MELANOCORTIN-5 RECEPTOR

ROLE IN ENERGY BALANCE

A Thesis

by

EMILY NELSON JORDAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2011

Major Subject: Nutrition

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Approved by:

Chair of Committee,	Caurnel Morgan
Committee Members,	Paul Wellman
	Chaodong Wu
Intercollegiate Faculty Chair,	Stephen Smith

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ABSTRACT

Melanocortin-5 Receptor Role in Energy Balance. (August 2011)

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Chair of Advisory Committee: Dr. Caurnel Morgan

Metabolic disorders, including obesity and fatty acid oxidation disorders (FODs), occur in approximately 34% of the American population and in 1 in every 10,000 Americans, respectively. Melanocortin-5 receptor (MC5R) is one of five G-protein coupled receptors that are known to mediate effects of melanocortins. Emerging evidence has linked MC5R to multiple metabolic disorders. Previous studies have provided evidence that MC5R helps to regulate lipid metabolism in the skeletal muscle, liver, and white adipose tissue, and in the skin and exocrine glands of vertebrates. We, therefore, tested the hypothesis that MC5R promotes FAO in skeletal muscle. In the present study, MC5R knockout (KO) mice and wild-type (WT) mice were placed on a low-fat or high-fat diet for 9 weeks. Methods including body weight gain and food intake determinations, behavioral testing to be assessed in future studies, insulin tolerance testing (ITT), and reverse transcription PCR (RT-PCR) were used to provide a picture of overall energy balance and metabolic activity in tissues. Results suggest that obesity induced MC5R (KO) mice, relative to WT mice, display a decrease in FAO not only in the skeletal muscle, but in the liver as well. We also show that to compensate for the loss of FAO in skeletal muscle, MC5R KO mice experience an increase in FAO in brown adipose tissue (BAT) and an

increase in adipogenesis in the liver. Not only does lipid metabolism activity shift to liver, data suggests that the glycolytic activity in skeletal muscle decreases and is again reallocated to the liver. Furthermore, we demonstrate that insulin sensitivity is maintained in MC5R KO mice on high-fat diet, in contrast to the decrease in sensitivity in WT mice on high-fat diet. These data collectively suggest that MC5R is extensively involved in overall lipid and glucose metabolism and not only shifts lipolysis and lipogenesis in the body, but may be involved in the dissociation of obesity and insulin resistance.

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CHAPTER I

INTRODUCTION

The *project goal* was to determine the role of MC5R in lipid metabolism. The *central hypothesis* that was tested in this project, is that MC5R activation stimulates lipid metabolism in skeletal muscle, was derived from preliminary data in the Morgan laboratory and the work of An and coworkers (2007) (1). The *general approach* that was used to test this hypothesis was to assess changes in biomarkers of metabolism using MC5R wild-type and knockout mice. This work is *significant* because it will advance the understanding of the role of MC5R in lipid metabolism and metabolic disorders. The approach was *innovative* because it used a unique MC5R mouse model on high-fat diet in order to make these advances. *The predicted outcome* was that the genetic deletion of MC5R globally will reduce fatty acid oxidation in skeletal muscle and, consequently, shift the burden of FAO to other tissues that do not express high levels of MC5R (e.g., liver). This outcome will have a positive impact because it helps to identify MC5R as a therapeutic target in metabolic disorders.

This thesis follows the style of *American Journal of Physiology-Endocrinology and Metabolism.*

CHAPTER II

LITERATURE REVIEW

RESEARCH JUSTIFICATION

Impact of Metabolic Disorders

According to the Morbidity and Mortality Weekly Report, 26.7% of adults in the United States are self-reportedly obese in 2009, and 33.8% in 2008, when surveyed by NHANES (19). Within the last 8 to 9 years, obesity among men and women, ages 40-59, increased approximately 35% (19). The obesity epidemic in the United States is beginning to affect both adults and children. Studies have shown a higher prevalence of obesity among adults who were obese as children or adolescents. Diet, parental obesity, and obesity-related gene polymorphisms contribute to the development of obesity (2). The repercussions of obesity does not simply affect quality of life, an issue of morbidity can be presented. Long-term obesity is often associated with the development of metabolic syndrome. One review defines metabolic syndrome as the presence of at least three of the following criteria: elevated triglycerides, low levels of high-density lipoproteins (HDL), abdominal circumference above the 90th percentile by sex, elevated fasting glucose, and high blood pressure (2). Additionally, obesity is frequently associated with the development of type-2 diabetes and the increased risk and worsened prognosis of many types of cancer (2, 28). With all factors combined, the risk of mortality and morbidity is higher with obesity.

Obesity and Diabetes

As mentioned above, there is significant association between obesity and the onset of type-2 diabetes (2, 7). The cycle can begin two ways: an increase in fat mass or the development of insulin resistance. First, the development of adiposity during the development of obesity can be the cause of systemic insulin resistance. The adipose tissue is enlarged and the tissue develops a low grade chronic inflammation (27). The inflammation allows macrophage infiltration and production of pro-inflammatory cytokines. The cytokine production, along with macrophage presence, causes adipose tissue dysfunction, increasing pro-hyperglycemic factor production and decreasing anti-hyperglycemic factor production (27). When combined, these aspects lead to systemic insulin resistance. Second, the cycle can begin by glucose uptake activated by insulin in the cell, the stimulation of lipogenesis, and the inhibition of lipolysis (57). An elevated fasting glucose level causes a cell to become insulin resistant, which disrupts glucose and lipid homeostasis pathways and produces elevations in fasting and postprandial glucose and lipid levels (57). The increase in lipid levels increases adiposity and eventually obesity. The relationship between obesity and insulin resistance has become a major issue in today's society. As obesity increases, so does the higher percentage of people who have type-2 diabetes. A further understanding of the mechanisms responsible for this association would be valuable, benefitting both the health status financial burden of the obese with type-2 diabetes.

Fatty Oxidation Disorders

Another set of metabolic disorders involve the disruption of lipid metabolism, as with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. These so-called FODs are common and inherited disorders that disrupt FAO. FODs stimulate hepatic ketogenesis, which becomes a major energy source when glucose is less available (41, 59). If FAO is insufficient, people diagnosed with FODs are unable to oxidize fatty acids for fuel, creating an energy shortage. Since MCAD deficiency is a genetic disorder, patients from childhood experience hypoketotic hypoglycemia, vomiting, and lethargy, and common infections (41). Liver disease and seizures may also develop. Eventually, the resultant metabolic stress can induce a coma and ultimately, death. The disease is usually detected between 3 months and 2 years of age. If MCAD deficiency is correctly diagnosed, the child can survive into adulthood by avoiding high fat foods and any prolonged period of fasting. If MCAD deficiency is not properly identified, at least 18% of affected individuals die during their first metabolic crisis (41). Increasing the understanding of FAO would expand the knowledge base from which effective treatment of FODs is expected to arise.

Melanocortins

The principal melanocortins include alpha-, beta-, and gamma-melanocortin stimulating hormones (α-MSH, β-MSH, and γ-MSH), as well as adrenocorticotropic hormone

(ACTH) (15). These melanocortin peptides are post translational products that are derived enzymatically from the pro-opiomelanocortin (POMC) precursor protein (15, 21). In the hypothalamic brain region, POMC neurons are sites of convergence of a variety of peripheral and central hormones, neurotransmitters, and nutrients involved in the regulation of feeding behavior (20, 45). Melanocortins, particularly α-MSH and ACTH, are also known to regulate metabolic functions in peripheral tissues (e.g., adipose) (1, 15).

Melanocortin Receptors

Five melanocortin receptor (MCR) sub-types are known to mediate biological actions of melanocortins (9). Melanocortin-1 receptor (MC1R) is expressed in epidermal and follicular melanocytes, and it stimulates melanogenesis (22, 39). MC2R, in the adrenal cortex, mediates the effects of ACTH on corticosteroid secretion (13, 51). MC3R,MC4R, and MC5R all are found in the brain (20, 37, 56). Central MC3R and MC4R are important for body weight regulation (5, 8, 16, 24). Functions for MC5R in the central nervous system have not been investigated extensively, but there is evidence that it may be involved in the control of pituitary hormone secretion (46).

