METABOLIC EFFECTS OF 17α-ESTRADIOL ARE GROWTH HORMONE INDEPENDENT AND SEX SPECIFIC

by

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ABSTRACT

Aging is a major risk factor for metabolic syndromes and type two diabetes. With growing elderly populations worldwide and increasing incidence of age-related diseases there is a great need to develop pharmacological interventions that would delay aging and protect from age-related diseases. 17-alpha estradiol (17α-E2) is an epimer of the primary female sex hormone estradiol and has been shown to extend lifespan and downregulate markers of age-related metabolic dysfunction in male mice. Because 17α-E2 does not induce feminization in males it holds potential as a novel therapeutic in humans for age-related metabolic dysfunction. Importantly, we have previously shown that 17α-E2 causes an increase of circulating and hepatic IGF-1 in aged mice, without any changes in GH release in treated animals. Based on this we propose a new hypothesis that 17α-E2 acts through a novel, GH-independent pathway stimulating production of IGF-1 and positively modulating metabolic function in a sex-specific manner. Here we studied 17α-E2 treated long-lived growth hormone receptor knockout (GHRKO) mice, characterized by severely reduced circulating and hepatic IGF-1 due to GH-resistance. We found increases in circulating IGF-1 after treatment in normal and GHRKO male mice, with no effect in female mice, which supports our hypothesis that 17α-E2 induces GH independent IGF-1 production. To determine novel genetic pathways activated by 17α-E2 we performed sequencing of hepatic RNA. Our analysis indicated differential regulation of steroid biosynthesis and insulin signaling pathways. The validation of our sequencing data using qPCR showed significant upregulation of genes involved in insulin action. Importantly, differential regulation of these pathways was present in normal male mice, with no changes in normal females or either male or female GHRKO animals. In summary, this new data supports our

hypothesis of a sex-specific effect of 17α-E2 treatment and differing mechanisms of action by which 17α-E2 upregulates IGF-1 independently of GH action.

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17-alpha Estradiol (17αE2)

Growth Hormone Receptor Knockout (GHRKO)

Growth Hormone (GH)

Growth Hormone Receptor (GHR)

Homeostatic Assessment of Insulin Resistance (HOMA)

Insulin-like Growth Factor-1 (IGF-1)

Insulin Receptor Substrate 1 (IRS1)

Janus Kinase (JAK)

Knockout Female Control (KOFC)

Knockout Male Control (KOMC)

Knockout Female Estradiol (KOFE)

Knockout Male Estradiol (KOME)

Normal Female Control (NFC)

Normal Male Control (NMC)

Normal Male Estradiol (NME)

Normal Female Estradiol (NFE)

Peroxisome Proliferation Factor Alpha (PPARα)

Peroxisome Proliferation Factor Gamma (PPARγ)

Phosphoinositide 3-kinases (PI3K)

Quantitative Polymerase Chain Reaction (qPCR)

RNA Integrity Number (RIN)

Signal Transducers and Activators of Transcription (STAT)

Suppressor of Cytokine Signaling (SOCS)

CHAPTER ONE: INTRODUCTION

Metabolic dysfunction, particularly type two diabetes and obesity prevalence, increases with age leading to increased healthcare costs and gives rise to numerous secondary health issues (1, 2). Interventions in the form of calorie restriction have proven effective in humans but pharmaceutical interventions are more desirable as they are easier to implement and have higher retention rates (3). With advances in healthcare the human population is living longer and thus there is a great need to develop new pharmacological interventions that would delay aging and protect from age-related metabolic diseases.

To further investigate possible treatments for age-related diseases and to examine how healthy lifespan (healthspan) can be extended in humans, the National Institutes of Aging (NIA) has created the Interventions Testing Program, which studies agents with the potential to extend lifespan and delay metabolic dysfunction with age. Agents of interest are tested at three separate sites using identical standardized protocols and results are pooled yielding robust statistical significance. One such agent from this program found to extend healthspan is 17α-E2. The female hormone 17α-E2 is a stereoisomer of the primary female sex hormone 17β-E2, the most potent estrogen hormone in circulation, differing from its epimer by the orientation of a hydroxyl group which causes a decreased affinity for the classical estrogen receptors, ERα and ERβ. 17α-E2 is naturally occurring in females and males and because of the aforementioned decreased affinity does not induce female characteristics in males. Studies have shown that 17α-E2 treatment alleviates age related metabolic dysfunction in mice (4) extends median lifespan in male mice (5, 6), and more recently the positive metabolic effects of 17α-E2 have been

associated with the production of testicular hormones in adult life (7). Since 17α-E2 is non-feminizing and given the link between lifespan extension and metabolism it holds potential as a novel therapeutic in humans for age-related metabolic dysfunction.

The focus of our research group is how modulation of the GH/IGF1 axis can contribute to life and health span extension. The GH/IGF1 axis has been shown to be the most conserved pathway affecting lifespan and manipulations to signaling have been shown to extend lifespan (BARTKE citation). Since 17α-E2 has been previously shown to extend lifespan it is of interest to us how 17α-E2 is affecting GH/IGF1 to extend lifespan.

We first studied the effects of 17α-E2 on GH/IGF1 signaling in C57B6 mice and observed a GH *independent* increase in IGF1 production with treatment. This is a seminal observation in the field of endocrinology as it is classically thought that IGF1 production is explicitly linked to GH release. In order to pinpoint how exactly 17-aE2 is in fact inducing an increase in IGF1 independent of GH we sought a model which has no GH. The growth hormone receptor knockout (GHRKO) mouse provides the ideal setting to study this. These mice were first described by Zhou et al in 1997 and are characterized by absence of the growth hormone receptor (GHR) and GH binding protein and thus are growth hormone resistant (16). Important to our study is the global lack of GHR as it guarantees that observed IGF1 increases with 17α-E2 treatment are independent from GH action and creates the ideal setting to test our hypothesis that 17α-E2 acts through a novel, GH-independent pathway stimulating production of IGF1 and positively modulating metabolic function in a sex-specific manner. Our research examined the effects of 17α-E2 treatment on the GH/IGF1 axis, genes downstream in the insulin and IGF1 signaling pathway and the extent of the effects of 17α-E2 on IGF1 production on hepatic tissue

to explore the mechanisms responsible for the previously observed lifespan extension and alleviation of age-related metabolic dysfunction.

Given the demographic trends of increased longevity and consequent rise of metabolic disorders with age and the important role that the GH/IGF1 axis plays in modulating lifespan an increased understanding of the mechanism of action of 17α-E2 is useful. Thus, the main focus of this study was to investigate the possible alternative regulation of GH/IGF1 signaling by 17α-E2.

CHAPTER TWO: LITERATURE REVIEW

Growth Hormone/IGF1 Axis and Aging

Growth hormone is secreted by the anterior pituitary gland in response to several stimuli which include growth hormone releasing hormone (GHRH), ghrelin and dietary stimuli. Its secretion is controlled by a negative feedback loop with increased levels of IGF1 stimulating decreased GH secretion. Briefly, GH release is stimulated by GHRH which is produced in the hypothalamus. GH travels to the liver where it binds growth hormone receptors (GHR) leading to the production of IGF1 by the liver. IGF1 travels through the bloodstream and acts on several tissues including muscle and bone to promote growth. There is a negative feedback loop where increases in IGF1 levels result in decreases in GHRH and thus decreases in release of GH. Somatostatin inhibits GH release in response to GHRH (6). Thus, there is a direct pathway, with regulation of hepatic IGF1 production by GH release and action.

It is important to note that GH is not continually secreted but secreted in bursts over the day and this sort of release pattern is termed “pulsatile.” The pattern of GH release also differs between females and males. In females, it is characterized by a continuous pattern of release but in males GH is released in bursts separated by two hour intervals where GH release levels drop to zero. (CITATION). These GH release cycles can be studied by drawing blood at consistent time intervals over the day for a measure of GH pulsativity (8). The liver is the principle source of IGF1 in the body with GH being the principle regulator of IGF1 production (9). IGF1 is a key player in muscle growth, stimulates amino acid uptake and protein synthesis. The production of IGF-1 has been classically inextricably linked with the action of GH. There have been

discussions in the field of the possibility of varying GH control of IGF1 in tissues other than the liver with some tissues displaying GH independent like production of IGF1, although possible sources of extrahepatic circulating IGF1 is likely small and not well studied (10).

