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Mechanisms mediating the anti-inflammatory effects of dietary methionine restriction

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Abstract

Introduction

Obesity is associated with inflammation, which contributes to several obesity-related comorbidities. This inflammation is characterized by increased inflammatory cytokine expression in adipose tissue (AT) depots and the liver. Dietary restriction of the essential amino acid methionine, in rodents, has been shown to improve body weight and adiposity, decrease high-fat diet- and age-induced inflammation, reduce hepatic steatosis, and increase insulin sensitivity despite inducing hyperphagia. Recent studies indicate that dietary methionine restriction (MR) rapidly and persistently increases tissue and circulating concentrations of the hormone fibroblast growth factor 21 (FGF21). In rodents, FGF21 has been shown to reduce body weight, increase insulin sensitivity, and recent reports indicate it may also decrease inflammation. STAT3 is a generally pro-inflammatory transcription factor which microarray analyses have identified as a potential "master regulator" of inflammatory processes in MR. We hypothesized that MR decreases inflammation by increasing FGF21 and downregulating STAT3 signaling.

Methods

24 wild type (WT) C57BL/6J and 24 *Fgf21* knock-out (KO) mice were fed a high-fat diet for four weeks, then randomized to a high-fat control (CON) diet or high-fat MR diet for 13 weeks. RT-PCR was used to measure markers of inflammation in the liver and epididymal white adipose tissue (EWAT). Western blot analyses were conducted to determine whether STAT3 activity was affected by MR.

Results

*Fgf21*KO mice had elevated hepatic expression of *Ccl2* (MCP-1), and MR significantly decreased its expression in *Fgf21*KO, but not WT mice. In EWAT, *Fgf21*KO mice had elevated expression of *Emr1* (F4/80), and MR reduced its expression in *Fgf21*KO mice, but not WT mice. MR reduced *Ccl2 expression* in EWAT of both genotypes. Expression levels of *Itgam* (CD11b) and IL-6 in EWAT did not differ amongst groups. MR decreased STAT3 phosphorylation at Tyr705 in both genotypes, but had no effect on Ser727 phosphorylation.

Conclusion

The RT-PCR results suggest that mice lacking FGF21 may be more susceptible to HFD-induced inflammation. Since MR induced comparable reductions in inflammatory markers in both genotypes, at first glance it appears that FGF21 does not play a role in the mechanism by which MR decreases inflammation. However, it should be noted that in *Fgf21*KO mice, MR induced an unexpected decrease in food intake that resulted in reduced body weight and adiposity compared to control-fed *Fgf21*KO mice. Western blot results support the hypothesis that MR decreases inflammation by downregulating STAT3 signaling.

MECHANISMS MEDIATING THE ANTI-INFLAMMATORY EFFECTS OF DIETARY METHIONINE RESTRICTION

by

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ABBREVIATIONS

AT	Adipose Tissue
CR	Calorie Restriction
CVD	Cardiovascular Disease
EE	Energy Expenditure
ERK1/2	Extracellular Mitogen-Activated Protein Kinase 1 & 2
EWAT	Epididymal White Adipose Tissue
FGF21	Fibroblast Growth Factor-21
*Fgf21*KO	Fibroblast Growth Factor-21 Knock-Out
HFD	High-Fat Diet
HTN	Hypertension
IWAT	Inguinal White Adipose Tissue
JAK	Janus Kinase
KOCON	Knock-Out Control Group
KOMR	Knock-Out Methionine Restriction Group
MR	Methionine Restriction
$pSTAT3^{Ser727}$	Phosphorylated STAT3 at Ser727
$pSTAT3^{Tyr705}$	Phosphorylated STAT3 at Tyr705
RER	Respiratory Exchange Ratio
RIPA	Radioimmunoprecipitation Assay
RPWAT	Retroperitoneal White Adipose Tissue
STAT3	Signal Transducer and Activator of Transcription
T2DM	Type 2 Diabetes Mellitus
WT	Wild-Type
WTCON	Wild-Type Control Group
WTMR	Wild-Type Methionine Restriction Group
VAT	Visceral Adipose Tissue

Chapter I

Introduction

The prevalence of obesity is on the rise in the US and is contributing to increased healthcare costs and the declining health of those with obesity. Obesity is associated with inflammation, which contributes to several obesity-related comorbidities. This inflammation is characterized by increased inflammatory cytokine expression in adipose tissue (AT) depots and the liver. Inflammatory markers often upregulated by obesity include MCP-1, IL-1β, TNF-α, IFN-γ, F4/80, ITGAM, and ITGAX.

Given the increasing prevalence of obesity, a common goal of researchers is to identify effective and safe methods for preventing or reversing obesity and metabolic disease. Dietary restriction of the essential amino acid, methionine, presents as an intriguing treatment for obesity and its comorbidities. Methionine restriction (MR) has been shown to improve several signs of metabolic health in rodents. MR reduces body weight and adiposity, decreases high-fat diet- and age-induced inflammation, reduces hepatic steatosis, and increases insulin sensitivity. All of these beneficial effects of MR occur despite an increase in energy intake.

Recent studies indicate that dietary MR rapidly and persistently increases tissue and circulating concentrations of the hormone fibroblast growth factor 21 (FGF21). FGF21 increases insulin sensitivity, reduces body weight, and has been shown to downregulate markers of inflammation in white adipose tissue (WAT) of obese mice and spleen of arthritic mice. This hormone is considered anti-obesogenic, however serum levels are increased in obesity, suggesting an FGF21-resistant state. Little is known about the role of FGF21 in inflammation, though recent studies show that treatment with the

inflammatory cytokine TNF-α reduces FGF21 signaling in adipocytes. Additionally, FGF21 was shown to reduce inflammatory cytokine expression in WAT of obese mice and in the spleen of arthritic mice.

STAT3, a generally pro-inflammatory transcription factor, is activated by JAK-mediated phosphorylation, which allows it to translocate to the nucleus and affect transcription, propagating the inflammatory response. Microarray analyses suggested that STAT3 signaling is downregulated by MR in liver and WAT, identifying STAT3 as a potential mediator of the anti-inflammatory effects of MR.

The purpose of the current study was to determine whether FGF21 mediates the anti-inflammatory effects of MR and to identify whether downregulation of STAT3 signaling is involved. Gene expression of inflammatory markers and phosphorylation of STAT3 was measured in AT and liver samples from wild-type (WT) and *Fgf21* knockout (KO) mice.