Peripherally, MC5R mRNA and protein are expressed abundantly in exocrine glands, including sebaceous, lachrymal, and preputial glands (8, 66). Chen and co-workers (1997), using mice that were genetically engineered to be MC5R-deficient, found that MC5R deficiency caused defective lipid metabolism in sebaceous glands (8). Morgan and co-workers (2004) found that MC5R deficiency reduced the content of specific li-

pid-based pheromones in the preputial glands of male mice (45). Although MC5R mRNA has been reported to be expressed in adipose, skin, muscle, liver, adrenal gland, and testis, in some tissues, the reports have been conflicting, and most have not been confirmed at the protein level (1, 9, 17, 35, 55). MC5R's metabolic activity has not been widely studied, but investigated primarily in relation to the skin, skeletal muscle, liver, and white and BAT (1, 8). As indicated in Figure 1, we confirmed by RT-PCR that MC5R is expressed in mRNA of BAT, skeletal muscle, testes, spleen, heart, omental fat, and may be marginally expressed in kidney and liver. In liver, we do not see the expected MC5R amplicon; instead we see genomic DNA amplification at approximately 900 base pairs. Genomic DNA can bind to primers, is intron spanning, and if amplified, results in an amplicon with a large molecular weight. When amplifying MC5R in the spleen, we see a non-specific amplicon at approximately 250 base pairs. This is most likely an alternate MC5R priming site binding the primers used.

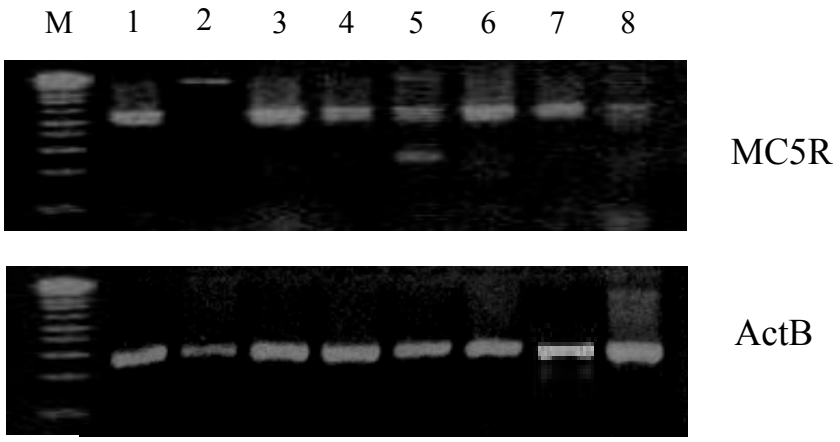


Figure 1. MC5R Tissue Distribution. Tissues obtained from wild-type mice, 18 weeks of age on LFD feeding, were used to analyze the presence of MC5R by RT-PCR: (1) BAT, (2) Liver, (3) Skeletal Muscle, (4) Testes, (5) Spleen, (6) Heart, (7) Omental Fat, (8) Kidney. Beta-actin (ActB) was used as the standard.

MC5R and Adipose Function

White adipose tissue (WAT) BAT and have been reported to express MC5R, and emerging evidence suggests that this MCR subtype might be upregulated during adipocyte differentiation or adipogenesis (29). Furthermore, the administration of α-MSH has been shown to alter lipid metabolism in adipose tissue by inhibiting adipogenesis, stimulating lipolysis, and inhibiting the expression and secretion of the adipocyte hormone, leptin, in the 3T3-L1 mouse adipocyte cell line (29).

In addition to this evidence suggesting direct regulation of lipid metabolism in adipocytes, melanocortins have been shown to alter the expression of genes that regulate lipid

metabolism. For example, one study investigated the relationship between the adipocyte cytokine, interleukin-6 (IL-6), and the melanocortin system (33). In addition to its role in the immune response, IL-6 influences lipid metabolism and insulin sensitivity (33). When 3T3-L1 adipocytes were incubated with α-MSH, IL-6 production increased. MC5R and MC2R are thought to be the most abundant MCR sub-types expressed in adipose, and α-MSH has low affinity for MC2R. Therefore, MC5R likely accounts for the IL-6 increase, and subsequent metabolic actions of IL-6 (33).

MC5R and Liver Function

MC5R function in the sea bass was found to stimulate hepatocyte lipolysis in this species (58). While all five melanocortin receptors are present in the tetrapod species, only 3 MCR's have been considered for function. MC1R has been studied in relation to the color patterns of fish, MC2R has been associated with cortisol secretion regulation in the fish, and MC4R has been linked to food intake and energy balance in the fish. MC3R and MC5R have not been characterized in relation to function in fish. This group found sea bass MC5R expressed in various isoforms, and that the administration of melanotan-II (MT-II), an agonist for MC5R, MC4R, and MC3R, increased the FAO in the liver. When sea bass liver cells were treated with MT-II, levels of non-esterified fatty acid (NEFA) increased within 4 hours. The increase in NEFA provides evidence of direct regulation of hepatic lipid metabolism by MC5R activation (58). Whether MC5R plays a

similar role in the livers of other vertebrates, including mammals, remains to be determined.

MC5R and Skeletal Muscle Function

MC5R is abundantly expressed in skeletal muscle, and its effect on lipid metabolism was examined *in vivo* and *in vitro*, using the C2C12 mouse skeletal muscle cell line (1, 8). First, mice injected with α-MSH, exhibited elevated activity of carnitine palmitoyltransferase-I (CPT-I), a rate limiting enzyme in FAO. Second, incubation of C2C12 cells with α-MSH increased the oxidation of the fatty acid, palmitate. To determine which MCR sub-type was responsible for this increase in FAO, different MCR modulators were administered. SHU9119 acts as an MC5R agonist and as an antagonist of MC3R and MC4R. Administration of SHU9119 increased palmitate oxidation in the cell line, suggesting modulation MC5R responsibility FAO. These results provide evidence that activation of MC5R in skeletal muscle increases FAO in the mouse and C2C12 skeletal muscle cells. These findings suggest that additional *in vivo* studies might provide additional physiological evidence of an association between MC5R and lipid metabolism.

MC5R in Skin Function

MC5R mRNA and protein have been reported in sebaceous glands of the skin (74). MC5R regulates lipid synthesis, storage, and secretion in sebaceous glands, as well as

other exocrine glands. MC5R's role in the mouse preputial gland, which is a modified and macroscopic sebaceous gland, was studied in relation to lipid metabolism (45). Earlier studies had shown that melanocortins increased preputial gland lipogenesis and the synthesis and release of lipid-based pheromones (9, 10). Melanocortin administration to mice stimulated the preputial biosynthesis and secretion of lipid-based pheromones that influenced aggressive behaviors in male wild-type mice, but not in MC5R null mutant mice (10). Moreover, MC5R deficiency reduced specific lipid-based compounds in the mouse preputial gland (44).

MC5R and Metabolic Disorders

Obesity and insulin resistance are important metabolic disorders that have been linked to MC5R expression. The Quebec Family Study investigated the relationship between metabolic defects and MC4R or MC5R (6). The subjects, French-Canadians from the greater Quebec City region, were divided into groups by weight, and various metabolic and genetic parameters were analyzed. A strong association between MC5R and obesity phenotypes was suggested. Among males, there appeared to be significant linkages between MC5R and body mass index, fat mass, and resting metabolic rate. Women only experienced a linkage between MC5R and body mass index (6).