GH signaling is primarily through the Janus Kinase/Signal transducer and activator of transcription (JAK/STAT) pathway and downstream of IGF1 signaling is the PI3-k/Akt pathway including IRS-1, SOCS family of genes. There is heavy crosstalk between IGF1 and insulin signaling.

With age, there are fluctuations in growth hormone levels and it is thought that decreases in growth hormone levels contribute to aging. However, there have been several studies which have attributed disruption in growth hormone (GH) signaling to lifespan extension, for instance in the growth hormone receptor knockout mouse (11).

17-alpha Estradiol

17α-E2 is a naturally occurring hormone in both females and males with endogenous levels being higher in females. 17α-E2 is weakly estrogenic and thus considered to be non-feminizing. This is due to its decreased binding affinity for the classical estrogen receptors ERα and ERβ. 17α-E2 needs to be distinguished from its isomer 17βE2 which is a potent feminizing hormone. 17βE2 has been the subject of studies which emphasize its neuroprotective effects and possible positive effects on cognition (12). 17α-E2 is a naturally occurring hormone and is termed non-feminizing in that it does not induce female characteristics in males. Several previous studies have examined the effect of 17α-E2 on lifespan as well as glucose homeostasis.

One such previous studies has shown up to a 20% increase in lifespan with 17α-E2 treatment (13, 14). In others, authors have demonstrated that 17α-E2 improves glucose tolerance and mTORC2 signaling (7). Of note, is that all these studies show positive effects of the 17α-E2 treatment only in males and not females.

Growth Hormone Receptor Knockout Mice

These mutant mice have a targeted inactivation of the *Ghr* gene which is generated by the deletion of the 3' portion of exon 4 and part of the downstream intron as pioneered by Zhou et al. (15). Mice lacking GHR function serve as a model of human Laron dwarf syndrome. Disruption of the GHR decreases IGF1 synthesis in the liver and stunts growth in these mice so they are smaller than their normal littermates. These mice have very low levels of IGF1 and increased levels of GH due to lack of negative feedback loop and subsequent overproduction by the pituitary gland and have increased lifespan (16). These mice have been studied previously are also characterized by numerous health benefits as previously described herein, and they are particularly useful in this study as their mutation prevents the presence of residual GH activity allowing us to pinpoint 17α-E2 regulation of IGF1 in these mice independently of GH action

CHAPTER THREE: MATERIALS AND METHODS

Animals –Growth hormone receptor knockout (GHRKO) and normal mice were produced in the lab of Dr. Andrejz Bartke of Southern Illinois University. C57B6 mice were housed in the lab of Dr. Michael Stout of Oklahoma Health Sciences Center. GHRKO mice were produced by mating heterozygous (GHR $^{+/-}$) female carriers of the disrupted GHR/GH-binding protein gene with knockout (GHR $^{-/-}$) males. Animal procedures were performed at OUHSC and SIU in accordance with their animal protocols.

17α-E2 Treatment– The first study was conducted in all male C57B6 mice beginning at 18 months of age and treatment continued for 15 weeks. Treatment in the GHRKO mice was initiated at six months of age. Treatment was administered for four months following which animals were euthanized and blood, plasma and tissue was collected and frozen at −80°C for analysis. In both cohorts of mice studied, they were fed a diet containing 17α-E2 continuously and had ad libitum access to food. 17α-E2 was mixed with their chow, prepared externally, at a dose of 14.4 milligrams per kilogram diet (14.4 ppm) based on previous work (13).

For the GHRKO treatment cohort there was eight experimental groups: Normal Male Control (NMC), Normal Male Estradiol (NME), GHRKO Male Control (KOMC), GHRKO Male Estradiol (KOME), Normal Female Control (NFC), Normal Female Estradiol (NFE), GHRKO Female Control (KOFC) and GHRKO Female Estradiol (KOFE). To each of these groups 12 mice were initially assigned.

GH Release Measurement - Pulsatile GH release was measured according to the protocol described by Steyn et. al. briefly, tail-tip blood samples were collected from the animals at regular intervals over the day and GH levels of whole blood were measured using a sensitive sandwich ELISA

qPCR –Briefly, total RNA was extracted from liver tissue using Trizol and reverse transcribed to cDNA using the iScript cDNA synthesis kit from Bio-Rad according to manufacturer's protocol. All the qPCR experiments were performed in replicates and beta-2 microglobulin (B2M) was used as an endogenous control. All results are reported as the mean ± standard error of the mean (SEM)

ELISA – Quantikine® ELISA Mouse/Rat IGF-1/IGF-1 Immunoassay from R &D Systems was used to measure IGF1 levels in plasma. Plasma insulin levels were measured with the Mouse Ultrasensitive Insulin ELISA from ALPCO. Hepatic total AKT1/2 and phosphorylated AKT (pS473) were measured using the Multispecies InstantOne^{TM} ELISA Kits, all from Thermo Fischer Scientific. All ELISA kits were used according to manufacturer's suggested protocol.

RNA Sequencing and Data Analysis – From each of the eight experimental groups, six animals were randomly selected and RNA extracted from liver tissue, in the same process described for qPCR analysis, for a total of 48 samples for RNA sequencing. RNA was extracted via a

Trizol/Chloroform extraction and externally tested for RNA Integrity with all samples having an RNA Integrity Number (RIN) greater than 6.9. Following quality control, library preparation and sequencing was performed by Novogene.

The mapping of sequencing reads to the mouse transcriptome (Illumina iGenomes annotation for UCSC mm10, http://support.illumina.com/sequencing/sequencing_software/igenome.html) was performed using HiSat2 (17).The number of reads aligned to its corresponding gene was calculated by HTSeq 0.6.1 (18)**.** Genes with an average FPKM lower than 1/100,000th of the total aligned reads in more than 50% of the samples were eliminated from further analyses. Statistical analyses for differentially expressed mRNAs was performed using the software R (3.2.2) and the Bioconductor package EdgeR (19) using the HTSeq output count. Read counts were normalized for library depth, and pairwise comparisons, measuring fold change, uncorrected P-values from the negative binomial distribution, and adjusted P values (false discovery rate; FDR) were obtained. Principal components analysis (PCA) was also performed using R to observe sample distribution in a two-dimensional plot and eliminate outliers. Unsupervised hierarchical clustering was performed also using the DESeq package to observe sample clustering. Genes with a FDR<0.05 and fold change (FC) >2.0 were considered as up-regulated; and with FDR<0.05 and FC<0.5 were considered as down-regulated. mRNAs were further processed for pathway analysis using the Generally Applicable Gene-set Enrichment (GAGE), which uses log- based fold changes as per gene statistics, and Pathview packages in R (20, 21)**.** Enrichment of gene ontology (GO) terms (biological processes, molecular function and cellular component) was also performed using the GAGE package. P values lower than 0.05 were considered as significant for pathways and GO Terms analysis.

Statistical Analysis – Data were compared using GraphPad Software (GraphPad Prism 8 Software for Windows) employing either a two-tailed Student's T test or three-way analysis of variance for genotype, treatment and interaction significance.

