greatest in the early morning and tapers to a nadir in the early night[9, 10]. A large number of Americans (5.8%) subject themselves to circadian dysregulation through shift work[11]. Shift work causes a misalignment between an individual's sleep/wake cycle and the natural light/dark cycle, leading to dysynchrony in their natural circadian rhythms. When individuals are subject to irregular schedules (i.e. shift work, jetlag, social jetlag), hormones peak at incorrect times or fail to oscillate at all, as is the case with GH [12], and these anticipatory benefits are lost and their risk of developing metabolic syndrome, diabetes, obesity, and CVD increases[8, 13-22]. Metabolic syndrome, diabetes, and obesity are major risk factors for the development of coronary heart disease, which is the greatest risk factor for the development of HF[1].

Mice lacking BMAL1 (a transcription factor critical for the function of the molecular clock; once called Mop3[23]) display varied pathologies including infertility, altered glucose metabolism[24], cardiomyopathy[25], accelerated aging[26, 27] and early

mortality[28]. The cardiomyocyte-specific BMAL1 knockout (CBK)-Cre(+) mouse was created to specifically determine the role of the circadian clock in the heart[29]. This animal developed altered metabolism and age-dependent cardiomyopathy including hypertrophy, decreased function, eventual failure, and early mortality[29, 30].

It is not known whether clock disruption in CBK-Cre(+) mice alters GH signaling at the heart. Herein we examine the effect of CBK on GH signaling in the mouse heart.

Methods

Animals

Generation of CBK-Cre(+) and –Cre(-) mice. B6.129(FVB)-Igf1tm1Dlr/J mice (IGF-$1^{(f/f)}$) were obtained from Jackson Laboratories and $Bmal1^{(f/f)}$ mice with C57B6 mouse background (of which we have an active colony) were crossed. The f1 generation was intercrossed to obtain the $Bmal1^{(f/f)}$//IGF-$1^{(f/f)}$ (I_2A_2) mouse. The I_2A_2 females were then crossed with CBK-Cre(+) males (described previously) to produce $Bmal1^{(f/f)}$//IGF-$1^{(f/+)}$ (CBKI) -Cre(+) and -Cre(-) males and females. CBKI-Cre(-) females were then crossed with CBK-Cre(+) males. This cross produced CBK-Cre(+), CBK-Cre(-), CBKI-Cre(+), and CBKI-Cre(-) littermates. All animal breeding and housing was done in accordance with local IACUC standards.

ZT 20 comparison of CBK-Cre(+) and –Cre(-) hearts. Ad libitum fed CBK-Cre(+) and -Cre(-) mice were anesthetized with pentobarbital (Fatal Plus;) prior to sacrifice at ZT 20 ± 1 hour. Hearts were collected and flash frozen in liquid nitrogen prior to biochemical analysis[31].

Time course qPCR studies. Following anesthesia with pentobarbital (Fatal Plus;), CBK-Cre(+) and -Cre(-) mice with ad libitum access to a standard rodent chow and water were sacrificed every three hours throughout the circadian day. Heart and livers were collected and flash frozen in liquid nitrogen prior to biochemical analysis.

GH injection experiment. At 15 weeks of age (+/- 3 days), male CBK-Cre(+) and CBK-Cre(-) control mice were individually housed in standard conditions under a 12hr:12hr light: dark cycle and had ad libitum access to standard rodent chow and water. After acclimatization to single housing, mice were placed in wire bottom cages without food 6 hours prior to either ZT 0 or ZT 12. Following the 6-hr fast, mice were anesthetized with 400 mg/kg chloral hydrate IP. An abdominal incision exposed the inferior vena cava. Either saline (control) or human recombinant GH (50 ng/g_{bw}; gift from Eli Lilly Co, Indianapolis, IN) was injected. Five minutes after saline or GH administration, heart and liver were rapidly excised and flash frozen in liquid nitrogen prior to biochemical analysis.

CBKI-Cre(+) and –Cre(-) molecular characterization. CBKI-Cre(+) and –Cre(-) mice with ad libitum access to standard rodent chow and water were anesthetized with pentobarbital (Fatal Plus;) prior to sacrifice at ZT6. Hearts and livers were collected and flash frozen in liquid nitrogen prior to biochemical analysis.

Immunoblotting

Protein lysates were prepared from powdered tissues (~20mg) using 300ml of tissue lysis buffer (50 mM Tris 7.3, 150 mM NaCl, 1mM EDTA pH 8.1, 1.5mM $MgCl_2$, 10% Glycerol, 1% Triton X-100, 10mM $Na_4P_2O_7$, 100mM NaF, 1mM Na_3VO_4, 1mM

phenylmethanesulfonyl fluoride, 5 mg/ml aprotinin, and 5 mg/ml leupeptin). Lysates were resolved under reducing conditions by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences), followed by blocking with 2% BSA. Membranes were immunoblotted with anti-phospho-STAT5 antibody (Y694; Cell Signaling; 9351L) (1:1000), anti-STAT5 antibody (Santa Cruz Biotechnology; sc-835) (1:1000), anti-phospho-Akt (S473; Cell Signaling; 9271S) (1:1000), anti-Akt (Cell Signaling; 9272S) (1:1000), anti-phospho-ERK1/2 (S217/221; Cell Signaling; 9121S) (1:1000), anti-ERK1/2 (Milipore; 06-182) (1:1000). Densitometry was performed using UVP Software 8.0.

Quantitative RT-PCR

RNA was extracted from hearts either via the standard methods described previously [32, 33], or using a QIAGEN RNeasy Mini Kit (Cat. No. 74104). Candidate gene expression analysis was performed by quantitative RT-PCR, using previously described methods (Gibson, et al. 1996; Heid, et al. 1996). For quantitative RT-PCR, a specific Taqman assay was designed for the GHR gene from mouse sequences available in GenBank. TaqMan Assays were ordered from Thermo Fisher Scientific; Igf-1 (Assay No. Mm00439560_m1), igf-1r (Assay No. Mm00802831_m1), socs2 (Assay No. Mm01233811_g1) All quantitative RT-PCR data are presented as fold change from littermate control.

Echocardiography

Echocardiography was performed on CBK-Cre(+), CBK-Cre(-), CBKI-Cre(+), and CBKI-Cre(-) littermates at 16 and 21 weeks of age. Data was obtained from analysis in M-mode using the Vevo 770 (Visual Sonics, Canada) high-resolution imaging system as described elsewhere[34].

Statistical analysis

One-way ANOVA was used for comparisons between genotypes at just one time of day using Excel. Comparisons comprising both genotypes and more than one time of day were done using 2-way ANOVA. For significant main effects, post hoc analyses were conducted via the Bonferoni method for pairwise comparisons or 1-way ANOVA (SPSS 24.0 Software). Analyses of rhythmicity were done using a Cosinor analysis (SPSS 24.0 Software).