Another study assessed in morbidly obese and lean Finnish subjects, relationships between polymorphisms in the genes for ectonucleotide pyrophosphatase phosphodieste-

rase-1 (ENPP1) and MCR sub-types, and obesity and insulin sensitivity. ENPP1, is an enzyme that has been implicated in obesity, insulin resistance, and type-2 diabetes (64). Twenty-five single-nucleotide polymorphisms (SNPs) for ENPP1 and each MCR sub-type were used as genetic markers. There was an association between SNPs in the MC5R and ENPP1 genes with obesity and insulin resistance, or type-2 diabetes (64). These findings suggest that MC5R might play a role in the development of metabolic disorders that affect the 36.6% and 26.5% percent of Americans who are overweight and obese, respectively (25).

SUMMARY

Defective lipid metabolism is crucial for the development of several metabolic disorders and, emerging evidence outline above suggests that melanocortins act through MC5R to regulate lipid metabolism. Most of this evidence, however, was generated using mammalian cells in vitro, and some of it was generated in fish. Our preliminary experiments suggested that MC5R might play a crucial role in the lipid metabolism in vivo, using the C57BL/6 strain of mouse, which is susceptible to the development of obesity during high-fat feeding. In addition, this obese mouse model can develop metabolic syndrome-like state, which can include obesity, insulin resistance, inflammation, cardiovascular disease, etc. We, therefore, sought to investigate the interactions between MC5R and high-fat feeding in MC5R wild-type and knockout mice on the C57BL/6 genetic background.

CHAPTER III

MELANOCORTIN-5 RECEPTOR ROLE IN ENERGY BALANCE

INTRODUCTION

Determining the mechanism by which MC5R stimulates lipid metabolism is a significant step in understanding metabolic disorders that involve the melanocortin system. Defective lipid metabolism is crucial for the development of these disorders, including medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, obesity, and diabetes, and emerging evidence suggests that melanocortins act through MC5R to regulate lipid metabolism. The effects of these disorders, or the disorder as a whole, could be alleviated if the role of MC5R in metabolism could be better understood. It has been suggested that central MC3R and MC4R are important for body weight regulation (5, 8, 16, 24). MC5R might be similarly involved in regulating genes responsible for lipid metabolism in different tissues. The findings that MC5R is involved in FAO in skeletal muscle by utilizing C2C12 mouse muscle cells suggests that supplementary *in vivo* studies might present additional physiological evidence of an association between MC5R and lipid metabolism (1, 3). The outcome of this project will have a positive impact because it is likely to identify MC5R as a therapeutic target for metabolic disorders. We, therefore, sought to investigate the interactions between MC5R and high-fat feeding in WT and MC5R KO mice.

MATERIALS AND METHODS

Animals and Diets

Virus free male mice of the C57BL/6J strain were obtained from Harlan (Indianapolis, IN) at 8 weeks of age. Male Melanocortin-5 Receptor (MC5R) knockout mice, which were originally generated and backcrossed for more than 12 generations at OHSU, were bred in the Kleberg laboratory animal facilities at Texas A&M University (43). At time of arrival, the mice were housed in individual cages in a temperature-controlled room with a 12-hour light/dark cycle. After 1 week of adaptation, the mice were randomly separated and allowed access to water and either a low-fat diet (LFD) or high-fat diet (HFD) ad libitum for 9 weeks. The low-fat diet, LFD, was composed of: 14% of calories in the form of fat, 33% in the form of protein, and 53% in the form of carbohydrates. The kilocalories contributed by fat were primarily contributed by soy bean oil. The high-fat diet, HFD, was composed of: 45% of calories in the form of fat, 20% in the form of protein, and 35% in the form of carbohydrates. The carbohydrates present were corn starch (21%), maltodextrin (29%) and sucrose (50%). The proportions of the various fats, making up 45% of kilocalorie content were soybean oil (12%) and lard (88%). Diets were obtained in pellet form from Research Diets (New Brunswick, NJ).

Procedure

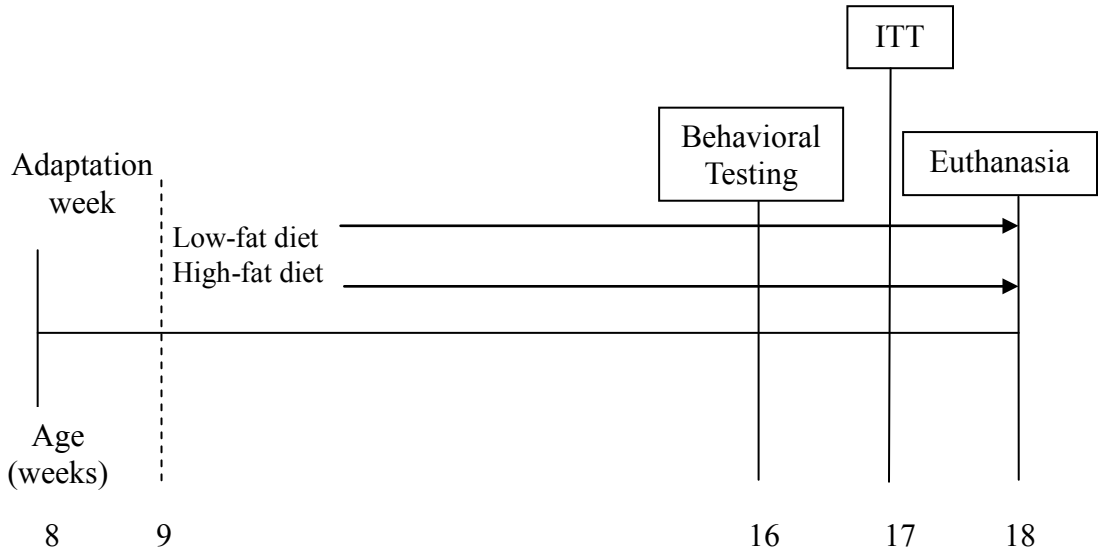


Figure 2. Timeline of Experiment

Timeline. An illustration of the study's timeline is shown in Figure 2. The duration of this particular study was 18 weeks. The Harlan WT mice arrived at 8 weeks of age and were allowed to acclimate for 1 week. The MC5R KO mice were age matched appropriately with the Harlan mice. At 9 weeks of age, all mice were placed on their respective diets for 9 weeks. Behavioral Testing, which will be analyzed in a future study, was performed on all mice at 16 weeks. Insulin Tolerance Testing (ITT) was performed on each animal at 17 weeks of age. The mice were given 1 week to recover, then were humanly sacrificed by euthanasia followed by decapitation. Multiple tissues were ex-

tracted; tissues analyzed include liver, skeletal muscle, omental (white) adipose, and brown adipose.

Assays

Body Weight Gain and Food Consumption Determination. Body weight gain and food intake were determined for each animal twice a week for the duration of the study. Body weight gain was determined by taking the final weight (to the nearest 0.1g) and subtracting the initial weight. Food consumption was determined by subtracting the weight of food left on the grid and the weight of spilled food from the initial weight of food supplied (to the nearest 0.1g). Water consumption was not recorded.

Behavior Testing. Mice were tested for behavioral differences at 16 weeks of age. Tail suspension was used to determine depressive-like behavior in the mice. To perform the test, the mice were suspended by their tails for 10 minutes and were recorded. The behavior analysis data will be used in a future study.

Insulin Tolerance Test (ITT) Analysis. Mice were fed their respective diet ad libitum during the testing. They were injected (i.p.) with 0.75m U/g body wt human insulin (Lilly, Indianapolis, Indiana). Blood samples were drawn from the tail vein at different time points: before insulin injection (0 min), 15 min, 30 min, 45 min, and 60 min after injection. Plasma was extracted and combined with a glucose reagent. Glucose concentrations

were quantified by measuring the absorbency of the plasma-glucose solution. Values were then calculated as a percentage of the initial time point (0 min) value.