Table 1 – Primer sequences used for gene expression analysis

Genes	**Primer Sequences (forward and Reverse)**
B2M	F: 5′-AAGTATACTCACGCCACCCA-3′
	R: 5′-CAG CGCTATGTATCAGTCTC-3′
IGF1	F: 5′-CTGAGCTGGTGGATGCTCTT-3′
	R: 5′-CACTCATCCACACCTGT-3′
PPARα	F: 5′-GTCAGTACTGTCGGTTTCAG-3′
	R: 5′-CAGATCAGCAGACTCTGGGT-3′
PPARγ	F: 5′-GGGAAAGACCAGCAACC-3′
	R: 5′-TGGAAGAATCGGACCTCTGC-3′
IRS1	F: 5'-AGCCCAAAAGCCCAGGAGAATA-3'
	R: 5'-TTTCGAGCCAGTCTCTTCTCTA-3'
Stat5b	F: 5'-AGA ATT TGC CAG GAC GGA ATT-3'
	R: 5'-GAT AGC CCC ATC ATT CCA GTG A-3'
SOCS2	F: 5'- CTG CGC GAG CTC AGT CAA A -3'
	R: 5'- ATC CGC AGGTTA GTC GGT CC -3'
mTOR	F: 5'- CGG CAA CTT GAC CAT CCT CT -3'
	R: 5'- TGCTGG AAG GCGTCA ATC TT -3'
Akt2	F: 5-GAGGACCTTCCATGTAGACT-3′
	R: 5′-CTCAGATGTGGAAGAGTGAC-3′

CHAPTER FOUR: RESULTS

Results

GH Independent Expression of IGF1 in C57B6 Male Mice Treated with 17α-E2 – To investigate the possible mechanism of 17α-E2 's lifespan extending properties and the interplay with the GH/IGF1 axis we examined the hepatic and circulating levels of IGF1 and pulsatile GH release in a cohort of C57B6 mice treated with 17α-E2 (Figure 1). In circulation, pulsatile release of GH in C57B6 mice at week 0 and 15 show no difference with 17α-E2 treatment. Since GH release is paired with IGF1 production we examined the IGF1 levels in circulation and hepatic IGF1 production. With 17α-E2 treatment we see, surprisingly a significant increase in IGF1 both in circulation and in the liver. This increase in IGF1 with 17α-E2 treatment is only seen in males and not in females. This is surprising as IGF1 production is directly linked with GH release.

GH Independent Expression of IGF1 in GHRKO and Normal Male Mice Treated with 17α-E2 – With the observed GH *independent* regulation of IGF1 production seen in the C57B6 mice with 17α-E2 treatment we sought to further investigate this independent regulation of IGF1 production using a special mouse model, the GHRKO mouse model. In the GHRKO mice, we first examined if hepatic and circulating levels of IGF1 levels were changed with the treatment regimen and if this change was visible in both normal and GHRKO mice. As expected, both circulating and hepatic IGF1 levels in both female and male GHRKO mice are diminished in comparison to normal mice (**Error! Reference source not found.**) due to global lack of *Ghr* in these mice. However, when examining treatment effects in male normal and GHRKO mice, we see that with 17α-E2 treatment there is a significant increase in circulating IGF1 (**Error!**

Reference source not found.A) We see also a similar result with hepatic IGF-1 mRNA expression, there is a significant increase in both the normal and the GHRKO male mice with 17α-E2 treatment (

Figure 2 - 17α-E2 Treatment Increases Circulating and Hepatic IGF1 Levels in Males

C). Of additional interest is the effect of 17α-E2 on female GHRKO and normal mice, there is no significant changes in IGF1 in circulation and in the liver in females with the treatment (

Figure 2 - 17α-E2 Treatment Increases Circulating and Hepatic IGF1 Levels in Males

A and D). This sex-specific effect of 17α-E2 has been previously observed and described by several others in the field (5, 13, 14, 22)

Impact of 17α-E2 Treatment on Signaling Downstream of Growth Hormone and IGF1 Receptors - To further investigate possible pathways involved in the GH independent increase in IGF1 seen with 17α-E2 treatment we examined genes downstream of the GHR in the JAK/STAT pathway, which GH primarily signals through, in attempts to pinpoint alternate regulation of the

classical pathway of IGF1 production. Starting with STAT5, treatment with 17α-E2 did not significantly change mRNA levels of STAT5B across all eight groups (

Figure 3 – No change in hepatic Stat5b expression and AKT phosphorylation with 17α-E2 treatment

). Since no difference was observed at this level we examined downstream activation of AKT, which was also found to have no differences with treatment across all eight groups as indicated by phosphorylation levels (

Figure 3 – No change in hepatic Stat5b expression and AKT phosphorylation with 17α-E2 treatment

). Downstream in the signaling cascade, it is known that GH induces SOCS2 which feeds-back to inhibit GH's transcriptional action, thus serving as a negative regulator of its action. It is expected then that levels of SOCS2 be quite low in GHRKO due to inactivation of GHR signaling. This is reflected in the decreased relative expression levels of SOCS2 seen in Figure 4 for both female and male GHRKO mice and significant treatment effects as calculated in the three-way ANOVA. However, we do not see any significant changes in SOCS2 expression with treatment in females or males (Figure 4). This suggest that 17α-E2 is acting independent of the classical GH signaling pathway through a novel pathway to increase IGF1 production.

Effect of 17α-E2 treatment on Insulin Sensitivity - Based on previous studies implicating 17α-E2 treatment in improvements in glucose tolerance and insulin sensitivity (4, 22) we examined plasma insulin levels at the end of the four month treatment as well as glucose levels and calculated the Homeostatic Assessment of Insulin Resistance (HOMA) score for a measure of insulin sensitivity. Plasma insulin levels in male GHRKO mice significantly decrease with 17α-E2 treatment (*Figure 5*A) and no change in normal males or female normal and GHRKO,

the latter consistent with previous observations that treatment effects are exclusive to males (*Figure 5*C and D). Examining blood glucose levels at the end of 17α-E2 treatment, there are significant decreases in both female normal and GHRKO mice with treatment (*Figure 5*D) and no change in in male normal and GHRKO mice (*Figure 5*C).

Calculation of the HOMA score based on the insulin and glucose data in the GHRKO and normal male and female mice show a significant decrease in HOMA score in the GHRKO male mice with 17α-E2 treatment and significant treatment effects based on the Three-way ANOVA in males (Figure 6A). Lower HOMA scores indicate increased insulin sensitivity and based on the decreased in HOMA score observed we examined hepatic IRS1 expression, which is downstream from the insulin receptor. We see that with 17α-E2 treatment there is significantly increased IRS1 expression in normal male mice (Figure 6A). There is no significant effect of treatment on IRS1 expression in male or female GHRKO or normal females (Figure 6B).

RNA-Sequencing Analysis of Liver - Based on the observed no difference in gene expression downstream of GH/IGF1 signaling this suggests that 17α-E2 is acting through a novel pathway yet to be elucidated to increase IGF1 production independent of GH. To further investigate the molecular basis of the observed upregulation of hepatic IGF1 abundance in male GHRKO mice, RNA analysis using next-generation sequencing was employed in liver from normal and GHRKO male and female mice. Since we are interested in the molecular mechanisms of 17α-E2 induced increase in GHR deficient livers and owing to the sex-specific effect of this treatment and thus to keep within the scope of this thesis, we examine here differentially regulated pathways that are altered in the GHRKO and show change with 17α-E2 treatment in male mice. For comparison purposes we show in (Figure 7) Venn diagrams of

differentially regulated pathways as well as up and down regulated genes across genders. We can see here that when comparing normal males to normal females the number of upregulated genes, interestingly enough, is relatively similar, with 86 and 83 genes significantly changed with 17α-E2 treatment, respectively.

There is a total of seven upregulated pathways in GHRKO males and a total of nine downregulated pathways in GHRKO with 17α-E2 treatment. In normal males, there are a total of six upregulated pathways and seven downregulated pathways with 17α-E2 treatment. All three pathways listed as common between normal and GHRKO males are commonly downregulated for both groups. In order to gain a broader understanding of the spread of the data for this study we generated a principle component analysis (PCA) plot for the RNAseq data which shows a clustering of the normal male treated and untreated in a sector on their own (Figure 8).

Impact of 17α-E2 Treatment on Peroxisome Proliferator Activated Receptors in Liver- In attempts to validate the upregulation of PPAR signaling with the RNAseq data previous observations regarding the positive effect of 17α-E2 on metabolism we examined the hepatic expression levels of the PPAR family of genes. The PPAR family of genes play pivotal roles in lipid sensing and metabolism (23). Interestingly enough, we see there is increased hepatic expression of PPARα in female GHRKO with treatment and no significant changes in PPARα expression in normal females or male GHRKO and normal mice (Figure 9). This observation is partially supported by the RNAseq data which showed upregulation of this pathway in normal male mice. These observations collectively provides an insight into the mechanistic effects of 17α-E2 treatment on insulin sensitivity and point to a broader more complex regulation.