Chapter II

Literature Review

Obesity-Induced Inflammation

Obesity is associated with chronic, low-grade, systemic inflammation.[1] Obesity-induced inflammation is similar to aging-related inflammation.[2] Both aging-related inflammation and obesity-associated inflammation are characterized by increased inflammatory cytokine expression and production of reactive oxygen species (ROS).[2,3] The inflammatory cytokines produced by obesity are found in circulation, characterizing the chronic inflammation as "systemic," and the inflammation is not associated with infection, characterizing it as "low-grade".[1] Though systemic, the inflammation is especially increased in adipose and liver tissues.[4] Cytokines produced in the AT as a result of obesity include IL-1β, IL-6, and TNF-α, which are thought to be regulated by the transcription factor NF-κB.[5-9]

Macrophages, cells involved in the body's immune responses, also play an important role in obesity-induced inflammation.[10] Polarization of macrophages influences their functions, with M1 polarization leading to phagocytosis of dead adipocytes and M2 polarization producing anti-inflammatory cytokines.[11,12] Obesity induces polarization toward the M1 (inflammatory) type, further contributing to an increase in production of pro-inflammatory cytokines.[10] Similarly to adipose tissue, the liver also experiences increased inflammation in the presence of obesity.[4] Kupffer cells, immune cells similar to macrophages but specific to the liver, will release cytokines, aiding in the development of inflammation.[4,13]

A high fat diet (HFD) is traditionally used to induce obesity and related inflammation in rodents.[14] In a study where a HFD and a normal fat diet (NFD) were fed, for 12 weeks, to mice and compared, the HFD-fed mice weighed more than the NFD-fed mice, despite similar calorie consumption.[14] The HFD also triggered inflammation, shown by increases in white adipose tissue (WAT) expression of F4/80, Cd11b, Cd11c, MCP-1, and TNF-α.[14]

Obesity-Related Comorbidities

Obesity can lead to a slew of health problems, including type 2 diabetes mellitus (T2DM), hypertension (HTN), dyslipidemia, hepatic steatosis, and cardiovascular disease (CVD).[1] Inflammation has been linked to the development and progression of insulin resistance, which can result in the development of T2DM.[1,15] Due to the increased prevalence of obesity, it is especially important to find safe and effective ways to combat excess adiposity, inflammation, and the comorbidities associated with obesity.

Obesity often leads to the development of T2DM, especially in those genetically prone to the disease. Obesity increases insulin resistance via inflammation. Specifically, pro-inflammatory cytokine release (TNF-α, IL-6 and MCP-1), amongst other pro-inflammatory factors from macrophages and AT, likely contribute to insulin resistance.[16-18] Insulin resistance causes increased production of insulin by pancreatic β cells. Insulin resistance leads to the development of T2DM when pancreatic β cells can no longer meet insulin production needs.[16,19,20]

CVD is linked to both systemic inflammation and obesity, independently.[21-23] As discussed previously, obesity leads to systemic inflammation. It is believed that this systemic inflammation is a risk factor for CVD.[21] Systemic inflammation likely causes or contributes to atherosclerosis.[21] Atherosclerosis causes arteries to narrow, which can contribute to development of HTN. Obesity can cause CVD through the development of atherosclerosis. Additionally, dyslipidemia, insulin resistance, and T2DM – all associated with obesity – can contribute to CVD.[21,24]

Dietary Methionine Restriction

In 1935 a group of scientists demonstrated that calorie restriction (CR) to 60% *ad libitum* intake could increase life span in rats.[25] In addition, this new dietary approach resulted in smaller sizes and body weights in the rats.[25] Though this research is over 80 years old, it is still relevant today. More recent studies have shown that CR produces life-span extension effects (30-40%) in multiple species.[2,26] However, it is not thought to be ideal for human applications because of the difficulty in adhering to such a restrictive diet.[2] In hopes of finding a diet that would work for humans and produce similar effects as CR, researchers searched for components of CR that lead to the enhanced longevity and decreased body mass benefits. While restriction of carbohydrates and fats did not seem to produce similar effects to CR, protein restriction did cause life-span extension, though not to the same degree as CR. Therefore, research continued into the restriction of various amino acids. In 1993, the restriction of the essential amino acid methionine was found to produce health benefits similar to those of CR in rats. It was discovered that an 80% reduction of methionine (by weight) in the diet resulted in 30% longer life span and

smaller body size and weight in rats.[27] Additionally, it appeared as though this diet may be more feasible for human application because, when fed *ad libitum*, energy intake actually increased in methionine-restricted animals.[27]

Since 1993, more benefits of dietary methionine restriction (MR) have been uncovered. Reduction in adiposity, especially in the visceral adipose tissue (VAT) depot, caused by MR has been seen in multiple studies,[27-30] which is likely a major contributor to lowered risk of developing obesity-associated co-morbidities. In fact, surgical removal of VAT in rats has been shown to increase insulin sensitivity,[31] delay diabetes onset,[32] and increase mean and maximal life span[33] despite similar amounts of total fat when compared to control groups whom had not had VAT surgically removed.[33] MR also increases insulin sensitivity.[28,30,34] Decreased pro-inflammatory marker expression in peripheral tissues as a result of MR[15] could be an effect of decreased adiposity and a cause of increased insulin sensitivity.

MR reduces hepatic lipid stores, reducing the risk of hepatic steatosis often seen with obesity,[28,35] and reduces serum lipid levels.[29] The reduction in hepatic lipid stores are due to changes in lipid metabolism. In a study on male F344 rats, MR caused an increase in hepatic fatty acid oxidation and a simultaneous decrease in hepatic fatty acid synthesis.[28]

The anti-obesogenic hormone, fibroblast growth factor-21 (FGF21), is increased by MR.[28,36-38] FGF21 has been shown to increase glucose uptake by adipocytes, independent of insulin activity, prevent obesity, and decrease blood glucose and triglyceride levels.[39]

What makes MR an especially unique dietary approach is its ability to increase energy expenditure (EE)[27,28,35,37] and induce a hyperphagic response.[28,29,37] Additionally, enhanced metabolic flexibility, shown by increased ranges in respiratory exchange ratio (RER), has been observed in rats.[28,37]