Tissue Sample Collection. Following anesthetization and termination by cervical dislocation, the following tissues were removed: liver, pancreas, brain, ears, preputial glands, kidneys, heart, skeletal muscle, inguinal adipose tissue, perigonadal adipose tissue, retroperitoneal adipose tissue, omental adipose tissue, BAT, spleen, testes, stomach, which was cut open and washed in PBS to remove food particles, and colon, which was rinsed through with PBS to remove digestive remnants. All tissues were immediately weighed, placed into liquid nitrogen, and stored at -80 degrees Celsius in foil packets.

RT-PCR. RNA was extracted (see Appendix B) from liver, skeletal muscle, omental (white) adipose, and BAT. RT-PCR was performed on these samples. Qualitative PCRs for various genes was carried out in a Mastercycler Thermal Cycler (Eppendorf AG, Hamburg, Germany). Two microlitres of the RT reaction was amplified in a total volume of 20 ml containing 50% REDTaq Ready Mix Reaction Buffer (Sigma Aldrich; St. Louis), two complimentary primers at a concentration of 10 nmol each and sterile water. The primer sequences were created and ordered from Integrated DNA Technologies (Coraville, Iowa). The PCR for all targets consisted of similar temperatures and time lengths, with varying number of cycles for amplification. PCR that was performed consists of initial denaturation at 95 C for 50 s, and a cycle of: denaturation at 94 C for 20s,

annealing at 60 C for 20s, and elongation at 72 C for 30-60s. Table 1 shows a list of primers used in RT-PCR for each tissue analyzed.

Table 1. List of Primers Used in RT-PCR on Various Tissues

Tissue	Skeletal Muscle	Liver	Omental Fat	Brown Fat
Primers	MCAD LCAD PPARα AKT1 PFK-1 AMPKa	MCAD LCAD PPARα AKT1 PFK-1 FGF-21 HSL DGAT2	MCAD LCAD PPARγ AKT1 SREBP1c	MCAD LCAD PPARγ AKT1 SREBP1c FGF-21

Statistical Analyses. WT and KO mice were fed either a LFD or a HFD. This resulted in the following treatment combinations: WT-LF (n = 5), KO-LF (n = 4), WT-HF (n = 5), and KO-HF (n = 4). Two-way analysis of variance (ANOVA) was used to determine the effects of HFD treatment. The body weight gain and food intake measurements and the ITT analysis represent a split-plot design. For post-test analysis, the Bonferroni t-test was used for multiple comparisons of means. Post hoc data were analyzed with Graph Pad Prism 4.0 (San Diego, CA) and SigmaStat 2.0 statistical software (SPSS; Chicago, IL). Differences with p-values less than 0.05 were deemed statistically significant. Results are presented as mean ± SEM.

RESULTS

Tissue Weights

MC5R plays a role in metabolism, and as a result, tissue weights of WT and MC5R KO mice are variable (Table 2).

Table 2. Animal Tissue Weights (mg)

Tissues	WT-LF	KO-LF	WT-HF	KO-HF
Liver (abs)	1479 ± 258	1118 ± 36*	1633 ± 246	1275 ± 31*††
Liver (norm)	48 ± 2.3	45 ± 1.4	42 ± 3.1	36 ± 1.4††
Pancreas (abs)	157 ± 22	149 ± 15	193 ± 38††	218 ± 31*††
Pancreas (norm)	5.1 ± 0.2	6 ± 0.6	5 ± 0.5	6.1 ± 0.9
BAT (abs)	154 ± 55	113 ± 21	360 ± 122††	252 ± 60†
BAT (norm)	4.9 ± 0.6	4.5 ± 0.8	9.2 ± 1.3††	6.9 ± 1.4
Preputial Glands (abs)	108 ± 21	87 ± 25	129 ± 11	89 ± 10**
Preputial Glands (norm)	3.5 ± 0.3	3.5± 0.5	3.3 ± 0.1	2.5 ± 0.3*
Kidney (abs)	410 ± 49	323 ± 9*	428 ± 25	418 ± 24†
Kidney (norm)	13.4 ± 0.4	12.9 ± 0.3	11 ± 0.4††	11.8 ± 0.7
Skeletal Muscle (abs)	1071 ± 459	1055 ± 20	1302 ± 85	1346 ± 9†
Skeletal Muscle (norm)	36.5 ± 8	42.1 ± 1	33.6 ± 1.4	38.1 ± 3.3
Inguinal Fat (abs)	420 ± 362	289 ± 71	1530 ± 663†	1226 ± 395†
Inguinal Fat (norm)	13 ± 4.6	11.5 ± 2.8	39 ± 7.8†	33.1 ± 8.8
Perigonadal Fat (abs)	630 ± 239	395 ± 38	2217 ± 422††	1945 ± 199††
Perigonadal Fat (norm)	20.1 ± 2.6	15.8 ± 1.5	56.9 ± 5††	54.3 ± 2.5††
Retroperitoneal Fat (abs)	230 ± 163	127 ± 48	828 ± 226††	741 ± 161††
Retroperitoneal Fat (norm)	7.1 ± 2	5.1 ± 2	21.2 ± 2.6††	23.6 ± 2.9††
Omental Fat (abs)	201 ± 198	114 ± 23	919 ± 242††	521 ± 141*†
Omental Fat (norm)	6.1 ± 2.6	4.5 ± 0.9	23.6 ± 2.9††	14.2 ± 3†

Genotype : * $P < 0.05$; ** $P < 0.01$ Diet : † $P < 0.05$; †† $P < 0.01$
Abs = mg tissue, Norm = mg tissue/kg body weight
Means ± SEM are shown

Post hoc analysis using Bonferroni's t-test indicated many significant differences in tissue weights; absolute and normalized to body weight (mg/kg).For example, the size of the livers varied with genotype and diet. On both diet groups, the average weight of the KO liver was statistically significantly less than the WT liver. Interestingly, the KO liver did increase in size when the animal was placed on high-fat diet, but the WT liver did not. In skeletal muscle, there was no genotypic effect, but there was a significant dietary effect in the KO mice. On high-fat diet, KO skeletal muscle was larger than low-fat diet, while WT skeletal muscle was not altered by high-fat diet. Lastly, all of the adipose tissue regions displayed statistically significant genotypic and dietary differences. While both genotypes on high-fat diet showed an increase in adipose tissue weight, the majority of WT high-fat diet adipose tissue areas had more statistical differences than KO high-fat, when compared to their low-fat diet counterparts. The perigonadal adipose region was the only fat region in which KO and WT mice exhibited similar statistical differences. These data suggest that not only didMC5R depletion in mice cause the liver to reduce in size; the deletion caused the liver and skeletal muscle size to be affected by diet. Only KO liver and skeletal muscle increased in weight when given high-fat diet, suggesting the two tissues were not burning fat but storing it. Furthermore, by measuring various adipose regions, it can be suggested that the mice on high-fat diet did become obese and that MC5R may play a role in the rate at which an animal deposits fatty acids in different adipose regions.

Body Weight and Food Intake Analysis

Body Weight Analysis. In relation to tissue weights, analysis of the final body weight gain and the body weight gain patterns of the four treatment groups showed significance differences. The average body weight gain from week to week is shown in Figure 3.

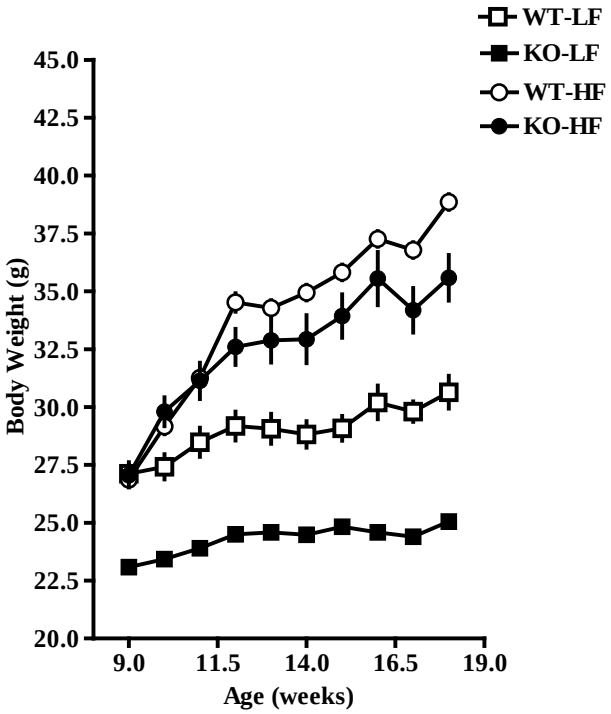


Figure 3. Body Weight. Findings: There was a genotypic effect, a dietary effect, and effect of time, a genotype by diet interaction, and a diet by time interaction. **Methods:** WT and MC5R KO animals (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Body weight was measured twice a week. **Statistics:** Means are shown (± SEM).