Results

CBK-Cre(+) hearts display pro-hypertrophic signaling

Previous findings in CBK-Cre(+) hearts describe a hypertrophic phenotype[30]. We, therefore, assessed the status of proteins known to promote hypertrophy in the hearts of 16 week-old male CBK-Cre(+) and CBK-Cre(-) mice and found that Akt phosphorylation (p) was elevated (pAkt/Akt) by 236% and pERK/ERK was increased 40% in CBK-Cre(+) hearts (relative to CBK-Cre(-) controls; Figure 1B-C). Because elevated GH/IGF-1 signaling is associated with cardiac hypertrophy, we investigated

whether these hearts also displayed signs of elevated GH/IGF-1 signaling. This revealed igf-1 mRNA to be elevated in the CBK hearts by 80% (relative to controls; Figure 1A).

Augmentation of GH/IGF-1Components In CBK-Cre(+) hearts

Elevated igf-1 gene expression prompted a more thorough assessment of genes related to GH/IGF-1 signaling beginning with GH receptor (ghr), the GHR-associated Janus Kinase 2 (jak2), which becomes active upon GH binding at the GHR and phosphorylates the GHR intracellular domain, signal transducer and activator of transcription 5b (stat5b) which binds to the phosphorylated GHR and becomes phosphorylated by JAK2 allowing it to homodimerize and activate transcription in the nucleus. Two genes activated by STAT5b are igf-1 and suppressor of cytokine signaling 2 (socs2), which directly inhibits signaling at the GHR by binding to and blocking signaling and also through participation as part of an ubiquitin ligase complex that marks GHR for degradation. Once secreted, IGF-1 binds to the IGF-1 receptor (IGF-1r) to promote somatogenesis in an autocrine, paracrine, and endocrine fashion. Therefore, gene expression levels of heart ghr, jak2, stat5b, socs2, igf-1, and igf-1r were assessed every three hours throughout the day in 12 week old CBK-Cre(+) and CBK-Cre(-) controls (Figure 2). Assessment of ghr mRNA revealed a significant increase in CBK-Cre(+) hearts (22%; main effect of genoype); a trend ($p=0.099$) for time-of-day main effect was also observed (Figure 2A). Jak2 had a significant time of day main effect, but no genotype main effect (Figure 2B). Assessment of stat5 and socs2 mRNA revealed no genotype or time of day main effects (Figures 2C-D). Igf-1 showed significant genotype main effect, which manifests as a 30% elevation in gene expression in CBK-Cre(+)

hearts, and significant time of day and interaction effects (Figure 2E). Expression of igf-1r revealed a significant main effect of genotype, manifesting as a 29% overall increase in gene expression in CBK-Cre(+) hearts (Figure 2F). Cosinor analyses revealed no significant expression rhythms in these genes.

Liver gene expression of ghr, and jak2 revealed no significant main effects (Figures 2G, H), and the main effects of genotype seen in heart igf-1 or igf-1r were not observed in the liver (Figures 2K-L). However, Stat5b ($p < 0.01$), socs2 ($p < 0.01$), igf-1 ($p = 0.013$), and igf-1r ($p < 0.01$) genes had significant time-of-day main effects. Subsequent cosinor analysis revealed significant 24-hour expression rhythms in stat5b ($p < 0.01$) and socs2 ($p = 0.036$) gene expression in CBK-Cre(-) mice and in 1gf-1 ($p = 0.01$) gene expression for CBK-Cre(+) mice, but not in igf-1r gene expression (Figures 2I-L).

Time-Of-Day Dependent GH Sensitivity In The Heart Is Lost In CBK-Cre(+) Mice

Based on the results so far, we hypothesized that elevated levels of ghr mRNA expression could result in increased GH sensitivity thereby causing elevations in igf-1 mRNA. Therefore, knowing that STAT5 phosphorylation is one of the primary pathways activated by GHR and that it is a good indicator of the degree of acute GH signaling, CBK and control mice were treated with intravenously administered GH (0.05 $\mu g/g_{(bw)}$) followed by collection of the heart and liver to assess the GH stimulated pSTAT5/STAT5 ratio (Figure 3). Two-way ANOVA revealed a significant interaction between ZT and genotype in the heart ($p < 0.05$; Figure 3A). Post hoc one-way ANOVA analyses revealed a significantly reduced cardiac pSTAT5 response to GH treatment at ZT 12 compared to

ZT 0 in CBK-Cre(-) mice ($p < 0.05$; Figure 3A). This time of day dependent difference was absent in CBK-Cre-(+) hearts and their pSTAT5 response was significantly elevated ($p < 0.05$; Figure 3A). When the livers were analyzed via two-way ANOVA there was no main effect of ZT, genotype, or interaction between ZT and genotype (Figure 3B).

Cardiomyocyte-Specific Bmal1 Knockout Igf-1 Hemizygous (CBKI) Cre(+) Mouse Characterization

To test the hypothesis that elevated igf-1 gene expression in CBK-Cre(+) mice, correlated with increased GH sensitivity at ZT12, is contributing to the cardiac hypertrophy and myopathy observed in CBK-Cre(+) mice, we created the CBKI-Cre(+) mouse ($Bmal1^{(-/-)}$//$IGF\text{-}1^{(-/+)}$//$Cre^{(+/-)}$), which is haplodeficient for IGF-1, to normalize igf-1 gene expression in the presence of CBK. To confirm this objective we collected hearts and livers from 16wk old CBKI-Cre(+) and CBKI-Cre(-) mice and assessed gene expression (Figure 4). We confirmed that Bmal1 had been knocked out in the heart, but not the liver of CBKI-Cre(+) mice compared to CBKI-Cre(-) controls (Figure 4A & 4B). Next, we assessed igf-1 mRNA levels in the heart and found no difference between CBKI-Cre(+) and controls (Figure 4C) in contrast to the 80% increase in igf-1 mRNA in 16 wk CBK-Cre(+) hearts compared to CBK-Cre(-) controls (Figure 1A). There was no genotype difference in liver expression of igf-1 mRNA between CBKI-Cre(+) and CBKI-Cre(-) mice (Figure 4D).

Serial echocardiography was performed to characterize the functional phenotype of CBKI-Cre(+) mice compared to CBK-Cre(+) and their Cre(-) controls (Figure 5). Left ventricle (LV) internal diameter (LVID) in diastole (d) increased in all mice between 16

and 21 weeks of age while the degree of increase was greater in CBK-Cre(+) mice (+ 0.407 mm) than in CBKI-Cre(+) mice (+ 0.226 mm) and CBK-Cre(-) and CBKI-Cre(-) controls (+ 0.087 and + 0.152 mm respectively; Figure 5A). Additionally, LVID;d was significantly greater in CBK-Cre(+) mice compared to CBK-Cre(-) hearts at 16 weeks, and while there was no genotype difference between CBKI-Cre(+) mice and Cre(-) controls, their LVID;d was greater at 16 and 21 weeks compared to CBK-Cre(-) controls (Figure 5A). LVID in systole (s) revealed similar findings as described in LVID;d with a greater increase in LVID;s in CBK-Cre(+) mice between 16 and 21 weeks (+ 0.579 mm) compared to CBKI-Cre(+) mice (+ 0.286 mm) and CBK- & CBKI-Cre(-) controls (+ 0.149 & + 0.069 mm respectively; Figure 5B). A parallel trend to that described above was observed in LV volume (vol);d and LV vol;s (Figure 5C and 5D respectively). Assessment of percent ejection fraction (EF) and fractional shortening (FS) revealed preserved EF and FS in CBK-Cre(-) and CBKI-Cre(-) controls between 16 and 21 weeks, while EF and FS decreased to a similar degree in both CBK-Cre(+) and CBKI-Cre(+) mice (Figure 5E & 5F). Cardiac output (CO) increased slightly in all but CBKI-Cre(+) mice, in which it decreased slightly (Figure 5G). Stroke volume (SV), likewise, only increased slightly in all genotypes (Figure 5H).