Plaisance *et al.* conducted a human study to investigate the effects of MR, implemented for 16 weeks, in adults with metabolic syndrome.[35] All participants consumed methionine-restricted shakes, unlimited fruits and vegetables (which contain very little methionine), and some grains (which also do not contain a lot of methionine) for the entire study. During that time, the control group received methionine supplements and the MR group received placebo capsules. At the conclusion of the study, the control and MR groups had comparable reductions in body weight, which caused improved biomarkers (fasting insulin, plasma adiponectin, plasma TG, FFA, total cholesterol, etc.) in participants from both groups. That the control group and the MR group both lost weight could be viewed as a weakness of the study, since it made it difficult to distinguish the effects of dietary MR. Two effects were unique to MR: 1) subjects fed the MR diet had significantly less lipid storage compared to the control group, and 2) subjects in the MR group oxidized more fat, as indicated by lower RQ values. Despite some promising results, the study design contained a major flaw. While methionine was restricted, the shakes contained cysteine, a metabolic product of methionine, which caused diminished efficacy of the restriction of methionine in the shakes. Studies have shown that when cysteine is added back to the MR diet, many of the beneficial effects of MR are reversed.[40] Cysteine addition to the MR diet in rodents ameliorated the ability of MR to decrease body weight, adiposity, and serum triglycerides.[40] Future clinical studies

must eliminate cysteine in addition to restricting methionine in order to collect non-confounded results.[35]

The multitude of benefits provided by this diet, in addition to the fact that it does not restrict food portions or calories, makes it a contender for human applications, specifically in populations suffering from metabolic syndrome and other obesity-associated morbidities. Additionally, further investigation into the mechanisms mediating the beneficial effects of MR could yield potential therapeutic strategies to treat obesity and related complications.

Anti-Inflammatory Effects of MR

MR induces anti-inflammatory actions in both liver and WAT.[15] These anti-inflammatory effects seem to not be a direct effect of decreased adiposity but, rather, from metabolic changes.[15] Using quantitative RT-PCR to measure expression of pro-inflammatory genes, Wanders *et al.* described transcriptional anti-inflammatory effects of MR in rats fed either MR or a control diet for 20 months. In retroperitoneal white adipose tissue (RPWAT), expression levels of Ccl2 (MCP-1), *Il1b*, *Tnf*, *Ifng*, *Emr1* (F4/80), *Itgam*, and *Itgax* were decreased in rats fed the MR diet. In inguinal white adipose tissue (IWAT) MR reduced *Ccl2* (MCP1) expression by 2.9-fold. Epididymal white adipose tissue (EWAT) analysis showed downregulation of *Emr1*, *Itgam*,and *Itgax* mRNA expression. Finally, hepatic expression of *Ccl2, Il1b, Tnf, Ifng, Emr1, Itgam,* and Itgax were reduced by MR. In addition to MR, a CR diet was assessed in the same study.

Results indicated that MR caused greater downregulation of inflammatory genes than CR in both liver and AT.[15]

Fibroblast Growth Factor 21 (FGF21)

FGF21 is a hormone secreted primarily by the liver, but also by AT.[41,42] In these organs, it works to regulate glucose and lipid metabolism and homeostasis.[41-44] Through PPARα-mediated activation, FGF21 binds to an FGF receptor with help from co-receptor βKlotho. Binding to the FGF receptor causes it to dimerize, autophosphorylate, and activate a signaling cascade. The signaling cascade causes the activation of extracellular mitogen-activated protein kinase 1 & 2 (ERK1/2), which allows it to activate several transcription factors.[43] FGF21 regulates glucose metabolism potentially through this mechanism. By upregulating GLUT1 expression in AT, glucose uptake is increased, independent of insulin action.[42,43,45]

Although FGF21 is considered anti-obesogenic, serum levels tend to be elevated in both obese mice and humans. Many scientists have proposed the idea of "FGF21-resistance," a concept similar to that of insulin-resistance, where the body is desensitized to the hormone and, therefore, increases its production with little benefit.[41-43,45] Scientists seem to agree that FGF21 resistance is a result, not the cause of obesity.[43,45] Despite resistance to the hormone, exogenous administration of FGF21 to obese rodents has shown improvements in weight with little to no side effects,[44] making administration of FGF21 a potential pharmacological therapy for obesity.

In addition to its work in improving glucose and lipid metabolism and homeostasis, FGF21 is associated with reduced inflammation. Treatment of adipocytes with TNF-α decreased FGF21 signaling by reducing the expression of its essential co-receptor βKlotho.[46] In addition to being affected *by* inflammation, FGF21 also reduces inflammation. A study using recombinant FGF21 showed that FGF21 reduced inflammatory cytokines IL-6 and TNF-α in WAT of rats with monosodium glutamate-induced obesity.[47] In arthritic mice, exogenous FGF21 administration caused downregulation of several pro-inflammatory cytokines in the spleen, including TNF-α, IL-1β, and IL-6.[48,49] Research on the role FGF21 plays in inflammation is limited but promising, highlighting the importance of further research into the hormone's anti-inflammatory properties.

One option for research into FGF21 is the use of *Fgf21* knock-out (*Fgf21*KO) mice in comparison to control wild-type (WT) mice. Fisher and colleagues sought to investigate similarities and differences between *Fgf21*KO and WT mice to establish that the two strains are phenotypically similar and comparisons between the two are valid.[43] While on chow-fed diets WT mice weighed less than *Fgf21*KO mice. Once put on a HFD that was intended to be obesogenic, the WT and *Fgf21*KO mice took on similar levels of adiposity. WT mice also experienced better glucose tolerance while on the chow diets, but these differences disappeared on the HFD. Circulating non-esterified fatty acids were also no longer different once the mice were put on the HFD. This research gives validation that WT and *Fgf21*KO mice can be appropriately compared when on a HFD.[43]

Signal Transducer and Activator of Transcription 3 (STAT3)