Various significant genotypic and dietary differences in body weight between the 4 treatment groups were evident. Three-way ANOVA indicated a genotypic effect ($F = 71.174$; $p < 0.001$), a dietary effect ($F = 350.907$; $p < 0.001$), an effect of time ($F = 9.256$; $p < 0.001$), a genotype by diet interaction ($F = 23.458$; $p < 0.001$), and a diet by time interaction ($F = 2.393$; $p = 0.015$), but no genotype by time interaction ($F = 0.469$; $p = 0.894$), or an interaction of the three factors ($F = 0.104$; $p = 1.0$). Although KO mice had an initial weight 4 grams less than WT, when fed the low-fat diet both WT and KO mice gained less than 2.5 grams, from initial weight, throughout the duration of the experiment. In contrast, WT mice on high-fat diet gained approximately 12 grams and KO mice on high-fat diet gained approximately 8.5 grams throughout the 9 weeks. On low-fat diet, both genotypes have similar weight gain, but when fed high-fat diet, WT mice exhibit a higher rate of weight gain than KO mice in the 9 week experiment. This suggests that the MC5R may assist in regulating metabolism when an animal if given excessive dietary fat.

In addition, there were various significant differences in body weight gain, as seen in (Figure 4) between the 4 treatment groups throughout the 9 week experiment.

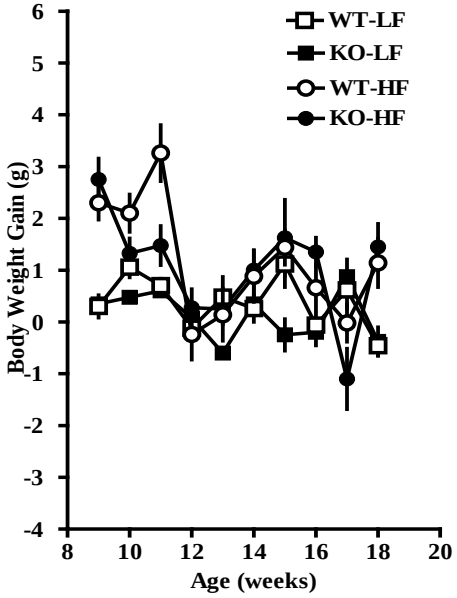


Figure 4. **Body Weight Gain. Findings:** There was a dietary effect, an effect of time, and a diet by time interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Body weight was measured twice a week. **Statistics:** Means are shown (± SEM).

Significant differences between treatment groups were seen in 8 of the 10 weeks (Table 3).

Table 3. Body Weight Gain (mg)				
Age	**WT/LF**	**KO/LF**	**WT/HF**	**KO/HF**
(weeks)				
9	300 ± 490	350 ± 160	2300 ± 730††	2750 ± 810††
10	1060 ± 450	470 ± 140	2100 ± 810†	1320 ± 580†
11	700 ± 220	600 ± 170	3260 ± 1210†	1470 ± 760
13	480 ± 870	-600 ± 170	140 ± 1130	250 ± 590†
15	1120 ± 1000	-250 ± 610	1440 ± 750	1620 ± 1470
16	-60 ± 500	-200 ± 510	660 ± 980	1350 ± 560††
17	620 ± 810	870 ± 670	-20 ± 810	-1100 ± 1170†
18	-460 ± 430	-370 ± 550	1140 ± 1050	1450 ± 890
Total	3960 ± 1080	1500 ± 430*	11000 ± 3300††	9050 ± 1200††
Diet : † P < 0.05; †† P < 0.01 Means ± SEM are shown				

Three-way ANOVA indicated a dietary effect ($F = 45.947$; $p < 0.001$), an effect of time ($F = 8.485$; $p < 0.001$), no genotypic effect ($F = 2.408$; $p = 0.123$), a diet by time interaction ($F = 6.596$; $p < 0.001$), but no genotype by diet interaction ($F = 0.290$; $p = 0.591$), genotype by time interaction ($F = 1.472$; $p = 0.164$), or an interaction of the three factors ($F = 1.673$; $p = 0.101$). All differences in body weight gain were seen between low-fat diet and high-fat diet groups of the same genotype. The majority of these differences are between the KO genotypes. The data suggests that MC5R may have an effect on body weight gain over time and again, on overall metabolic regulation. Additionally, the decline in weight gain seen from week 16 to 18 could be attributed to testing done on the mice. Week 16's behavioral testing and week 17's ITT may have negatively affected weight gain for both genotypes, with KO weight gain decreasing more severely than WT weight gain. The excess insulin in the mice's system may have harmfully affected KO food intake further than the WT food intake. This suggests that different stimuli affects KO mice, in both diet groups, more largely than WT mice.

Total body weight gain (Figure 5) not only varied as suspected between diets, WT and KO mice gained significantly different amounts of weight on low-fat diet.

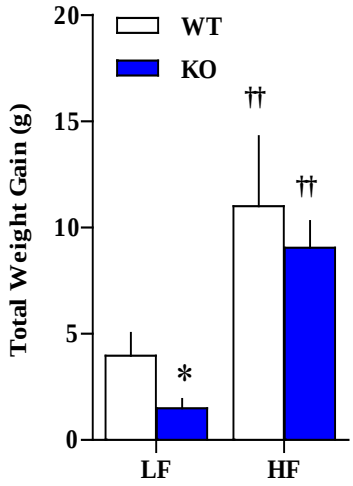


Figure 5. Total Body Weight Gain. Findings: There was a dietary effect. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Body weight was measured twice a week. **Statistics:** Means are shown (± SEM). For genotype: *p < 0.05. For diet: † p < 0.05; ††p < 0.01.

For overall weight gain, two-way ANOVA indicated a dietary effect ($F_{1,14}$= 42.769; p < 0.001), no effect on genotype x diet interaction ($F_{1,14}$ = 0.0523; p = 0.822), and a trend for a genotypic effect ($F_{1,14}$ = 3.907; p = 0.068). First, on low-fat diet, WT mice gained significantly more weight during the experiment than KO mice. Second, on high-fat diet, the average body weight gain was higher in WT mice than for KO mice, but there was no statistically significant difference. It is probable that having a greater sample size would have reduced the variance, and would have revealed a statistically significant difference in weight gain. Additionally, in both genotypes, weight gain increased approximately 700% on high-fat diet when compared to low-fat diet. These data suggest that, through excessive weight gain, both genotypes may have developed obesity.

When analyzing the data, contrasting trends were apparent from weeks 9-11 and weeks 12-18 of age, especially pertaining to high-fat diet. Figure 6 and Figure 7 show analyzed data from the two time periods where different trends became apparent.