Figures

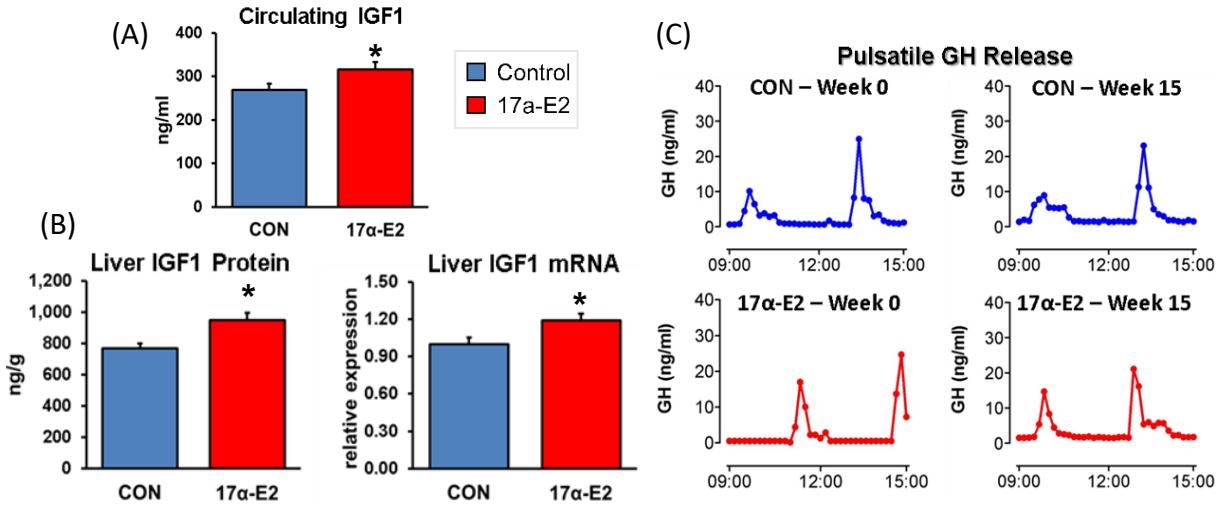

Figure 1- 17α-E2 Treatment increases IGF1 levels in C57BL6 Mice with no change in GH pulsativity

Circulating IGF1 and hepatic protein and mRNA IGF1 expression and pulsatile GH release in control and 17α-E2 treated male C57B6 mice. A. Circulating IGF1 in control (CON) and 17α-E2 treated mice as measured by ELISA. B. Hepatic protein and mRNA levels of IGF1 as measured by ELISA and qPCR, respectively. C. Pulsatile GH release in control and 17α-E2 treated mice at week 0 and week 15. Results are presented as the mean ±SEM. Bars labeled with asterisk show p values using Student's t-test; *$p<0.05$

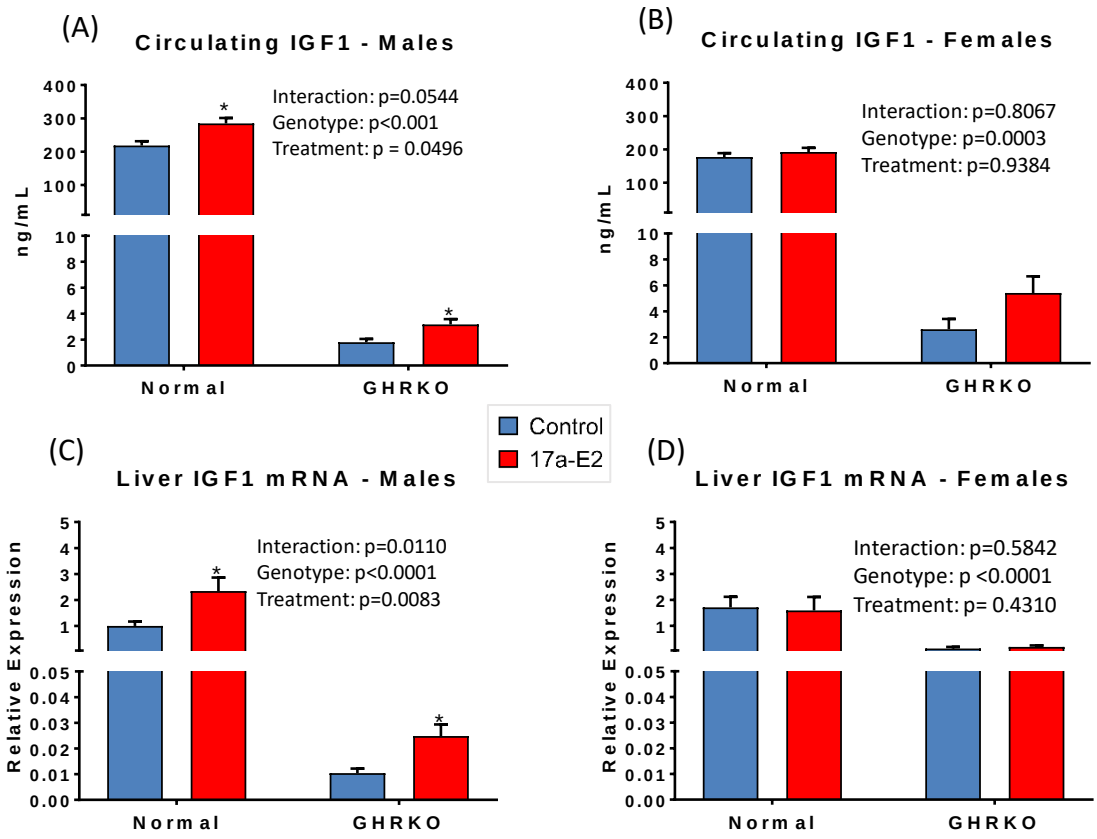

Figure 2 - 17α-E2 Treatment Increases Circulating and Hepatic IGF1 Levels in Males

Circulating and Hepatic expression of IGF1 in GHRKO and normal Male and Female Mice. A. Circulating IGFI levels in normal untreated (n=8) and treated (n=12) male mice and GHRKO treated (n=10) and untreated (n=9) male mice as measured by ELISA. B. Circulating IGFI levels in normal untreated (n=11) and treated (n=11) female mice and GHRKO treated (n=9) and untreated (n=9) female mice as measured by ELISA. C. Hepatic IGFI levels in normal untreated and treated male mice and GHRKO treated and untreated male mice as measured by qPCR. D. Hepatic IGFI levels in normal untreated and treated male mice and GHRKO treated and untreated male mice as measured by qPCR. All results are presented as the mean ±SEM. Bars labeled with asterisk show p values using Student's t-test; *p<0.05.