The STATs are a set of transcription factors that are activated through the phosphorylation mediated by janus kinases (JAKs) and, once activated, dimerize, move to the nucleus, and affect gene transcription.[30,50] Activators of the JAK-STAT pathway include cardiotrophin-1 (CT-1),[51-53] growth hormone (GH),[51,54] interferon gamma (IFN-γ),[51,55-57] interleukins 6 and 11 (IL-6 and IL-11),[55,58,59] leukemia inhibitory factor (LIF),[51,55,57,59,60] neuropoietin (NP),[51,61-63] and oncostatin M (OSM).[51,55,57,59,64] STAT3 is widely recognized for its role in propagating inflammation, but has been studied primarily for its role in cancer-related inflammation. Two phosphorylation sites have been identified on STAT3: Tyr705 and Ser727.[50,65,66] The actions of and differences in phosphorylation sites are not completely understood. Phosphorylation at Tyr 705 appears to promote the dimerization of STAT3 and is essential for STAT3 activation,[50,66,67] while phosphorylation at Ser727 seems to enhance the transcription effects of STAT3.[50,66] STAT3 is found in adipocytes[68,69] and evidence suggests that phosphorylated STAT3 (pSTAT3) plays a role in adipogenesis and preadipocyte differentiation.[68,70-73] pSTAT3 also upregulates the expression of pro-inflammatory markers.[2,74]

MR and STAT3

Microarray analyses conducted on tissue samples from rats fed a control, MR, or 40% calorie-restricted (CR) diet for 20 months showed that both MR and CR decreased expression of inflammatory genes in liver and WAT of rats.[15] In fact, despite similar reductions in body weight and adiposity between the MR and CR rats, MR had a

significantly greater anti-inflammatory effect in liver and adipose tissue than CR. Furthermore, analysis using Ingenuity Pathway Analysis (Qiagen) software identified a downregulation of STAT3 signaling in liver and WAT of rats fed the MR diet.[2] These data suggest that STAT3 may be the mechanism behind the MR-induced downregulation of age- and obesity-induced inflammation.

Chapter III

Methods

Animal Study

Animal studies were conducted at Pennington Biomedical Research Center, and tissues were generously provided by Dr. Tom Gettys. All animal experiments were reviewed and approved by the Pennington Institutional Animal Care and Use Committee using guidelines established by the National Research Council, the Animal Welfare Act, and the PHS Policy on humane care and use of laboratory animals. Male WT C57BL/6J mice were obtained from Jackson Labs (Bar Harbor, ME, USA), and *Fgf21*KO (KO) mice were kindly provided by Dr. Steve Kliewer (UT Southwestern). 24 WT C57BL/6J and 24 *Fgf21*KO mice, age 12 weeks, were fed a HFD for four weeks. After those four weeks, 12 WT and 12 KO mice were placed on high-fat methionine-restricted (MR) diets while the other mice remained on the control HFD (CON). The CON diet contained .86% methionine, by weight, and the MR diet contained .17% methionine and no cysteine (an 80% reduction, typical for MR diets in mice). The WT and KO mice were fed the CON or MR diet for 13 weeks after randomization. After these 13 weeks, mice were euthanized and tissues were collected and flash frozen in liquid nitrogen.

RNA Extraction and Real-time PCR

RNA was extracted from EWAT and liver samples using the Qiagen RNeasy Lipid Tissue Mini Kit 50. DNAse digestion was performed to improve purity of RNA.

RNA concentrations and purity were verified by nanodrop. From isolated RNA, reverse transcription was performed to make cDNA.

Real-time PCR (RT-PCR) using SYBR-green reagents was performed on Liver samples to measure expression of the inflammatory marker genes for *Ccl2* (MCP-1) and *Emr1* (F4/80) and on EWAT samples to measure expression of the inflammatory marker genes for *Emr1* (F4/80), *Itgam* (CD11b), *Il6* (interleukin-6), and *Ccl2* (MCP-1). Gene expression measures were normalized to cyclophilin.

Gene	Forward Primer	Reverse Primer
Cyclophilin	5'-CTT CGA GCT GTT TGC AGA CAA AGT-3'	5'-AGA TGC CAG GAC CTG TAT GCT-3'
Ccl2	5'-GGA GAG ACA GCG GTC GTA AG-3'	5'-CCA GCC GGC AAC TGT GA-3'
Emr1	5'-GTG CCA TCA TTG CGG GAT TC-3'	5'-GAC GGT TGA GCA GAC AGT GA-3'
Itgam	5'-CCA CAC TAG CAT CAA GGG CA-3'	5'-CCC TGA TCA CCG TGG AGA AG-3'
Il6	5'-ATG GAT GCT ACC AAA CTG GAT-3'	5'-TGA AGG ACT CTG GCT TTG TCT-3'

Table 1. Forward and reverse primer sequences used in RT-PCR.

Protein Extraction and Immunoblot Analysis

To determine if MR decreases inflammation through downregulation of STAT3 signaling and to determine the role of FGF21 in anti-inflammatory effects of MR, western blot analyses of STAT3 phosphorylation at Tyr705 and Ser727 were conducted. Protein extracts from tissues were made by homogenizing ~100mg of each sample in 300uL of radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. Lysates were incubated in lysis buffer on ice for 20 minutes and

were then centrifuged for 30 minutes at 13,200 RPM at four degrees Celsius. The infranatant was collected and assayed for protein concentrations. Protein concentrations were determined by Lowry Protein assay. Protein samples were combined with laemmli buffer and boiled for five minutes. Protein from each sample were loaded onto a 10% Criterion Precast (Bio-Rad Laboratories) gel. Proteins were separated via electrophoresis at 90-110V for 2.5 hours. Proteins (total amounts varied between membranes) were transferred to a PVDF membrane overnight at 10V. Membranes were rinsed with deionized water the following day and were incubated in blocking buffer (5% BSA in TBSt) for 1 hour. Primary antibody (pSTAT3^{Tyr705} and pSTAT3^{Ser727} in 5% BSA and total STAT3 in 1% BSA) was added to a pouch with the membranes and incubated for either about one hour or overnight (varied by membrane and by antibody) at four degrees Celsius. Afterward, the membranes were washed in TBSt on a shaker for five minutes, three times. Secondary antibody was added to the membranes (anti-rabbit, in 1% BSA) and incubated for one hour, followed by another three washes in TBSt. The membrane was then covered with ECL for five minutes and immediately imaged using ImageQuant LAS4000 equipment and software (GE Healthcare). pSTAT3^{Tyr705} and pSTAT3^{Tyr705} antibody from Cell Signaling Technology (Danvers, MA) were used; STAT3 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Band densities of pSTAT3 were quantified using ImageJ software and normalized to total STAT3.

Statistical Analysis

RT-PCR data were analyzed by one-way ANOVA. Tukey's multiple comparisons test was used to identify significant differences between groups. Western blot data were

analyzed by unpaired t-test. Data was analyzed by Prism software (version 7.00, GraphPad, La Jolla, California).