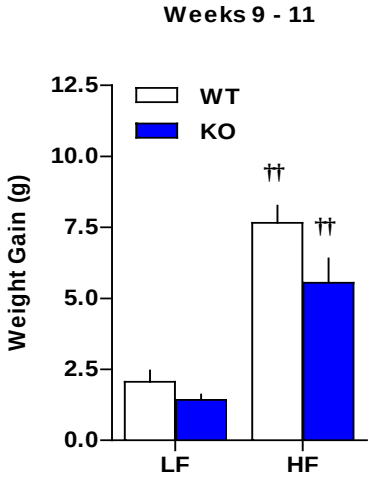


Figure 6. Total Body Weight Gain: Weeks 1-3. Findings: There was a genotypic effect and a dietary effect **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Body weight was measured twice a week. **Statistics:** Means are shown (± SEM). For diet: † $p < 0.05$; ††$p < 0.01$.

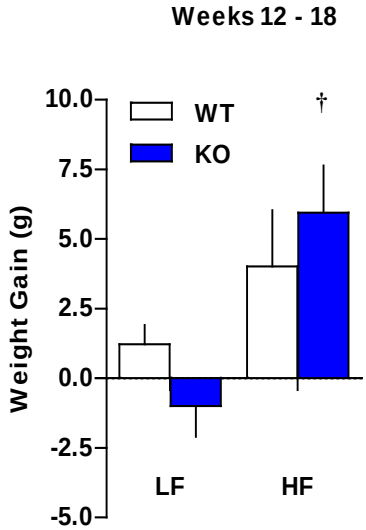


Figure 7. Total Body Weight Gain: Weeks 4-9. Findings: There was a dietary effect. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Body weight was measured twice a week. **Statistics:** Means are shown (± SEM). For diet: †$p < 0.05$.

When evaluating total body weight gain in weeks 9 through 11, two-way ANOVA indicated a genotypic effect ($F_{1,14}$= 5.931; p = 0.029), a dietary effect ($F_{1,14}$ = 74.447; p < 0.001), but no effect on genotype x diet interaction ($F_{1,14}$ = 1.173; p = 0.212). On both diets, there was a decreasing trend of KO mice weight gain when compared to WT mice. When compared to low-fat diet, both genotypes had a 400% higher weight gain on high-fat diet.

In contrast, Figure 7 shows differing trends compared to weeks 9-11.In regard to weeks 12 through 18, two-way ANOVA indicated no genotypic effect ($F_{1,14}$= 0.009; p = 0.924), no effect on genotype x diet interaction ($F_{1,14}$ = 1.927; p = 0.187), but there was a dietary effect ($F_{1,14}$ = 10.636; p = 0.006). Similar to previous weeks, KO mice on low-fat diet had tended to have a lower overall weight gain than WT mice on low-fat diet, with KO mice even experiencing a negative value in weight gain. In contrast to weeks 9-11, KO mice on high-fat diet tended to have a higher overall weight gain than WT mice on high-fat diet. Additionally, the weight gain between WT mice on low-fat diet compared to high-fat diet was not statistically significantly different; however, the difference in weight gain between the KO groups was deemed statistically significant. These data suggest that short-term, WT mice have a higher propensity for weight gain than KO mice, on either diet. In contrast, when placed on high-fat diet, long-term, KO mice may gain more weight than WT mice.

Food Intake Analysis. Average food intake of the four treatment groups, as seen in Figure 8, was analyzed over the 9 week experiment.

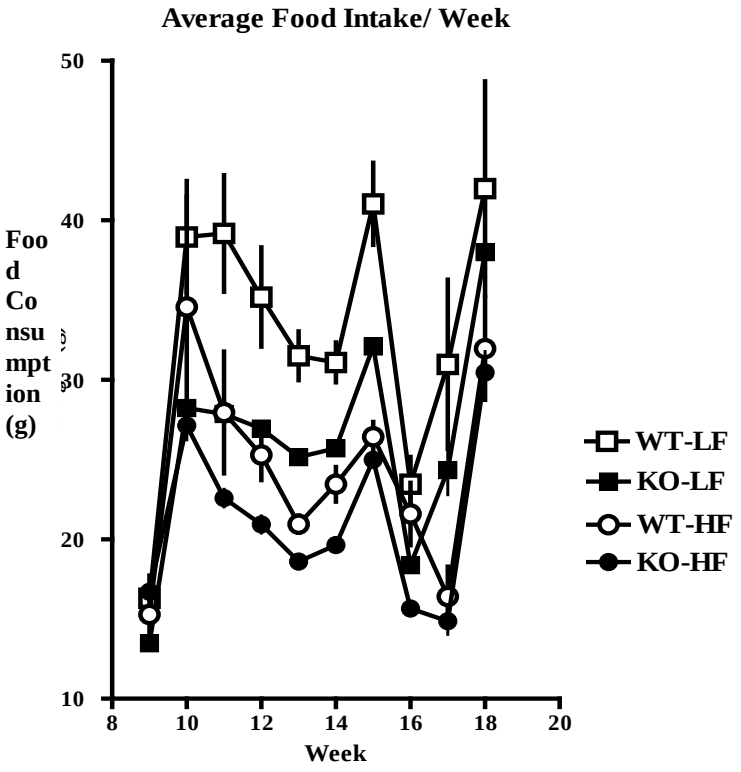


Figure 8. **Food Intake. Findings:** There was a genotypic effect, a dietary effect, an effect of time, and a genotype by diet interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM).

Three-way ANOVA indicated a genotypic effect ($F = 21.179$; $p < 0.001$), a dietary effect ($F = 43.859$; $p < 0.001$), an effect of time ($F = 14.488$; $p < 0.001$), and a genotype by diet interaction ($F = 4.003$; $p = 0.047$), but no genotype by time interaction ($F = 0.584$; $p = 0.809$), no diet by time interaction ($F = 1.374$; $p = 0.205$), and no interaction of the three factors ($F = 0.064$; $p = 1.00$). Food intake values were expected to be lower for high-fat diet, seeing as the food contains a higher caloric density. Interestingly,

on both diets, WT mice had higher food consumption than the KO mice. Table 4 illustrates significant differences at various weeks in the experiment. On a low-fat diet, KO mice consumed significantly less food than WT mice five out of the nine weeks of the experiment. While on high-fat diet, a similar difference was only seen at week twelve.

Table 4. Food Intake (mg/week)

Age (weeks)	WT/LF	KO/LF	WT/HF	KO/HF
10	38.94 ± 3.41	28.23 ± 1.11*	34.56 ± 6.69	27.13 ± 3.58
11	39.16 ± 3.55	27.85 ± 0.75*	27.94 ± 3.70	22.58 ± 2.24
12	35.18 ± 3.02	26.93 ± 1.49	25.28 ± 1.53	20.93 ± 2.17*
13	31.50 ± 1.48	25.15 ± 0.64*	20.94 ± 0.48††	18.60 ± 1.55††
14	31.08 ± 1.21	25.70 ± 1.17*	23.44 ± 1.06††	19.63 ± 1.63†
15	41.02 ± 2.49	32.08 ± 1.32*	26.42 ± 0.90††	24.98 ±1.32††
16	23.44 ± 1.66	18.38 ± 1.03	16.40 ± 1.83†	14.85 ± 3.29
17	30.96 ± 5.15	24.33 ± 6.14	16.40 ± 1.83†	14.85 ± 3.29
Total	329.56 ± 43.3	260.1 ± 7.03*	238.84 ± 25.12†	211.48 ± 15.18†

Genotype : * P < 0.05 Diet : † P < 0.05; †† P < 0.01
Means ± SEM are shown

Total food intake was calculated for each treatment group (Figure 9) and there were significant differences between dietary and genotypic groups.

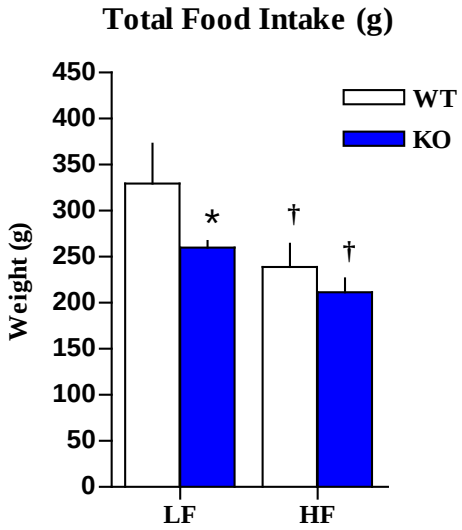


Figure 9. **Total Food Intake. Findings:** There was a dietary effect. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Food intake was measured twice a week. **Statistics:** Means are shown (± SEM). For genotype: *$p < 0.05$. For diet: † $p < 0.05$.