Discussion

The purpose of this current study was to describe the effect of CBK on GH/IGF-1 signaling in mouse hearts and to explore whether hemizygous igf-1 knockout in the CBKI-Cre(+) mouse protected against HF development seen in CBK-Cre(+) animals. Here we report that CBK results in elevated Akt and ERK phosphorylation as well as

increased ghr, igf-1 and igf-1r gene expression suggesting increased cardiac GH/IGF-1 signaling and increased GH sensitivity. When GH response was assessed biochemically, CBK-Cre(-) control hearts displayed a significant time-of-day variation in GH sensitivity with decreased sensitivity at ZT 12 compared to ZT 0. This time-of-day variation in GH sensitivity was lost in CBK-Cre(+) hearts, resulting in elevated GH sensitivity at ZT 12 compared to CBK-Cre(-) controls. Increased GH sensitivity could be contributing to elevated igf-1 mRNA expression. Hemizygous knockout of the igf-1 gene in the CBKI-Cre(+) mice appears to partially protect against certain aspects of the cardiomyopathy observed in the CBK-Cre(+) hearts including LVID in both systole and diastole as well as LV diastolic and systolic volume.

That the circadian clock causes a rhythm in cardiac GH sensitivity is not without precedent in other hormones. It has been shown that the circadian clock controls hormone signaling via a number of different mechanisms. Circulating levels of corticosterone display a powerful diurnal rhythm[35], however this rhythm does not appear to be dependent on a rhythm of the upstream hormone adrenocorticotropin hormone (ACTH) alone, but also on on direct suprachiasmatic nucleus (SCN) input to the adrenal gland via the thoracic splanchnic nerve influencing a rhythm in response to ACTH[36, 37] including hormone production[38-40]. Additionally, there is evidence that the circadian clock directly influences sensitivity of peripheral tissues to corticosterone via directly modulating expression of the glucocorticoid receptor[36, 41]. Furthermore, it has been shown that insulin sensitivity is rhythmic, peaking in the morning and decreasing throughout the day [9, 10]. Rhythmic sensitivity is even observed in the case of nicotine[42].

It has been well documented that elevated GH and IGF-1, as is the case in acromegaly, leads to cardiac hypertrophy[2, 43-51]. And while echocardiographic analysis of the effect of CBKI-Cre(+) on the cardiomyopathy phenotype suggest a partial rescue, it is clear that there are also other factors at play. Recently McGinnis, et al [31] reported increased insulin sensitivity throughout the day in CBK-Cre(+) mice accompanied by an in crease in protein synthesis and an overall reduction in autophagy. The simultaneous signaling of GH and insulin usually indicates a positive energy balance and promotes anabolism[52], consistent with these findings. This echocardiographic data describes the progression of cardiac form and function from 16 to 21 weeks of age. However, it would be important and informative to do serial echocardiography tracking the development of cardiomyopathy for an extended period of time so as to more fully appreciate the influence of CBKI-Cre(+).

In conclusion, our data show that loss of the cardiac circadian clock via CBK results in the loss of a GH sensitivity rhythm and increased GH sensitivity at ZT 12. Additionally, ghr, igf-1, and igf-1r gene expression is elevated. These increases in the magnitude of growth signal in the heart combined with the evidence that the CBKI-Cre(+) mouse appears to be partially protected against the development of cardiomyopathy, strongly suggest that elevated GH/IGF-1 signaling contributes to the development of hypertrophy and heart failure in CBK mice and possibly the increased risk of CVD in humans experiencing chronic circadian clock disruption (i.e. shift workers).

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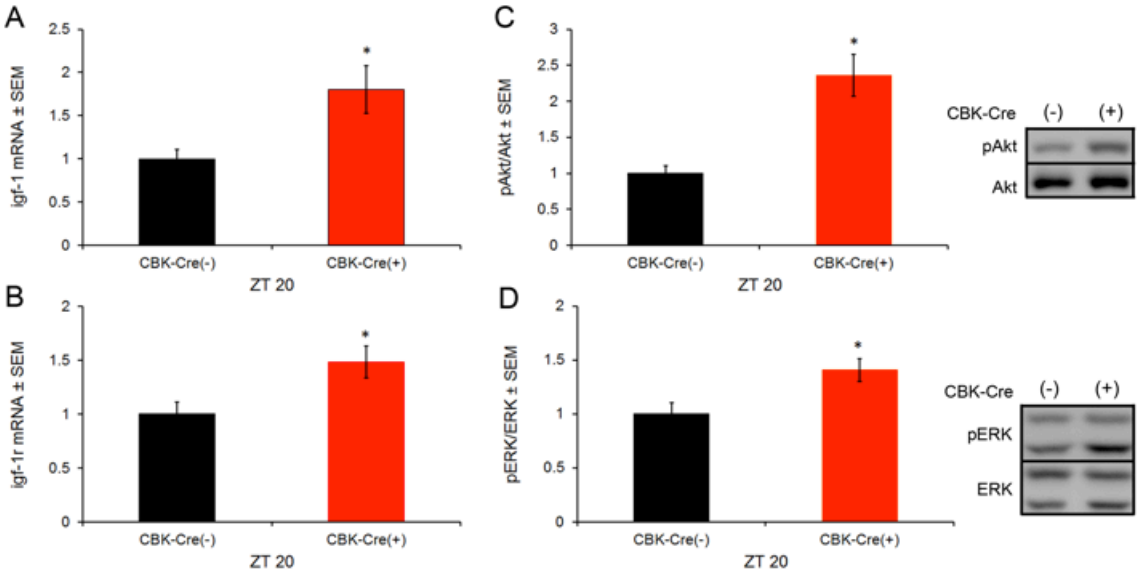


Figure 1 Increased gene expression and signaling activation in CBK hearts. Hearts from CBK-Cre(+) and CBK-Cre(-) mice at 16 weeks of age at ZT 20 were assessed for igf-1 (A) and igf-1r (B) mRNA expression, as well as for Akt (C) and ERK (D) phosphorylation (n = 6; * $p < 0.05$). For un-cropped blots from which representative images were derived see SI Figure 1.