Chapter IV

Results

Liver expression of MCP-1 and F4/80

KOCON mice had significantly increased expression of the inflammatory chemokine *Ccl2* (MCP1) in the liver compared to WT mice (**Fig. 1**). MR decreased MCP-1 expression in KO mice ($p<0.0001$), and tended to do so in WT mice. However, WT mice fed the Con diet had significantly less MCP-1 expression in their liver compared to KO mice. Expression of the inflammatory macrophage marker, F4/80 expression was unaffected by diet and genotype (**Fig. 2**).

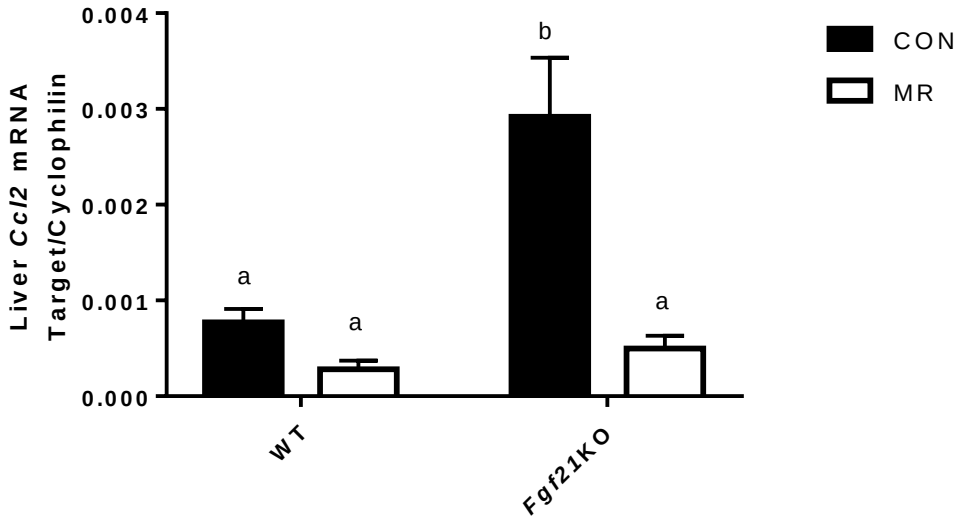

Figure 1. Hepatic *Ccl2* (MCP1) mRNA expression in WT and *Fgf21*KO mice. Values are means ± SEM, n=7-10.

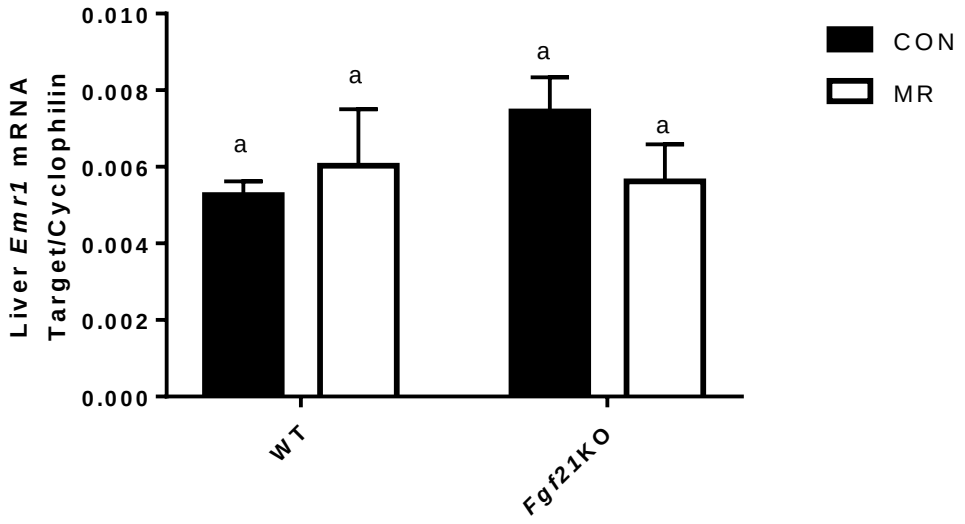

Figure 2. Hepatic *Emr1* (F4/80) mRNA expression in WT and *Fgf21*KO mice. Values are means ± SEM, n=6-9.

MR decreased EWAT MCP-1 expression in both WT and Fgf21KO mice

MR decreased MCP-1 expression in EWAT of WT and *Fgf21*KO mice. No significant differences in EWAT MCP-1 mRNA expression were found between genotypes (**Fig. 3**).

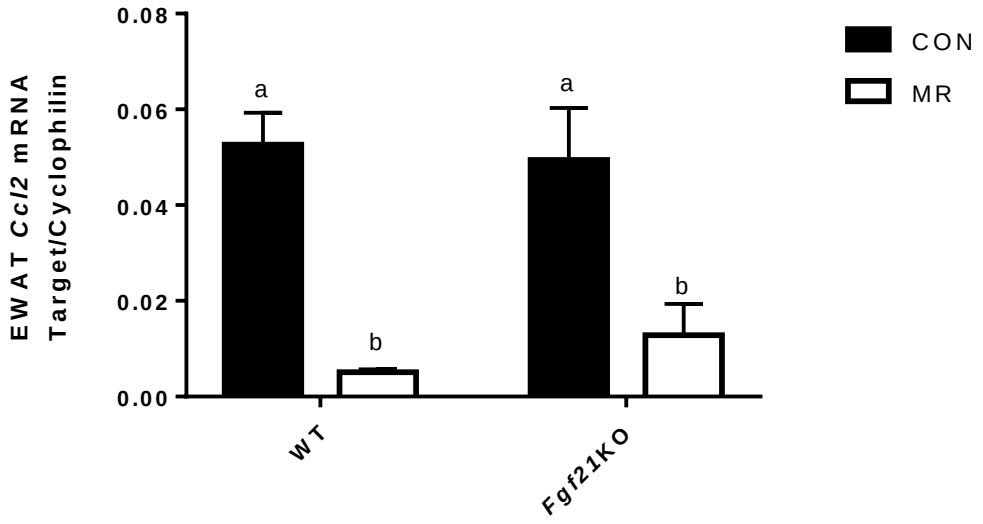

Figure 3. EWAT *Ccl2* mRNA expression in WT and *Fgf21*KO mice. Values are means ± SEM, n=8-10.