For total food intake, two-way ANOVA indicated no genotypic effect ($F_{1,14}$= 2.73; p = 0.1209), no effect on genotype x diet interaction ($F_{1,14}$ = 0.52; p = 0.4846), but there was a dietary effect ($F_{1,14}$ = 5.65; p = 0.0323). Compared to WT on low-fat diet, KO on low-fat diet had a 30% lower food intake. Additionally, both genotypes experienced a significant decrease in total grams of food intake when on high-fat diet. Under normal conditions on a low-fat diet, KO mice consumed less food than WT mice. When fed a high-fat diet, there was no difference between WT and KO mice, but both genotypes experienced a decrease in food intake compared to low-fat diet.

Caloric Intake. Caloric intake was calculated by multiplying the food intake by kcal per gram of diet (Figure 10).

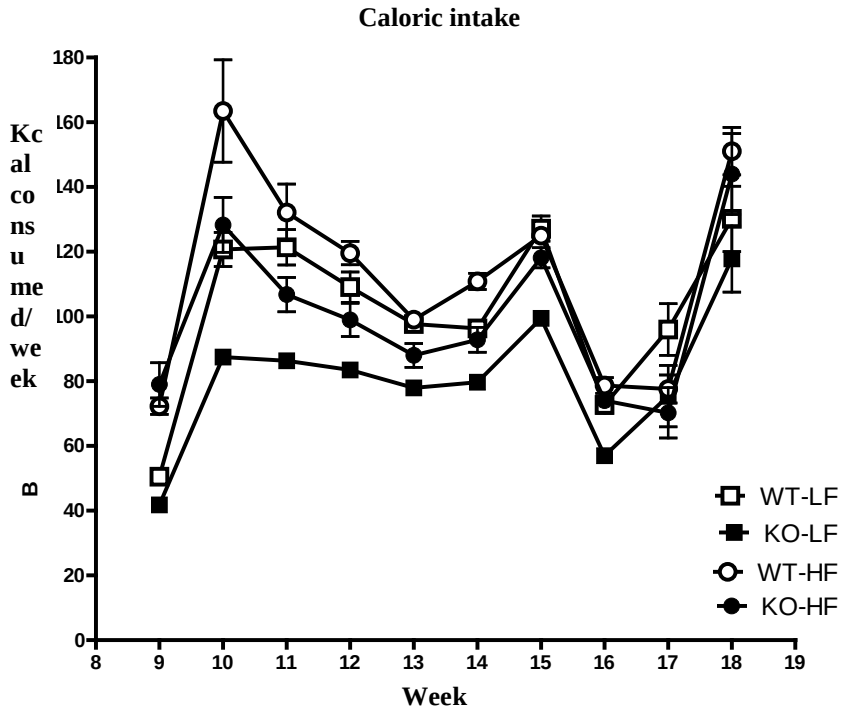


Figure 10. Caloric Intake. Findings: There was a genotypic effect, a dietary effect, an effect of time. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Food intake was measured twice a week. **Statistics:** Means are shown (± SEM).

When analyzing caloric intake data, three-way ANOVA indicated a genotypic effect ($F = 17.973$; $p < 0.001$), a dietary effect ($F = 13.795$; $p < 0.001$), an effect of time ($F = 14.127$; $p < 0.001$), but no genotype by diet interaction ($F = 41.115$; $p = 0.293$), genotype by time interaction ($F = 0.587$; $p = 0.806$), diet by time interaction ($F = 1.255$; $p = 0.267$), and no interaction of the three factors ($F = 0.078$; $p = 1.00$). On either diet, the MC5R KO mice did not appear to have as high a caloric intake as WT mice. Differ-

ences between genotypes on the same diet were evident until week 15. The data suggests that MC5R and/or body size caused the KO mice to consume fewer calories than WT mice. Considering overlapping began to occur after week 15, we can speculate that if the experiment were lengthened, we might see the KO mice begin to consume similar or more calories than WT mice. Table 5 displays the genotypic and dietary significant differences which appeared in 7 of the 9 weeks of the experiment.

Table 5. Caloric Intake (kcal/week)

Age (weeks)	WT/LF	KO/LF	WT/HF	KO/HF
9	50.53 ± 4.77	41.77 ± 3.36	72.27 ± 5.65†	78.99 ± 13.47†
10	120.71 ± 11.82	87.5 ± 3.45*	163.47 ± 35.4	128.3 ± 16.92
11	121.4 ± 12.29	86.34 ± 2.34*	132.16 ± 19.59	106.78 ± 10.6
13	109.06 ± 10.45	83.47 ± 4.62	119.57 ± 8.1	98.98 ± 10.26
14	97.65 ± 5.15	77.97 ± 1.2*	99.04 ± 2.56	87.98 ± 7.34
15	96.35 ± 4.21	79.67 ± 3.64*	110.87 ± 5.6	92.83 ± 7.69
16	127.16 ± 8.63	99.44 ± 4.1*	124.97 ± 4.73	118.12 ± 6.24†
Total	1021.64 ± 72.75	806.31 ± 22.52*	1129.71 ± 64.4	1000.28 ± 74.16†

Genotype : * $P < 0.05$ Diet : † $P < 0.05$
Means ± SEM are shown

Total caloric intake (Figure 11) was then analyzed over the course of the 9 week experiment.

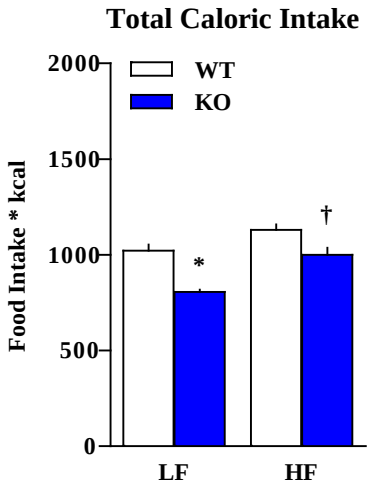


Figure 11. Total Caloric Intake. Findings: There was a dietary effect and a genotypic effect. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Food intake was measured twice a week. **Statistics:** Means are shown (± SEM). For genotype: *p < 0.05. For diet: † p < 0.05.

For total caloric intake, two-way ANOVA indicated a genotypic effect ($F_{1,14}$= 6.63; p = 0.022), a dietary effect ($F_{1,14}$ = 5.089; p = 0.041), but no genotype by diet interaction ($F_{1,14}$ = 0.411; p = 0.532). On low-fat diet, KO mice had a 20% lower caloric intake than WT mice, while there was no difference between genotypes on high-fat diet. Additionally, there was a 20% increase in caloric intake from KO mice on low-fat diet to KO mice on high-fat diet, but WT caloric intake was not altered by diet. This suggests that caloric intake levels are affected by MC5R, on low-fat diet and on high-fat diet.