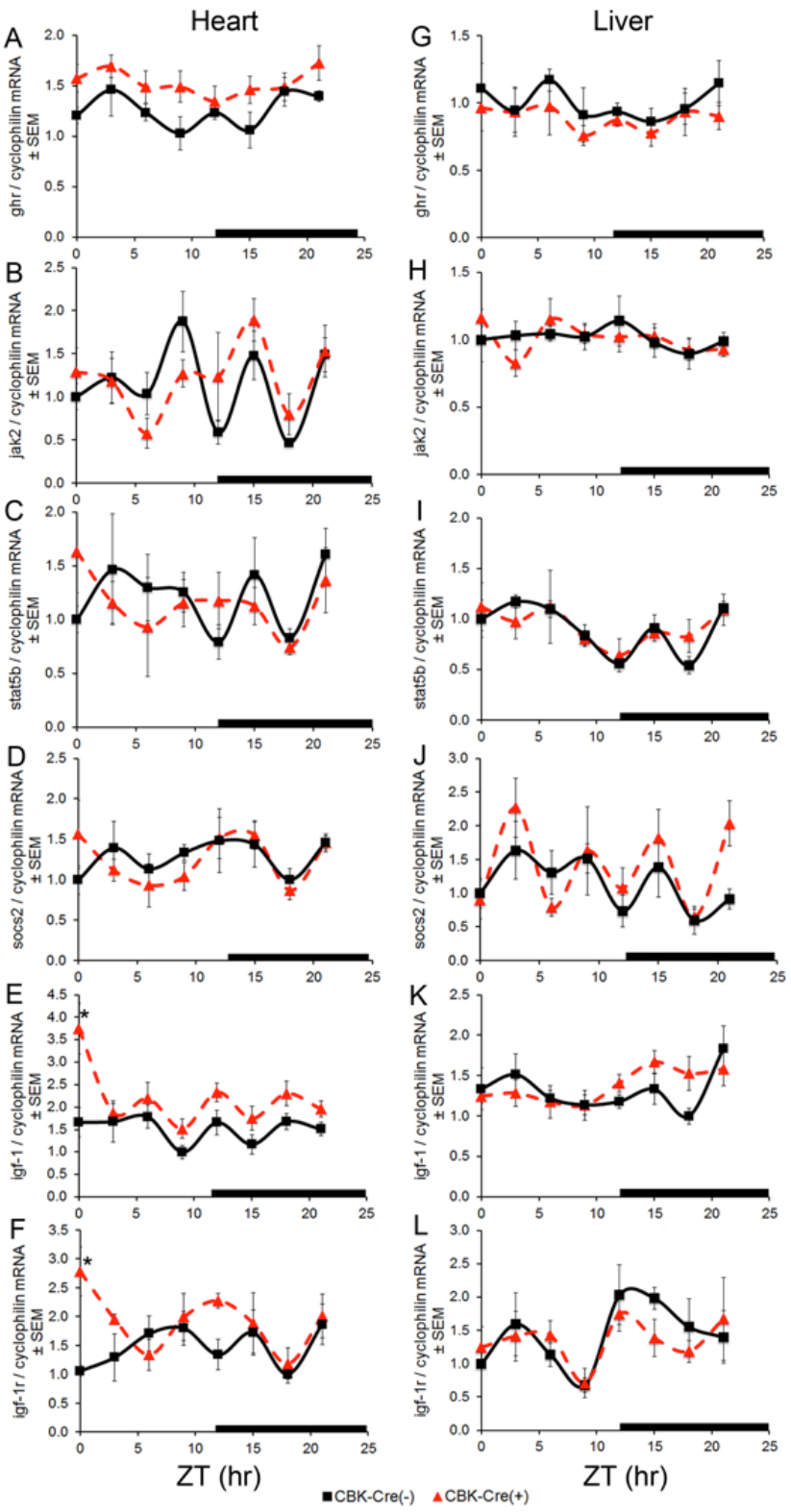

Heart
Liver
A
B
C
D
E
F
G
H
I
J
K
L
ghr / cyclophilin mRNA ± SEM
jak2 / cyclophilin mRNA ± SEM
stat5b / cyclophilin mRNA ± SEM
socs2 / cyclophilin mRNA ± SEM
igf-1 / cyclophilin mRNA ± SEM
igf-1r / cyclophilin mRNA ± SEM
*
*
ZT (hr)
ZT (hr)
CBK-Cre(-)
CBK-Cre(+)

Figure 2

Figure 2 Expression of GH signaling pathway genes throughout the day in 12 week old CBK-Cre(+) and CBK-Cre(-) mice. Heart and liver expression of ghr (A, G), jak2 (B, H), stat5b (C, I), socs2 (D, J), igf-1 (E, K), and igf-1r (F, L) mRNA was assessed (n = 5-6; *p < 0.05 between genotypes at given ZT).