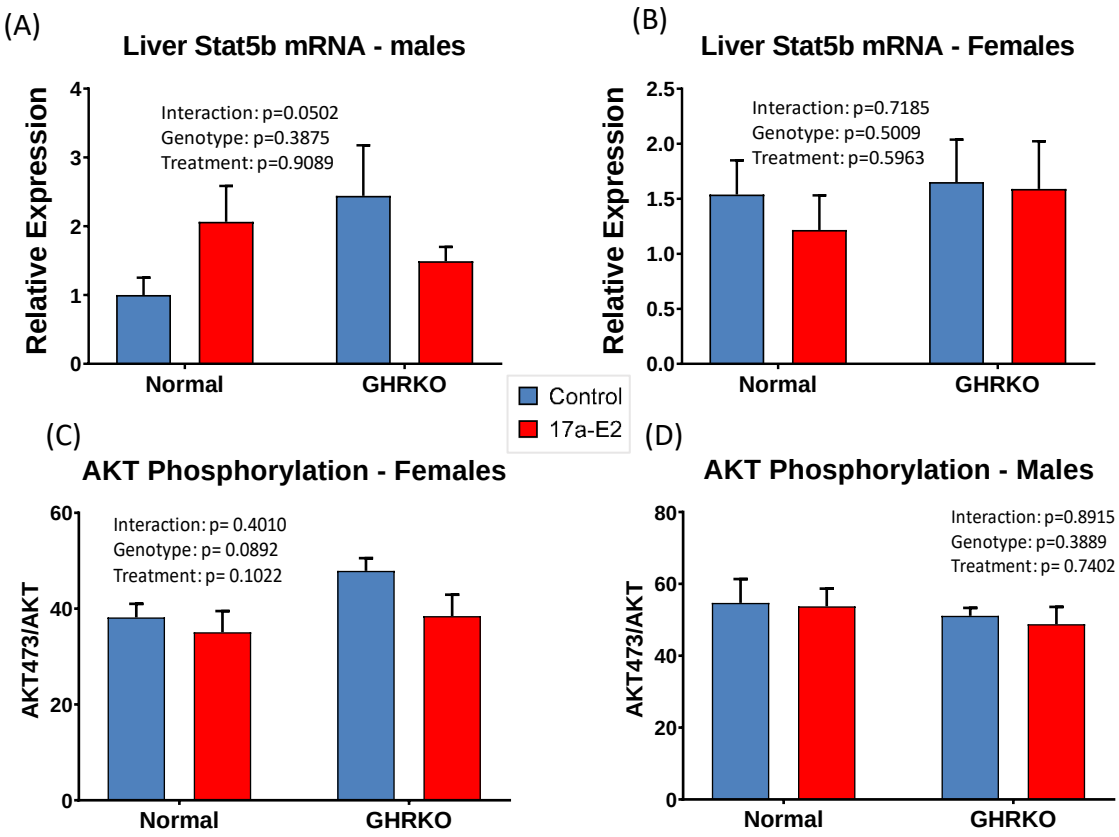

Figure 3 – No change in hepatic Stat5b expression and AKT phosphorylation with 17α-E2 treatment

Hepatic Stat5b expression and AKT phosphorylation (S473) levels in treated and untreated male and female GHRKO and normal mice. A. Relative expression levels of Stat5b in liver of normal and GHRKO male mice with and without 17α-E2 treatment. B. Relative expression levels of Stat5b in liver of normal and GHRKO female mice with and without 17α-E2 treatment. C. AKT phosphorylation levels in liver of normal and GHRKO male mice with and without 17α-E2 treatment. D. AKT phosphorylation levels in liver of normal and GHRKO female mice with and without 17α-E2 treatment. All results are presented as the mean ±SEM with n equivalent to that described in the caption of Figure 1 this is true for Figure 4 and Figure 5 as well.

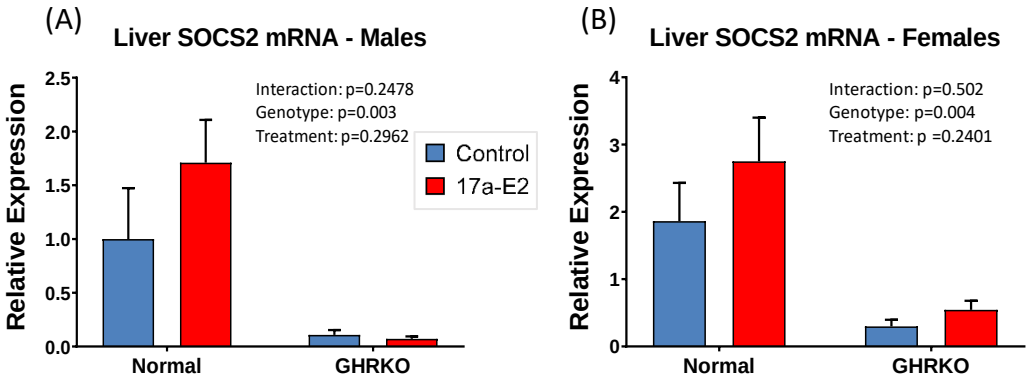

Figure 4 - Downregulation of SOCS2 in Liver of GHRKO Mice

Hepatic SOCS2 expression in treated and untreated male and female GHRKO and normal mice. A. Relative expression levels of SOCS2 in liver of normal and GHRKO male mice with and without 17α-E2 treatment. B. Relative expression levels of SOCS2 in liver of normal and GHRKO female mice with and without 17α-E2 treatment. All results are presented as the mean ±SEM.

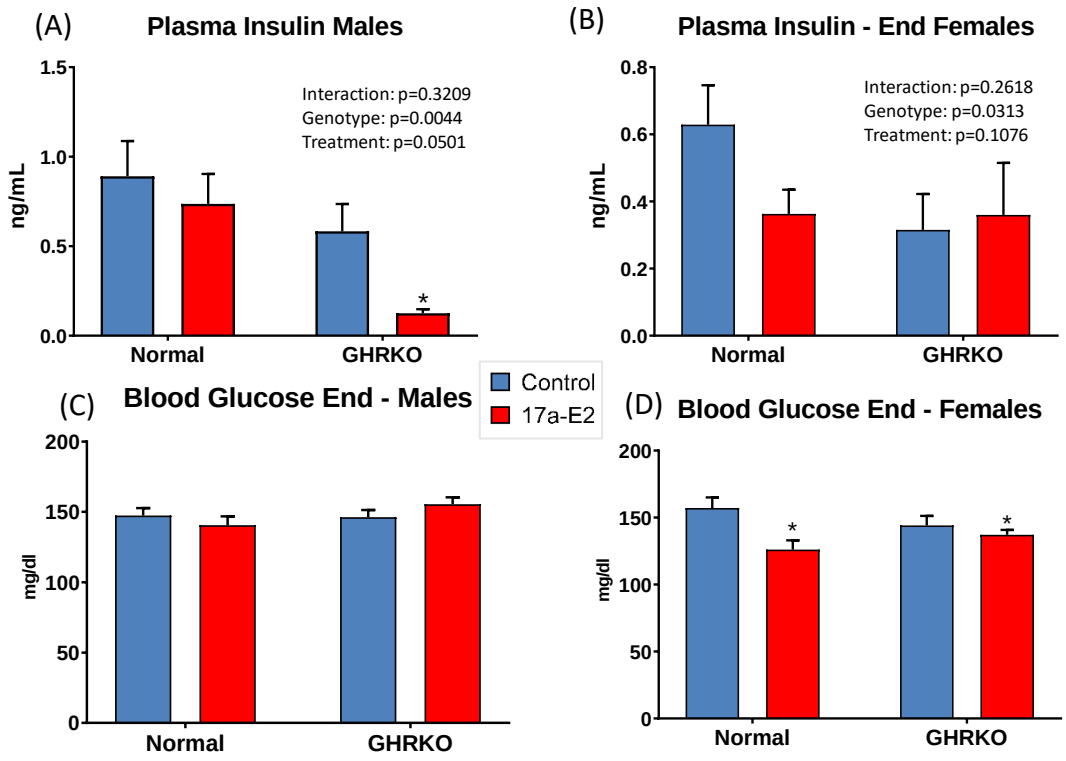

Figure 5- Glucose levels decrease and Insulin levels decrease in males with 17α-E2 Treatment

Blood glucose levels and plasma insulin levels measured after the four months of treatment with 17α-E2. A. Plasma insulin levels in normal and GHRKO male mice with and without 17α-E2 treatment. B. Plasma insulin levels in normal and GHRKO female mice with and without 17α-E2 treatment. C. Blood glucose levels in normal and GHRKO male mice with and without 17α-E2 treatment. All results are presented as the mean ±SEM. Bars labeled with asterisk show p values using Student's t-test; *$p<0.05$