MR decreased EWAT F4/80 expression in Fgf21KO mice

*Fgf21*KO mice had elevated *Emr1* (F4/80) expression in EWAT compared to WT mice, and MR decreased its expression in *Fgf21*KO mice, but not in WT mice (**Fig. 4**).

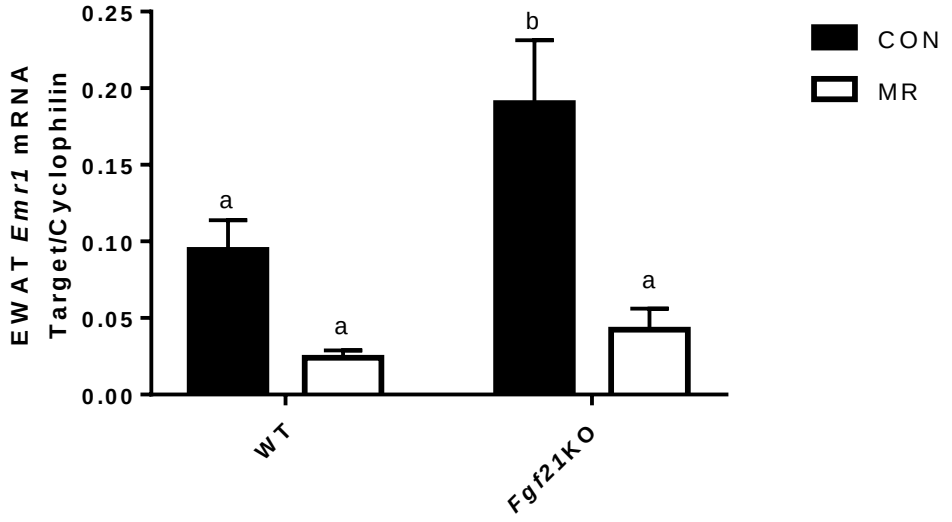

Figure 4. EWAT *Emr1* mRNA expression in WT and *Fgf21KO* mice. Values are means ± SEM, n=8-10.

EWAT Itgam and Il6 expression did not differ between groups

mRNA expression of *Itgam*, an inflammatory macrophage marker, in EWAT was similar between all four groups (**Fig. 5**). Additionally, mRNA expression of the inflammatory cytokine *Il6* in EWAT was similar between all four groups of mice. Though trends seen in the figures are similar to those of *Ccl2* and *Emr1*, differences between groups were not statistically significant (**Fig. 6**).

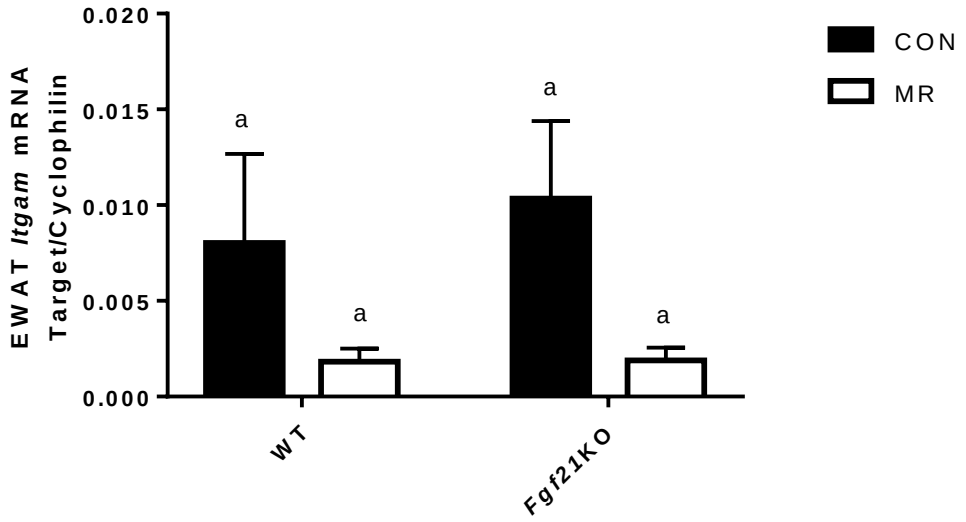

Figure 5. EWAT *Itgam* mRNA expression in WT and *Fgf21*KO mice. Values are means ± SEM, n=5-9.

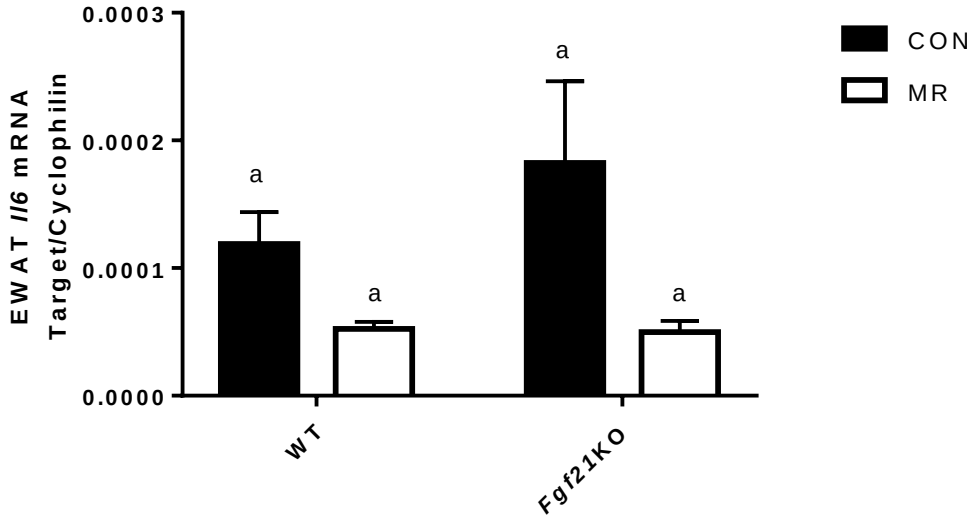

Figure 6. EWAT IL-6 mRNA expression in WT and *Fgf21*KO mice. Values are means ± SEM, n=8-9.