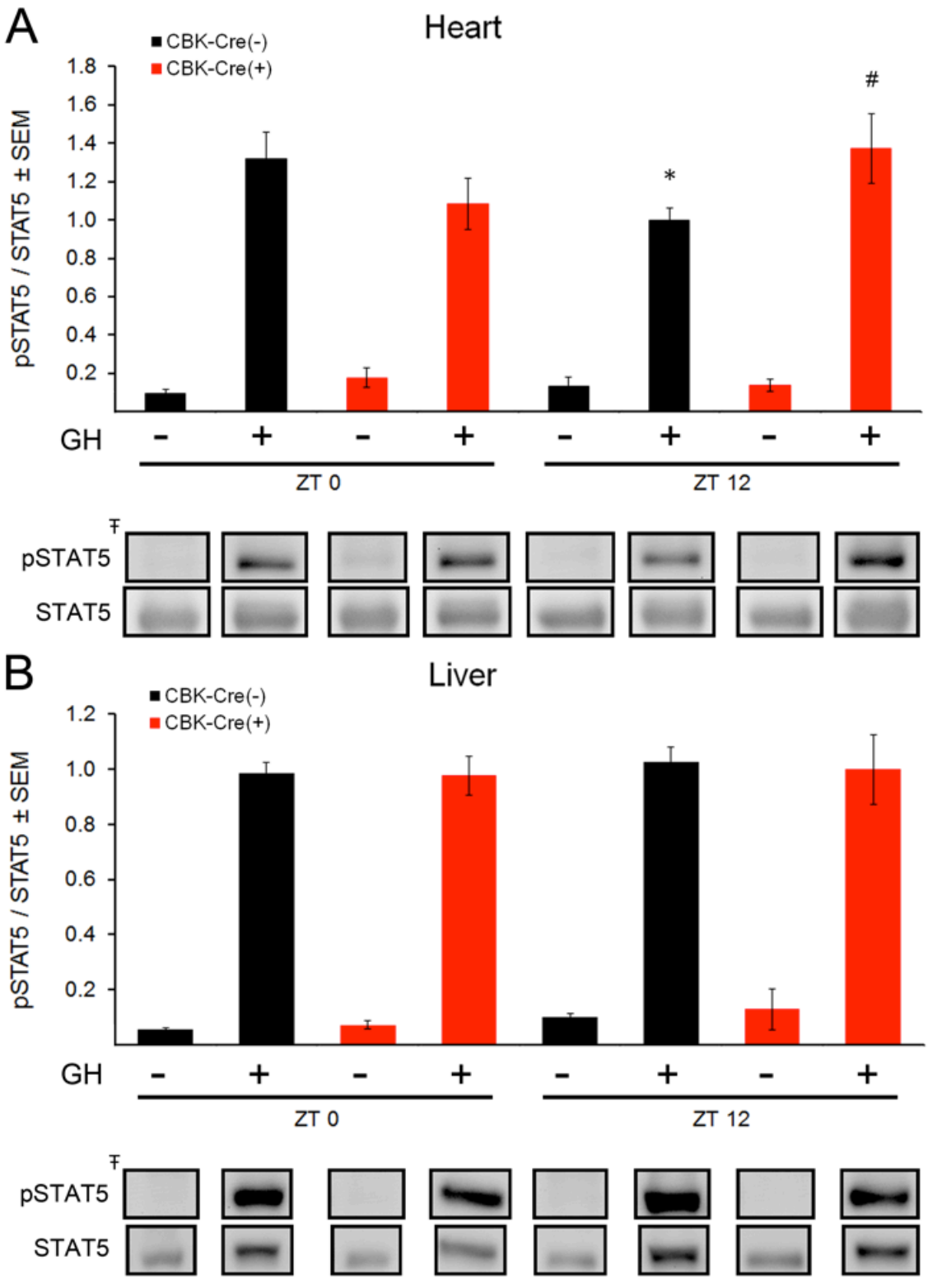

A
Heart
CBK-Cre(-)
CBK-Cre(+)
pSTAT5 / STAT5 ± SEM
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
#
GH
-
+
-
+
-
+
-
+
ZT 0
ZT 12
pSTAT5
STAT5
B
Liver
CBK-Cre(-)
CBK-Cre(+)
pSTAT5 / STAT5 ± SEM
1.2
1.0
0.8
0.6
0.4
0.2
GH
-
+
-
+
-
+
-
+
ZT 0
ZT 12
pSTAT5
STAT5

Figure 3

Figure 3 STAT5 phosphorylation in Heart (A) and liver (B) in response to a five-minute intravenous GH or Saline treatment in CBK-Cre(+) and CBK-Cre(-) mice at ZT 0 and ZT 12. (n = 5-9); * $p < 0.05$ compared with CBK-Cre(-) at ZT 0; # $p < 0.05$ compared with CBK-Cre(-) at ZT 12. (Ŧ – representative blots were created by rearranging bands from the same blot into a more meaningful order for easier visualization. Original blots can be found in SI Figure 2.)

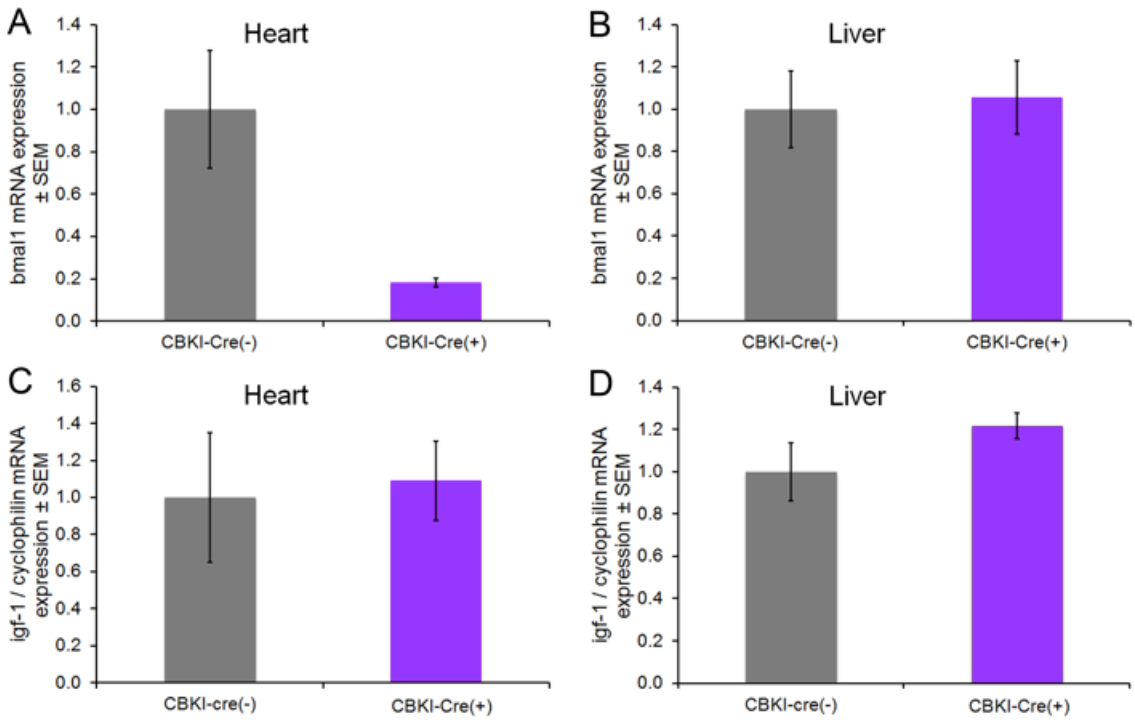


Figure 4 Basic molecular characterization of 16 week old CBKI-Cre(+) and –Cre(-) mice sacrificed at ZT 6. Gene expression of bmal1 mRNA in heart (A) and liver (B) and igf-1 in heart (C) and liver (D) was assessed. Phosphorylation of Akt was assessed in heart (E) and liver (F) (n = 2-4).