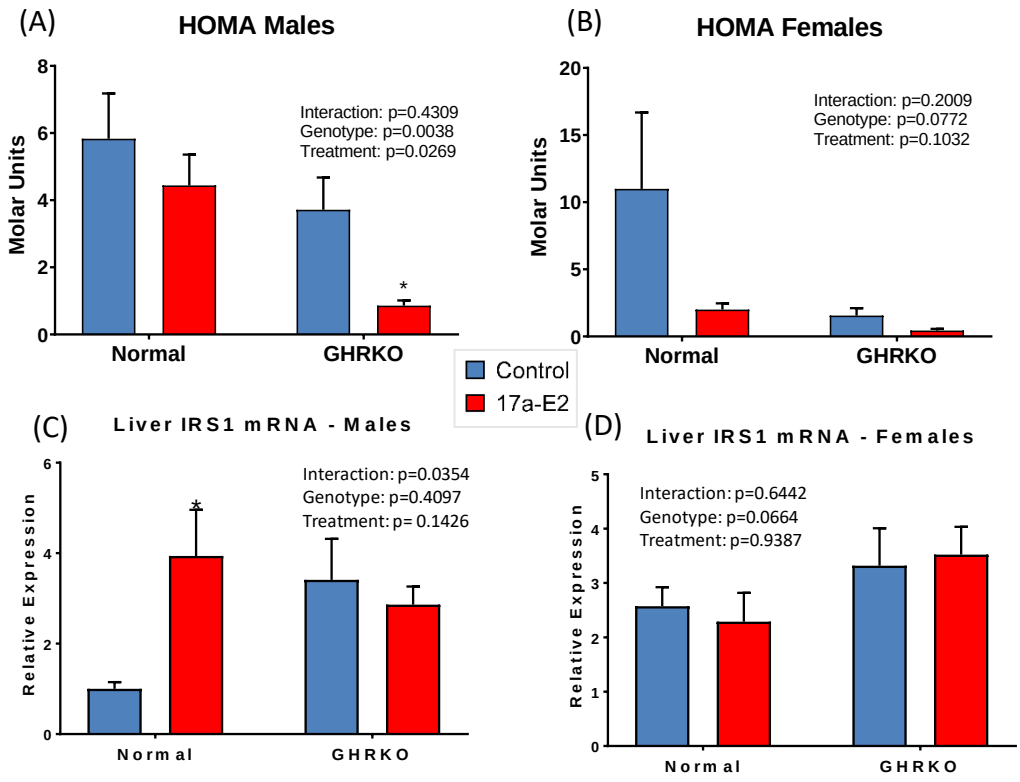

Figure 6 - Effect of 17α-E2 on insulin signaling

Homeostatic Assessment of Insulin Resistance and Hepatic IRS1 expression levels in treated and untreated male and female GHRKO and normal mice C. Relative expression of IRS1 in liver of normal and GHRKO male mice with and without 17α-E2 treatment. D. Relative expression of IRS1 in liver of normal and GHRKO female mice with and without 17α-E2 treatment. Lower HOMA score indicates higher insulin sensitivity and is calculated by multiplication of fasting insulin and fasting glucose and dividing by 22.5. All results are presented as the mean ±SEM. Bars labeled with asterisk show p values using Student's t-test; *$p<0.05$.

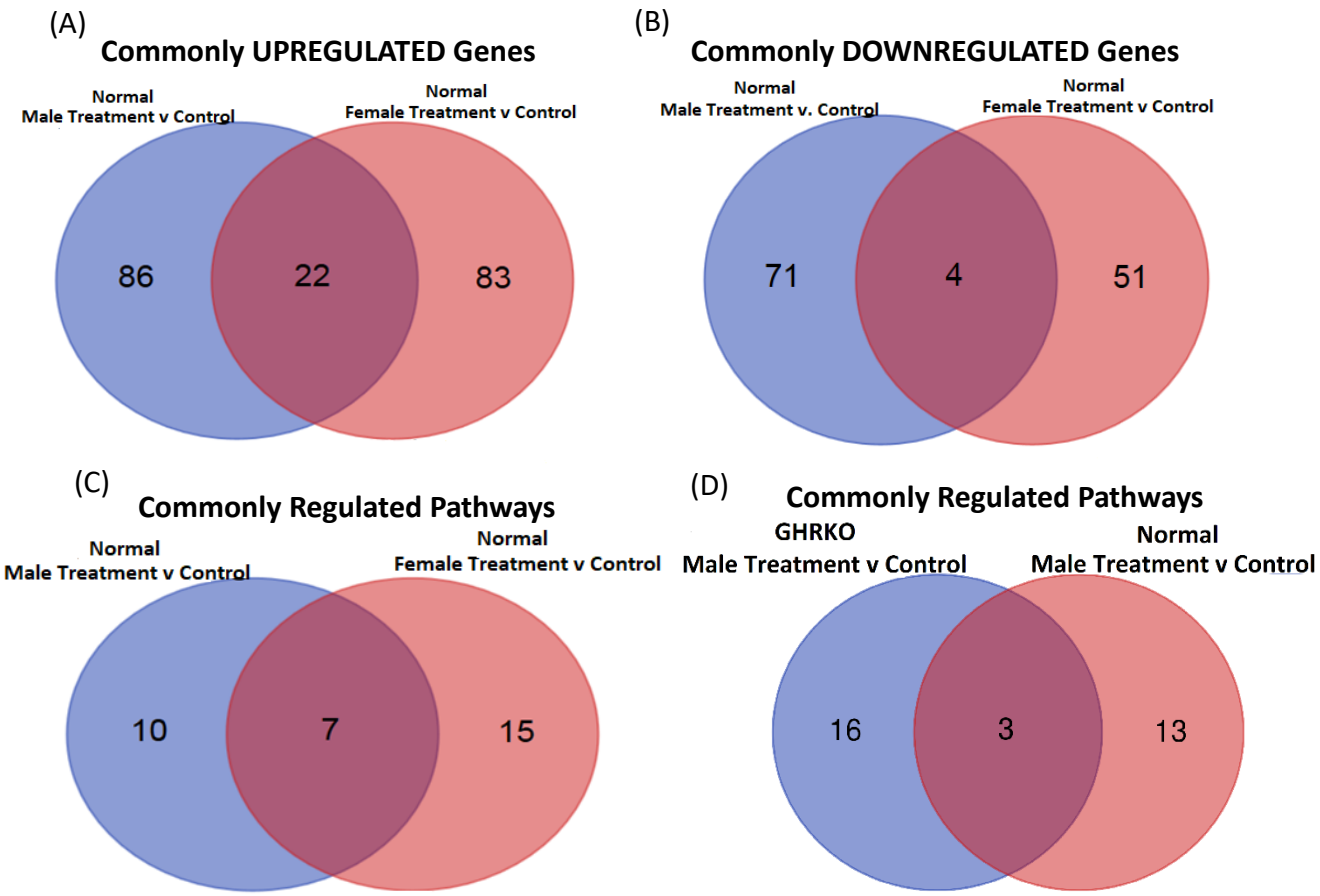

Figure 7 - Differentially Regulated Genes and Pathways Between Normal Males and Females in when Comparing Treated and Untreated Mice

Genes and pathways altered with 17α-E2 treatment, a schematic comparison across groups. A. Number of genes upregulated in normal male and female mice with 17α-E2 treatment and those shared by the two groups. B. Number of genes downregulated in normal male and female mice with 17α-E2 treatment and those shared by the two groups. C. Number of pathways differentially regulated in normal male and female mice with 17α-E2 treatment. D. Number of pathways altered with 17α-E2 treatment a comparison between GHRKO and normal male mice.