EWAT pSTAT3Tyr705, but not pSTAT3Ser727, was reduced by MR in WT mice

Since STAT3 is a transcription factor known to propagate inflammation and microarray analyses identified STAT3 as a potential master regulator of the anti-inflammatory effects of MR, STAT3 phosphorylation in EWAT was measured. Western blot analyses of EWAT indicated that MR significantly decreased phosphorylation of STAT3 at Tyr705 in WT mice (p=0.0118) (**Fig. 7**). The analyses also showed no significant differences in phosphorylation of STAT3 at Ser727 between the two groups (**Fig. 7**).

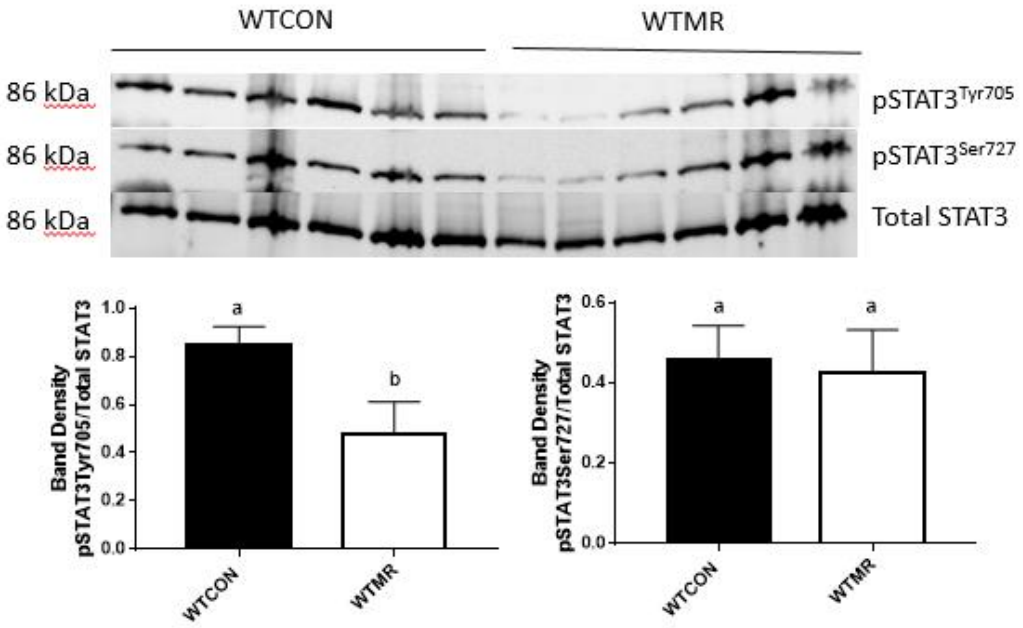

Figure 7. EWAT pSTAT3^{Tyr705} and pSTAT3^{Ser727} in WTCON and WTMR mice. Values are means ± SEM, n=6.

EWAT pSTAT3^{Tyr705}, but not pSTAT3^{Ser727}, was significantly reduced by MR in Fgf21KO mice

Similarly to the WT mice, MR decreased phosphorylation of STAT3 at Tyr705 in the *Fgf21*KO mice (p=0.009) (**Fig. 8**). However, phosphorylation of STAT3 at Ser727 was not significantly different between the two groups (**Fig. 8**).

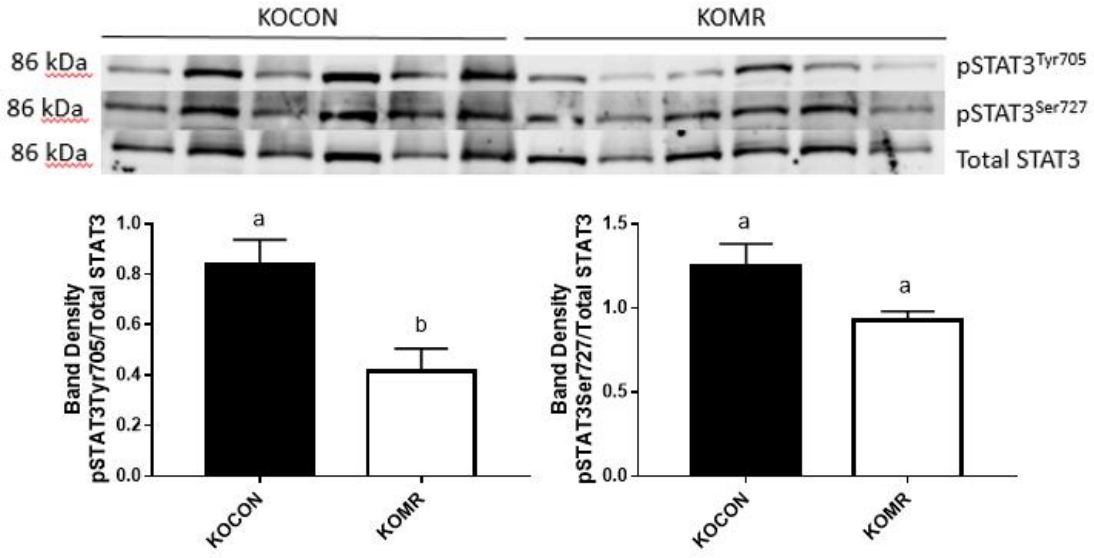

Figure 8. EWAT pSTAT3^{Tyr705} and pSTAT3^{Ser727} in KOCON and KOMR mice. Values are means ± SEM, n=6.

EWAT pSTAT3^{Tyr705} was affected by absence of FGF21 while pSTAT3^{Ser727} was not

WTCON and KOCON were compared to assess the effects that absence of *Fgf21* has on STAT3. Western blot analyses indicated that phosphorylation of STAT3 at Tyr705 was increased in *Fgf21*KO mice compared to WT mice (p=0.0177) (**Fig. 9**). Phosphorylation of STAT3 at Ser727, however, was not significantly different between WTCON and KOCON (**Fig. 9**).