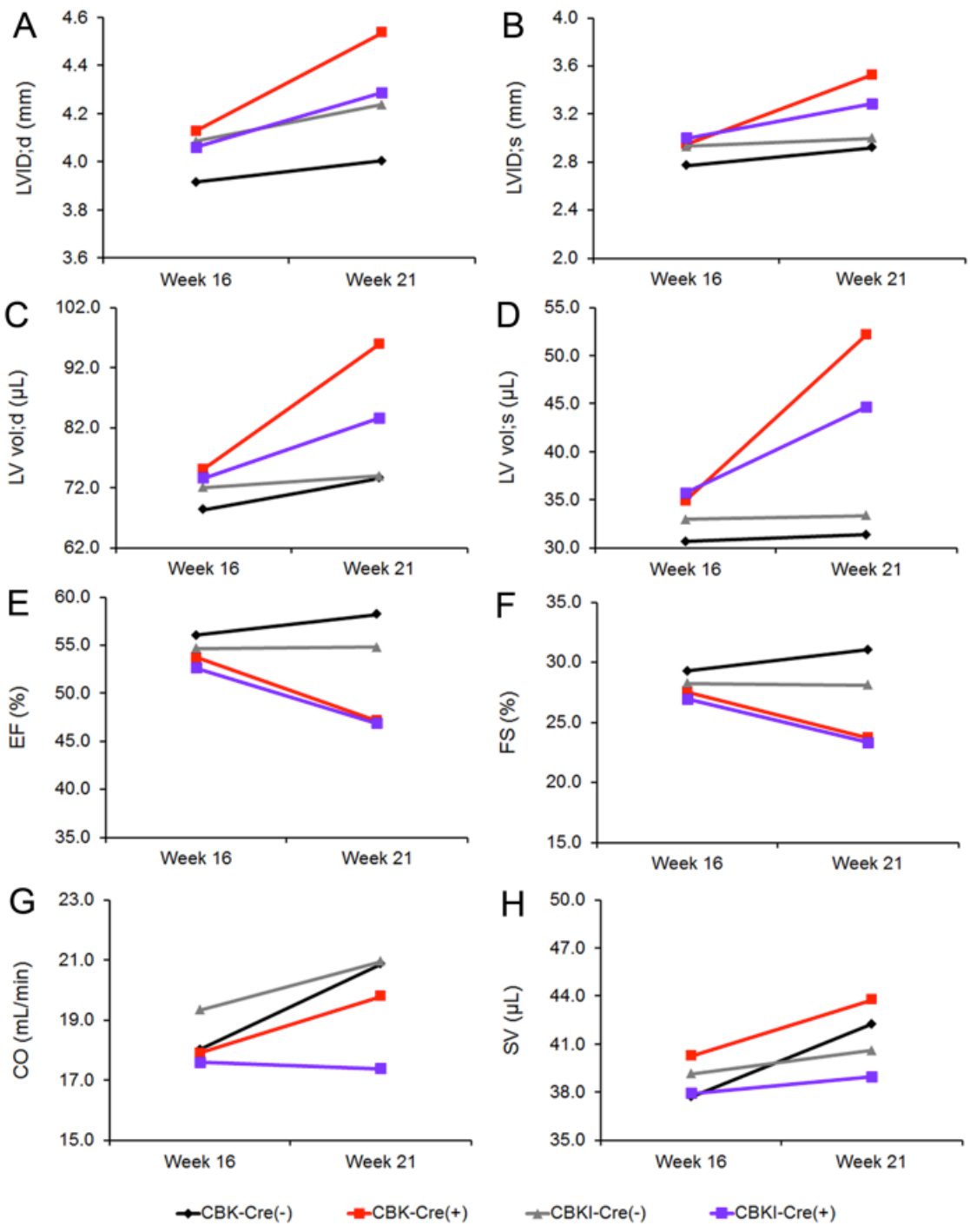
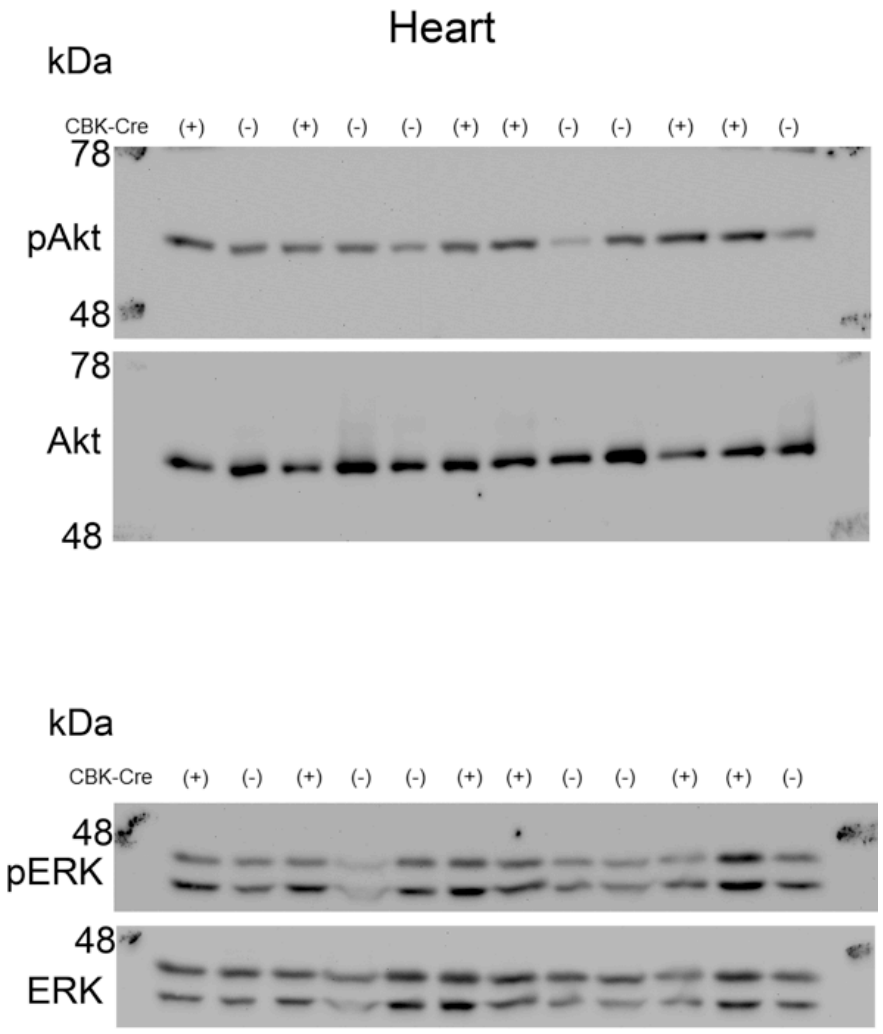
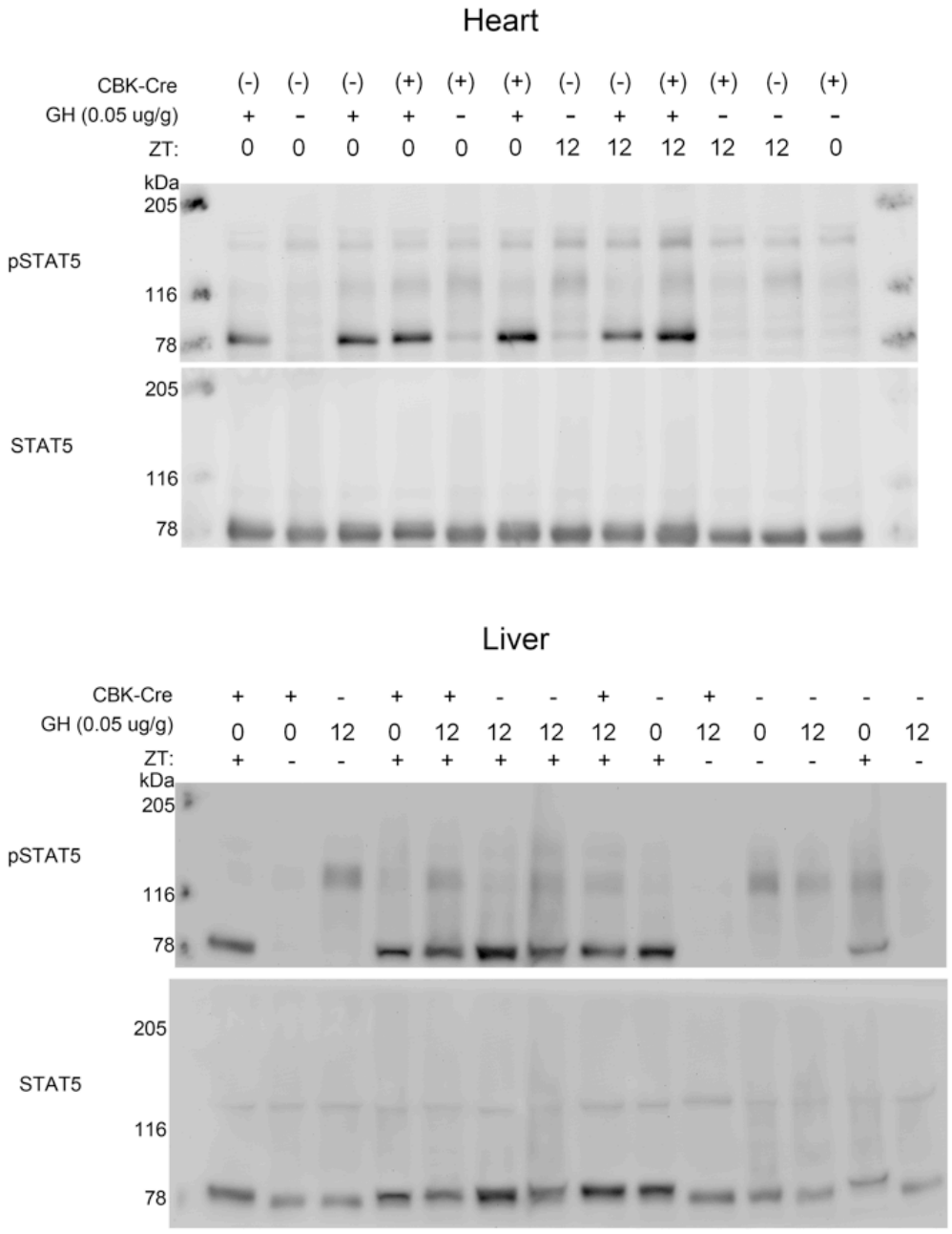