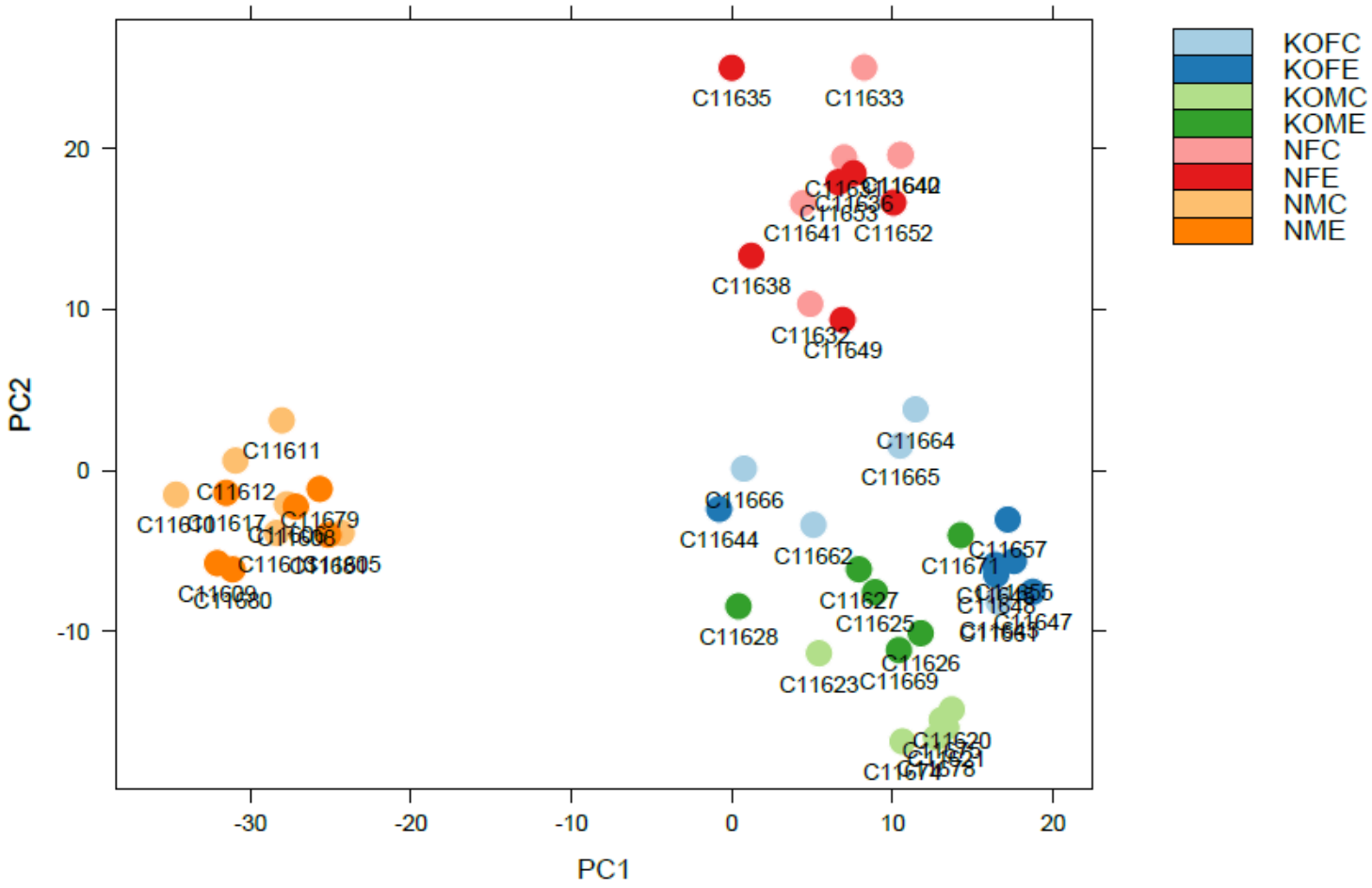

Figure 8 – Principle Component Analysis of RNA-sequencing data of the eight experimental groups

Table 2- Differentially Regulated Pathways in GHRKO and Normal Male Mice with 17α-E2 Treatment (arrows indicate up or down regulation of the pathway)

Regulated Pathways in Male GHRKO Mice		
mmu00230	**Purine metabolism**	↓
mmu00480	**Glutathione metabolism**	↓
mmu00500	**Starch and sucrose metabolism**	↓
mmu00590	**Arachidonic acid metabolism**	↑
mmu04110	**Cell cycle**	↓
mmu03420	**Nucleotide excision repair**	↓
mmu03440	**Homologous recombination**	↓
mmu04020	**Calcium signaling pathway**	↑
mmu04270	**Vascular smooth muscle contraction**	↑
mmu04514	**Cell adhesion molecules (CAMs)**	↑
mmu04610	**Complement and coagulation cascades**	↑
mmu04630	**Jak-STAT signaling pathway**	↑
mmu04640	**Hematopoietic cell lineage**	↑
mmu04660	**T cell receptor signaling pathway**	↑
mmu04672	**Intestinal immune network for IgA production**	↑
mmu04973	**Carbohydrate digestion and absorption**	↓
Commonly Regulated Pathways between Male GHRKO and Normal Mice		
mmu00100	**Steroid biosynthesis**	↓
mmu00900	**Terpenoid backbone biosynthesis**	↓
mmu03030	**DNA replication**	↓
Regulated Pathways in Normal Male Mice		
mmu00140	**Steroid hormone biosynthesis**	↑
mmu00190	**Oxidative phosphorylation**	↓
mmu00240	**Pyrimidine metabolism**	↓
mmu00250	**Alanine, aspartate and glutamate metabolism**	↓
mmu02010	**ABC transporters**	↑
mmu03040	**Spliceosome**	↓
mmu03050	**Proteasome**	↓
mmu04012	**ErbB signaling pathway**	↑
mmu04070	**Phosphatidylinositol signaling system**	↑
mmu04122	**Sulfur relay system**	↓
mmu04141	**Protein processing in endoplasmic reticulum**	↓
mmu04614	**Renin-angiotensin system**	↑
mmu04976	**Bile secretion**	↑
mmu03320	**PPAR signaling pathway**	↑

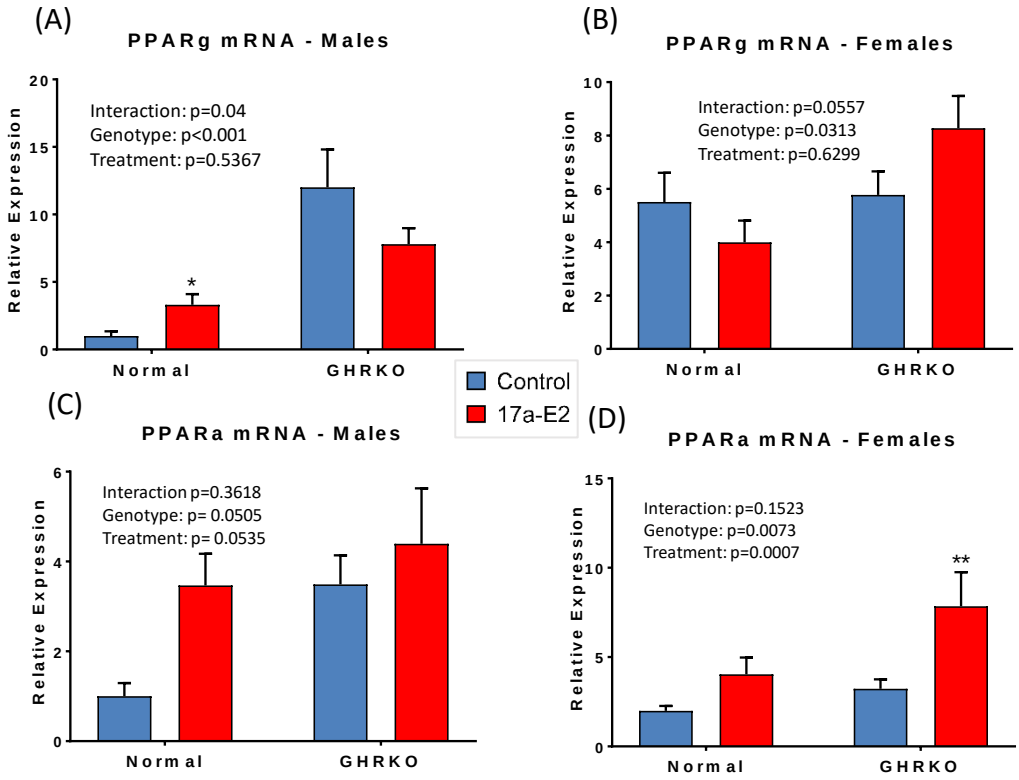

Figure 9 -Regulatory Role of 17α-E2 on Peroxisome Proliferator Activated Receptors in Liver

PPARγ and PPARα expression in levels in treated and untreated male and female GHRKO and normal mice. A. Relative expression of PPARγ in liver of normal and GHRKO male mice with and without 17α-E2 treatment. B. Relative expression of PPARγ in liver of normal and GHRKO female mice with and without 17α-E2 treatment. C. Relative expression of PPARα in liver of normal and GHRKO male mice with and without 17α-E2 treatment. D. Relative expression of PPARα in liver of normal and GHRKO male mice with and without 17α-E2 treatment. All results are presented as the mean ±SEM. Bars labeled with asterisk show p values using Student's t-test; *p<0.05, **p<0.01.