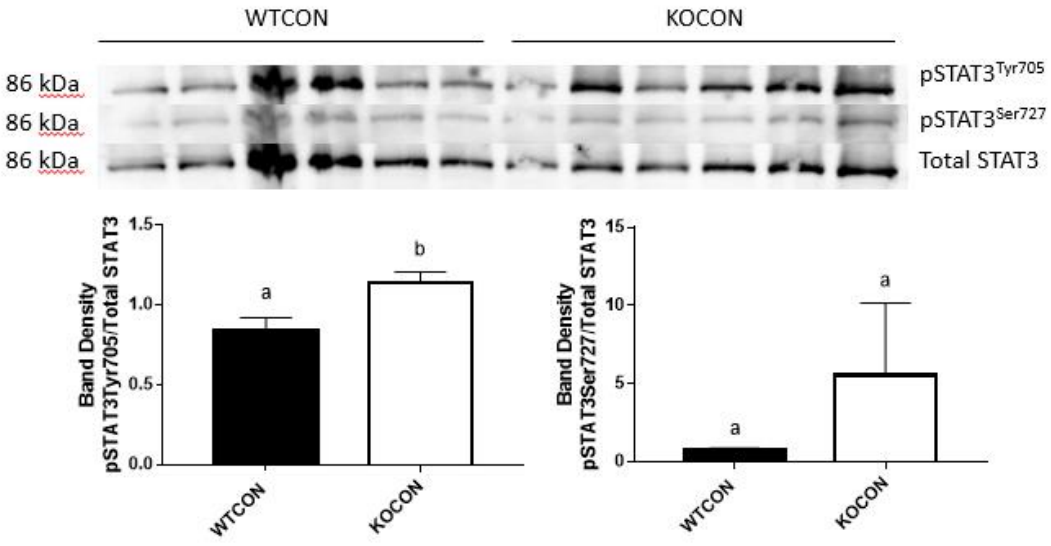

Figure 9. EWAT pSTAT3^{Tyr705} and pSTAT3^{Ser727} in WTCON and KOCON mice. Values are means ± SEM, n=6.

Chapter V

Discussion

Obesity increases inflammation throughout the body.[1] This inflammation is characterized by increased inflammatory cytokine expression and increased ROS production, similar to inflammation observed in aging. [2,3] Inflammatory markers often upregulated in obesity include MCP-1, IL-1β, TNF-α, IL-6, IFN-γ, F4/80, ITGAM, and ITGAX. [15]

NF-κB, a proinflammatory transcription factor, is activated by hypoxia in adipocytes and AT macrophages. This hypoxia response is initiated by adipocyte hypertrophy.[1,75] Activation of this transcription factor is to blame for the upregulation of many pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and MCP-1.[1,14,75] In addition to NF-κB activation, polarization of macrophages to the M1 type causes upregulation of pro-inflammatory cytokines.[4,10,13]

CR has gained attention for its ability to increase lifespan and adiposity in rodents.[25] However, use of this dietary approach is not ideal for humans.[2] MR has been shown to produce similar benefits as CR,[27-30] but, interestingly, induces a hyperphagic response with simultaneous increased energy expenditure.[27-29,35,37] MR also increases insulin sensitivity and decreases pro-inflammatory cytokine expression, reduces hepatic lipid stores, and reduces serum lipid levels.[15,28,29,31,35] In the present study, both WTMR and KOMR mice weighed less than the CON groups. This reduction in weight was accompanied by a reduction in adiposity in WTMR and KOMR. Food intake increased in WTMR mice in comparison to WTCON, however KOMR mice did not develop a

hyperphagic response in comparison to KOCON (unpublished data from Gettys and colleagues).

MR has been shown to decrease the pro-inflammatory cytokines MCP-1, IL-1β, TNF-α, IFN-γ, F4/80, ITGAM, and ITGAX in WAT and liver.[15] In the present study, measures for MCP-1 and F4/80 in the liver and MCP-1, F4/80, ITGAM, and IL-6 in EWAT were gathered. Our data for MCP-1 were in agreement with other data that suggests downregulation of the gene in EWAT and liver of mice on dietary MR. EWAT F4/80, ITGAM, and IL6 were not in agreement with other data, but the data were trending toward downregulation of these pro-inflammatory genes in MR. A flaw of our study and similar studies is that only portions of each tissue from each mouse is taken and used for RNA isolation and subsequent reverse transcription and RT-PCR. Fluctuations of gene expression in various portions of the same tissue are common. Because we can only feasibly take a portion of each tissue, gene expression levels may be slightly different from total tissue gene expression. This may be why we found trends between groups without significant differences. Additionally, increasing the number of samples used in the study could help determine significance by limiting the SEM.

FGF21 is a hormone that increases glucose uptake by adipocytes, prevents obesity, decreases blood glucose, and decreases triglyceride levels.[39] Studies show that MR increases levels of FGF21 in circulation.[28,36-38] Inflammation (specifically, the presence of TNF-α) decreases FGF21 signaling in adipocytes through the reduced expression in co-receptor βKlotho.[46] FGF21 serum levels are higher in obese rodents and humans, possibly as a result of FGF21 resistance. Exogenous administration, however, has been shown to improve weight in obese rodents and decrease pro-inflammatory

cytokine expression in the spleen of arthritic mice, suggesting its potential use as a pharmacologic agent.[44,48,49] Additionally, recombinant FGF21 reduced expression of pro-inflammatory cytokines in mouse WAT.[47] Our data showed increased expression of Liver MCP-1 and EWAT F4/80 in KOCON mice when compared to all other groups. This suggests the *Fgf21*KO mice were more susceptible to HFD-induced inflammation. Western blot analyses showed increased phosphorylation of STAT3 at Tyr705 in KOCON when compared to WTCON. This suggests that the increased inflammation seen in the absence of FGF21 is potentially mediated by the phosphorylation of STAT3 at Tyr705.

STAT3, a generally pro-inflammatory transcription factor, is activated at two phosphorylation sites: Tyr705 and Ser727.[50,65,66] Our data indicate that phosphorylation of STAT3 at Tyr705 is downregulated by MR in both WT and KO mice. Additionally, phosphorylation of STAT3 at Tyr705 is upregulated in KOCON mice when compared to WTCON mice. These data suggest that the mechanism by which MR downregulates phosphorylation of STAT3 at Ser727 is independent of *Fgf21*. Phosphorylation of STAT3 at Ser727 was unaffected by both MR and lack of FGF21. However, Tyr705 is the main site of phosphorylation on STAT3 while Ser727 is the secondary maximal activation site. The data which show downregulation of phosphorylated STAT3 at Tyr705 are in agreement with our hypothesis that STAT3 is a potential master regulator of inflammation in MR.

In conclusion, MR decreased inflammation in both WT and *Fgf21*KO mice, indicating that *Fgf21* does not play a role in the mechanisms by which MR reduces

inflammation. Western blot results support the hypothesis that MR decreases inflammation by downregulation of STAT3 signaling.

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