A
LVID;d (mm)
4.6
4.4
4.2
4.0
3.8
3.6
Week 16
Week 21
B
LVID;s (mm)
4.0
3.6
3.2
2.8
2.4
2.0
Week 16
Week 21
C
LV vol;d (µL)
102.0
92.0
82.0
72.0
62.0
Week 16
Week 21
D
LV vol;s (µL)
55.0
50.0
45.0
40.0
35.0
30.0
Week 16
Week 21
E
EF (%)
60.0
55.0
50.0
45.0
40.0
35.0
Week 16
Week 21
F
FS (%)
35.0
30.0
25.0
20.0
15.0
Week 16
Week 21
G
CO (mL/min)
23.0
21.0
19.0
17.0
15.0
Week 16
Week 21
H
SV (µL)
50.0
47.0
44.0
41.0
38.0
35.0
Week 16
Week 21
CBK-Cre(-)
CBK-Cre(+)
CBKI-Cre(-)
CBKI-Cre(+)

Figure 5

Figure 5 Echocardiographic characterization of CBKI-Cre(+) and –Cre(-) and CBK-Cre(+) and –Cre(-) mice using M-mode at 16 and 21 weeks. Parameters characterized are LVID;d (A), LVID;s (B), LV vol;d (C), LV vol;s (D), EF (E), FS (F), CO (G), and SV (H). At 16 weeks, n = 3-10. At 21 weeks, n = 2-8.



SI Figure 1 Original immunoblots from which representative blots for figure 1C-D were derived.



SI Figure 2 Original immunoblots from which representative blots for figure 3A-B were derived.

CONCLUSION

Extensive research has been done to elucidate GH signaling pathways and to describe the effects and role of GH in mammals systemically as well as in individual organs. Nevertheless, differences in GH sensitivity and responsiveness between major metabolic organs have not been described. Herein, we describe the relative rank order of tissue GH sensitivity and responsiveness in heart, liver, kidney, gonadal WAT, and gastrocnemius muscle. The descending order for sensitivity is liver = kidney > gonadal WAT = heart = gastrocnemius. The descending order for responsiveness is gastrocnemius > heart > liver > gonadal WAT > kidney. Additionally, responsiveness had a rough positive correlation with STAT5 protein level.

This research addresses a barren zone in the center of GH research that has previously been largely ignored. This area has been has been explored only incidentally when in 1984 D'Ercole, et al [64] described the change in IGF-1 peptide concentration over time in the liver, kidney, heart, lung, brain and serum in rats in response to an injection of GH. Their principle objective was to assess the timing of IGF-1 increase of the various tissues and the serum in relation to the liver to determine whether IGF-1 was being produced in extrahepatic tissues. Incidentally, they also found that the maximum IGF-1 concentration achieved as a result of their treatment differed by tissue with kidney achieving a concentration of 3.5 U/g, followed by lung (~1.2 U/g), then heart and liver (~0.8 U/g), and finally brain, which did not increase much above baseline. Additionally, they measured dose response in liver and lung using four different doses of GH and fit

the data using linear regression. Major differences between this paper and our experiment (in addition to the difference in animal model) are the number of GH doses used in the dose response curve (4, D'Ercole et al and 9, Berry et al), the number of tissues analyzed (2, D'Ercole et al, and 5, Berry et al), and the magnitude of the doses used. In their dose response curve the lowest dose equates to ~ 100ng/g, greater than our constrained EC50 estimate for gastrocnemius, the least sensitive of the five tissues we assessed; and roughly two times the concentration at which maximal pSTAT5/STAT5 is achieved in mouse liver. This limits the meaningfulness of the dose response information produced by D'Ercole et al. Another important difference is that we used a more acute measure of GH signaling (pSTAT5 compared to IGF-1 peptide), thereby avoiding potential secondary effects of the GH treatment. However, measuring a more distal output of GH signaling may provide more insight into the functional consequences of differential GH sensitivity. Regardless, our results differ from those of D'Ercole et al in that they report that maximal distal response (tissue IGF-1 concentration) to their extremely high dose of GH is 3x higher in kidney than it is in liver, while we report the acute maximal response (pSTAT5) in kidney to GH to be nearly 100x lower in kidney than in liver. This major difference may be the result of secondary effects, differences between mice and rats, or simply due to increased amplification of the downstream signal in kidney compared to the liver.

The second part of this work focuses on GH signaling in the heart, the crosstalk that occurs between this and the circadian clock, and the influence of GH signaling on the cardiac phenotype in the CBK-Cre(+) mice. The key findings from these experiments are that CBK-Cre(+) hearts have increased ghr, igf-1, and igf-1r mRNA expression compared to controls, that CBK-Cre(-) hearts display a time of day dependent difference in the

acute response to GH treatment, and that CBK-Cre(+) hearts lack this time of day difference in response and have overall greater response to GH treatment than CBK-Cre(-) hearts. Furthermore, we show that cardiomyocyte-specific hemizygous IGF-1 knockout in the CBKI-Cre(+) animals partially protects against the development of hypertrophy and heart failure compared to CBK-Cre(+) mice. Recently, McGinnis et al showed a number of genes involved in the shared IGF-1/Insulin signaling pathways to be upregulated in CBK-Cre(+) hearts compared to controls and that Akt phosphorylation in response to 5 minutes insulin treatment was greater in CBK-Cre(+) hearts than in CBK-Cre(-) controls. We also corroborate their findings that pAkt is elevated by roughly two fold in CBK-Cre(+) hearts injected with saline and in those not receiving any treatment at all[378].