CHAPTER FIVE: DISCUSSION

The well-established idea in the field of GH/IGF1 research is the inextricable link between IGF1 production by the liver, which is the primary source of IGF1 production, to GH signaling. This signaling cascade and the downstream effects of it are responsible in maintaining glucose homeostasis and imbalance causes a myriad of metabolic disorders and dysregulation of the signaling is common with age and age-associated obesity. Traditionally, negative modulation of GH/IGF1 signaling increases lifespan and this is apparent in studies involving long-living models for aging studies including GHRKO and Ames Dwarf mice. These long-lived models are characterized by decreased levels of GH, IGF1 and insulin. In this study, we see IGF1 increases while GH remains the same with 17α-E2 treatment in the GHRKO mice and yet there is increased insulin sensitivity. This questions traditional ideas about the effects of negative modulation of GH/IGF1 signaling in increasing lifespan. In both normal and GHRKO male mice with 17α-E2 treatment IGF1 increases, supporting our hypothesis. Throughout this study we see a sex-specific divergence in 17α-E2 treatment effects, with effects seen only in males. This was previously observed by others in the field in the context of lifespan and aging and one possible explanation for the lifespan extension aspect in response to the treatment is that it is well known that females across several species live longer than males (24) and it is likely due to increased endogenous levels of estrogens compared to males so treatment at this dosage does not render any effects.

Based on our results it can be said that the 17α-E2 treatment is increasing IGF1 in GHRKO mice. This is of particular interest because it has been well established that IGF1

expression is practically completely dependent on GH action and the visible increase in IGF1 expression in GHRKO mice would then suggest an alternate pathway of IGF1 production as mediated by 17α-E2 treatment. These results are also consistent with the relative gene expression data on genes downstream of classical GH, IGF1 and Insulin signaling in which we find no significant changes including SOCS2, STAT5b and AKT activation, pointing again to a novel pathway which 17α-E2 acts through to increase IGF1 production (Figure 3 and Figure 4). The GH independent IGF1 production with 17α-E2 treatment is also seen in C57B6 mice and further supported by data the growth-hormone resistant GHRKO mice (Figure 1 and Figure 2).

Examining one previous study which described the treatment of GHRKO mice with 17βE2 in the context of bone growth (6) Here they describe an increase in mRNA IGF1 levels which is similar to what we observed here with the estrogen treatment and an increase in STAT5 activation which is not consistent with our data. This is likely because they used the enantiomer of 17α-E2, 17βE2 for treatment. Although, this lends support to an underlying mechanism of estrogen action on the GH/IGF1 axis which may underlie sex differences in metabolism with aging.

Using this as a springboard for the underlying sexual dimorphisms with treatment, comparison of our sequencing data with metabolomics data from a recent study treating castrated males and ovariectomized females with 17α-E2 we see some overlap. They describe a change in the metabolites associated with the urea cycle including glutamine and alanine in mice treated with 17α-E2. When comparing this to our RNAseq output we also see a change in Alanine, aspartate and glutamate metabolism (Table 2) they noted that the positive effects of treatment are

ablated when gonads are removed in the males pointing to a complex interaction of sex hormone production in the mice with the beneficial effects of 17α-E2 treatment (5).

The principle component analysis of the RNAseq data uncovers an interesting relationship with all the normal male treated and untreated mice clustering on their own, this is possibly due to the differential regulation of IGF1 production through a novel pathway in these mice and a dependence of treatment on male sex hormones as described by Michael et al. Upon further examination of the regulated pathways in our data with treatment in males, in the pathways distinct to GHRKO mice we see there is positive regulation of calcium signaling and JAK/STAT signaling. This can be explained by the signaling pathway by which estrogens act, they initiate signaling via MAPK and PI3K to increase intracellular calcium. With regard to the JAK/STAT pathway upregulation this could provide a clue as to the alternative regulation of IGF1 production as GH signals through this pathway. Going along with this pathway in the context of regulated pathways in normal mice with treatment we see there is upregulation of the PI3K pathway. This observation in the sequencing data is supported by the increase in mRNA IRS1 expression levels (Figure 4A) which is only seen in normal males. IRS1 connects insulin receptor activation to downstream PI3K signaling cascades and subsequent transport of glucose. Increased PI3K activation in the normal mice can suggest increased insulin signaling and may contribute to increased insulin sensitivity. This increase in insulin sensitivity with 17α-E2 treatment is further supported by the decrease in HOMA scores in male mice (Figure 6) In addition, results seen with increases in PPARγ with treatment in normal males suggests enhanced lipid metabolism in these animals lending itself to the idea that 17α-E2 enhances metabolic function (Figure 5A).

This can lead us to imagine that 17α-E2 is working on different parts of pathways to lead to a similar result overall male mice regarding positive metabolic effects. It is important to note that when comparing GHRKO males to females, the only pathway common between the two with treatment is the calcium signaling pathway and this could suggest again that since estrogens work to increase intracellular calcium the exogenous origins of estrogen via 17α-E2 treatment worked to increase levels in females to account for this upregulation. Although the PI3K pathway is only upregulated with treatment in normal males, it can lend support to previous findings in the field showing positive metabolic effects of treatment only in males.

Turning to other alternatively regulated pathways with 17α-E2 treatment in the normal male mice, we see an upregulation of the Renin-Angiotensin pathway. This pathway is thought to negatively affect insulin signaling but upon closer examination of the individual genes upregulated in the Renin-Angiotensin pathway in the Normal male mice with treatment we see those that are upregulated are involved with Angiotensin (1–7), which is derived from the metabolism of Angiotensin I. In contrast, other parts of the pathway including Angiotensin II which negatively modulates insulin signaling on multiple levels including IRS1, AKT, and PI3K (25, 26) were unchanged. Given that there exists a crosstalk between renin-angiotensin and insulin signaling, and the upregulation of this system could be indicative of enhanced insulin signaling, it is plausible that 17α-E2 promotes insulin sensitivity. Upregulation of the proteasome pathway in normal male mice could account for the no visible change in STAT5b observed in the qPCR data as it would indicate increased proteasome mediated protein degradation and subsequent downregulation of STAT5 (27).

This observation suggests a highly complex mechanism of action that could point to an interplay of 17α-E2 treatment with other sex hormones as was recently published about in mice whose gonads were removed (5). Many of the other pathways up and downregulated in the male GHRKO and normal mice have an unclear purpose in the range for which this study was conducted and we have included them here in the hope that this study will lay the ground work for a better understanding of the 17α-E2 mechanisms of action as it poises itself as a possible therapeutic for age-related metabolic dysfunction.

Ultimately, to determine the origins of this sex-specific effect of 17α-E2 on GH independent IGF1 synthesis future studies with the removal of gonads in a cohort of female and male GHRKO mice similar to work which was conducted by Garratt et. al. is recommended. This would provide further information regarding the pathways with which 17α-E2 acts to increase IGF1 independent of GH and isolate the sex-specific factors contributing to the positive metabolic effects seen with treatment. Given that 17α-E2 has been shown to extend lifespan preferentially in males it would be interesting to conduct a long-term treatment, again with a cohort of GHRKO males and females to investigate the longevity aspect as this study was limited to a four-month period of treatment. Studying the long-term effects of 17α-E2 in GHRKO mice can give insight into how this hormonal treatment can affect these mice with regard to metabolic function with age and given the complex interplay with aging IGF1/GH secretion can raise questions about how 17α-E2 affects their levels over a lifespan.

The present study establishes an upregulation of IGF1 by a hormone other than GH which is a novel and groundbreaking observation in the field. Based off of these findings that 17aE2 works through a novel GH independent pathway to positively regulate insulin sensitivity

and other findings in the field regarding the lifespan extending potential and positive metabolic effects and given its nature as a non-feminizing estrogen, 17α-E2 has promising potential as a therapeutic retaining the positive metabolic effects while reducing potential complications associated with feminizing hormones.

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