Cytokine receptors have been shown to interact with one another and even influence ligand sensitivity of neighboring receptors. For example, the absence of IGF-1R leads to a blunting in GH stimulated pSTAT5 that can be restored by reintroducing the IGF-1R[32-35]. It has also been shown that skeletal muscle-specific GHR knockout leads to an insulin resistant state with as evidenced by increased peak serum glucose following a glucose tolerance test and failure of an insulin tolerance test to produce a reduction in serum glucose levels[379]. Here we have reported elevated GH sensitivity in CBK-Cre(+) mice compared to controls accompanied by elevated ghr and igf-1r gene expression. In these same animals, McGinnis et al have reported increased insulin sensitivity as reported by elevated Akt phosphorylation. It is reasonable to hypothesize that elevated insulin sensitivity in CBK-Cre(+) hearts may be due, in part, to elevated IGF-1R.

Autophagy is also impacted in CBK-Cre(+) hearts and they experience an overall reduction in autophagy and increase in protein synthesis via increased Akt and mTOR phosphorylation[378]. While this finding was originally presented in the context of insulin signaling, GH has also been shown to have powerful anti-apoptotic effects in cardiomyocytes[380]. However, the anti-apoptotic effects of GH in cardiomyocytes appear to be IGF-1 independent and to function via a calcineurin-dependent mechanism, involving mitogen activated protein kinase p38 (MAPK p38) [380].

We also report that our CBKI-Cre(+) mouse partially rescues the dilated cardiomyopathy (DCM) phenotype observed in CBK-Cre(+) mice. This is not entirely surprising, however, because, while the literature is conflicting as to whether GH treatment is beneficial in heart failure, there is a consistent observation that normalizing elevated GH levels in acromegalic patients results in reduced heart size[375]. Therefore, normalizing elevated igf-1 gene expression results in some reduction in heart size, or rather, prevention of heart enlargement.

Further studies in this area are needed to better understand the relationship between the observed alterations in both the insulin and GH signaling pathways. Possible approaches should include cardiomyocyte-specific Bmal1(-/-)//GHR(-/-) mice to view the effect on the DCM phenotype as well as the effect on cardiac insulin sensitivity. Additionally, a cardiomyocyte-specific Bmal1(-/-)//IGF-1R(-/-) mouse would be helpful in dissecting the contributions of direct and indirect (IGF-1 mediated) GH effects. Corroboration of the relationship between the cardiomyocyte circadian clock and the GH/IGF-1 signaling pathway should also be described using methods of circadian clock disruption, including the cardiomyocyte-specific CLOCK mutant (CCM) mouse, which

overexpresses a dominant negative form of the clock gene, as well as more physiological methods including simulated shift work and reverse restricted feeding. Such strategies will help determine whether the observed effects are dependent on the circadian clock in general or whether they are Bmal1 specific.

The effects of the cardiac circadian clock on GH/IGF-1 and insulin-mediated signaling have been well described both here and elsewhere[378]. Nonetheless, the mechanism by which the clock exerts these effects remains somewhat elusive and our knowledge is limited to the observation of altered gene and protein expression patterns and levels. What remains to be examined is whether expression of these genes and their respective proteins is controlled directly by the clock or indirectly by clock controlled transcription factors, and in both cases, which clock components or secondary transcription factors are directly responsible for said regulation. This endeavor would be much facilitated by a tractable in vitro model. This, however, has proved to be a challenge. We have found that the process of immortalization of many cardiomyocyte cell lines (i.e. AC-16 cells), results in either the loss of GHR expression, the loss of GH responsiveness, or both. However, the HL-1 cell line, a line of adult atrial cardiomyocytes, expresses GHR, JAK2, and STAT5 and has immunodetectable increases JAK2 and STAT5 phosphorylation in response to GH treatment [380]. Such a system would enable more facile experimentation, including ChipSeq studies, to more completely probe the connection between the clock and these signaling pathways.

In this body of work we have characterized GH sensitivity in heart, liver, kidney, gonadal WAT, and gastrocnemius muscle at one time of day. We have also described time-of-day dependent differences in cardiac GH response or sensitivity to GH treatment

in CBK-Cre(-) control animals that are abolished in the CBK-Cre(+) mice. These findings underlie the need for further studies to characterize tissue-specific differences in GH sensitivity and responsiveness throughout the day to better understand this time of day dependent difference in GH sensitivity in the heart and to assess whether other tissues also display time of day differences in GH sensitivity. An understanding of when tissue-specific GH sensitivity is maximal would greatly influence our understanding of the role of GH signaling in each tissue. In our sensitivity and responsiveness study we found that inter-tissue differences in sensitivity and responsiveness were only loosely correlated with STAT5 abundance. It is possible that intra-tissue time-of-day differences in GH sensitivity may be dependent on different mechanisms, which could vary from tissue to tissue. Additionally, we did not observe dose dependent induction of either ERK or Akt phosphorylation, in any of the tissues analyzed, under the conditions used (5 minute GH treatment; data not shown). Optimization of the conditions needed to observe GH dose-dependent ERK and Akt phosphorylation and assessment of tissue-specific differences will also greatly enhance our understanding of the role of GH in different tissues.

In recent decades, the heart has become appreciated as a secretory organ with the discovery of its secretion of brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP)[381], which are important regulators of blood pressure. Interestingly, Iglesias et al [382] showed that the heart may be responsible for feedback onto the hypothalamus and GH secretion. Specifically, they reported that cardiomyocytes synthesize and secrete ghrelin, a major stimulant of GH secretion. Their model was in vitro, and it is difficult to know exactly how well their findings will translate in vivo. Investigating this finding

further in the CBK-Cre(+) mouse will also prove beneficial in understanding how the cardiomyocyte circadian clock is intertwined with the GH/IGF-1 signaling axis.

Overall, our studies have expanded the understanding of how cardiomyocyte-specific Bmal1 knockout influences cardiac physiology and subsequent pathological changes leading to DCM and heart failure through regulation of GH signaling. Additionally, we have provided a detailed description of tissue-specific GH sensitivity and responsiveness, which, along with our finding of differential time-of-day dependent GH sensitivity in control mouse hearts that disappears in the CBK-Cre(+) mouse, has revealed the need for more extensive characterization of tissue-specific GH sensitivity and responsiveness throughout the day. This description of GH dose responsiveness has provided a reference that can be used to interpret the results of other GH studies in mouse models including by providing an understanding of the GH dose that will achieve a maximal or sub-maximal response in a given tissue. Additionally, it provides a valuable reference for planning of future studies assessing GH signaling in heart, liver, kidney, white adipose tissue, and skeletal muscle.

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APPENDIX

IACUC APPROVAL LETTER

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 24-Jul-2017

TO: Frank, Stuart J

FROM:

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 24-Jul-2017.

Protocol PI:	Frank, Stuart J
Title:	The Role of the Cardiac Circadian Clock on Growth Hormone/Insulin-Like Growth Factor 1 Signaling and the Development of Heart Failure (Ryan Berry)
Sponsor:	UAB DEPARTMENT
Animal Project Number (APN):	IACUC-20156

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 07-Jul-2017.

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