Feed Efficiency and Caloric Efficiency. Feed efficiency was calculated by taking the average weight gain divided by the average food intake. In Figure 12, feed efficiency is represented in grams**.**

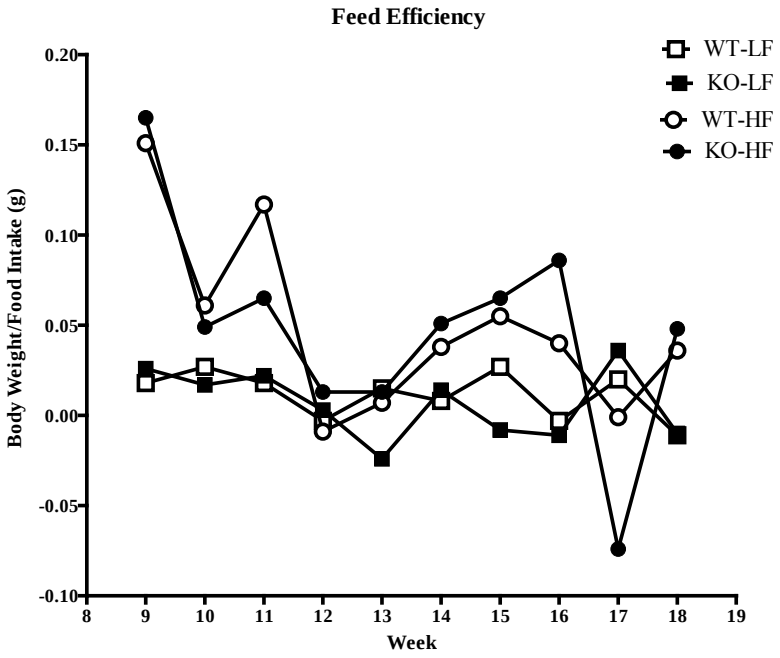


Figure 12. **Feed Efficiency (grams). Findings:** There was a dietary effect, an effect of time, a diet by time interaction, and an interaction of the three factors. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Feed efficiency is calculated by body weight gain (g)/food intake (g). **Statistics:** Means are shown (± SEM).

When analyzing feed efficiency over the 18 week experiment, three-way ANOVA indicated dietary effect ($F = 37.283$; $p < 0.001$), an effect of time ($F = 9.679$; $p < 0.001$), a diet by time interaction ($F = 4.003$; $p = 0.047$), and an interaction of the three factors ($F = 2.091$; $p = 0.034$) . There was no genotypic effect ($F = 0.566$; $p = 0.453$), no genotype by diet interaction ($F = 0.036$; $p = 0.850$), and no genotype by time interaction ($F = 1.366$; $p = 0.209$). Again, there appears to be a decline in feed efficiency from week 16 to 18. This could be attributed to behavioral and/or ITT performed on the mice, with KO high-fat feed efficiency being most severely affected.

To normalize the feed efficiency results, caloric efficiency was calculated. The feed efficiency data was then calculated by taking body weight divided by food intake, then multiplying the outcome by the calories provided by 1 gram of food (Figure 13).

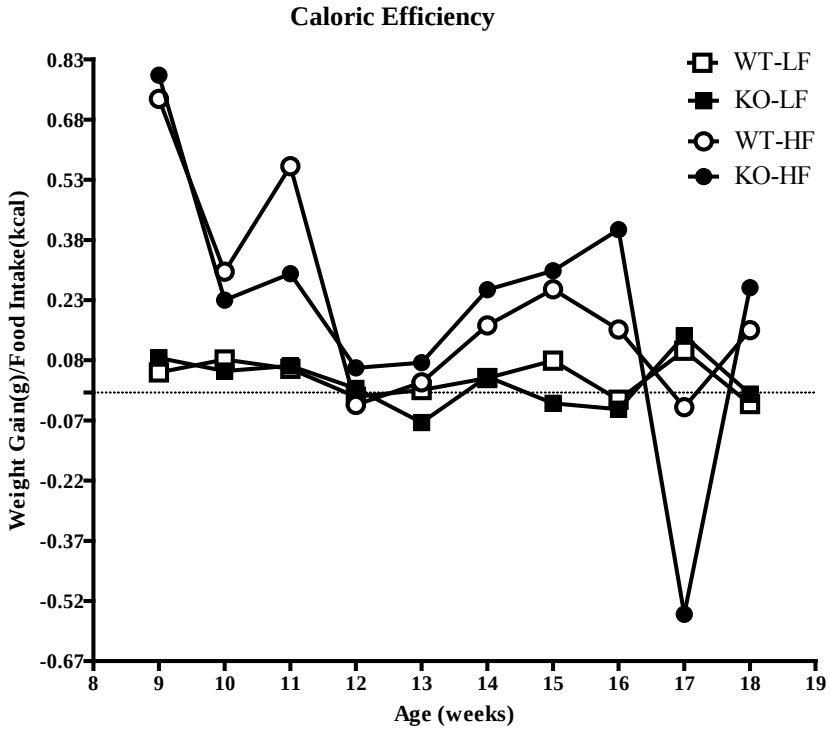


Figure 13. Caloric Efficiency (kcals). Findings: There was a dietary effect, an effect of time, a diet by time interaction, and a diet, genotype, and time interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Feed efficiency is calculated by (body weight gain (g)/food intake (g)) * kcal**. Statistics:** Means are shown (± SEM).

Three-way ANOVA indicated dietary effect ($F = 49.75$; $p < 0.001$), an effect of time ($F = 10.62$; $p < 0.001$), a diet by time interaction ($F = 10.919$; $p < 0.001$), and an interaction of the three factors ($F = 1.968$; $p = 0.047$) . There was no genotypic effect ($F = 0.256$; $p = 0.614$), no genotype by diet interaction ($F = 0.01$; $p = 0.919$), and no geno-

type by time interaction (F = 1.527 ; p = 0.144). Similar outcomes to the feed efficiency measured in grams occurred.

Significant difference in feed efficiency between the treatment groups were seen 8 out of the 9 weeks (Table 6). Genotypic differences were only displayed in the low-fat diet mice.

Table 6. Feed Efficiency

Age (weeks)	WT/LF	KO/LF	WT/HF	KO/HF
9	0.02 ± 0.01	0.03 ± 0.008	0.15 ± 0.03††	0.17 ± 0.02††
10	0.03 ± 0.00	0.02 ± 0.003	0.06 ± 0.01†	0.05 ± 0.01†
11	0.02 ± 0.00	0.02 ± 0.003	0.12 ± 0.02††	0.06 ± 0.02†
13	0.02 ± 0.01	-0.02 ± 0.004*	0.01 ± 0.03	0.02 ± 0.02
15	0.03 ± 0.01	-0.01 ± 0.01*	0.05 ± 0.01	0.06 ± 0.03
16	-0.01 ± 0.01	-0.01 ± 0.02	0.03 ± 0.03	0.09 ± 0.02††
17	0.03 ± 0.02	0.05 ± 0.027	-0.01 ± 0.02	-0.12 ± 0.07
18	-0.01 ± 0.00	-0.001 ± 0.01	0.03 ± 0.02††	0.06 ± 0.02††
Total	0.13 ± 0.09	0.07 ±0.09	0.48 ± 0.20††	0.44 ± 0.24††

Genotype : * P < 0.05 Diet : † P < 0.05; †† P < 0.01
Means ± SEM are shown

Total feed efficiency was calculated in grams for the entirety of the experiment. There was indication of only significant differences between dietary groups.

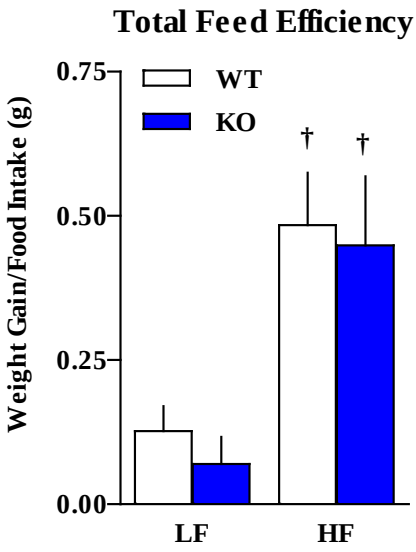


Figure 14. **Total Feed Efficiency. Findings:** There was a dietary effect **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. Feed efficiency is calculated by body weight gain (g)/food intake (g). **Statistics:** Means are shown (± SEM). For diet: † $p < 0.05$.

Analysis of the total feed efficiency (Figure 14) calculated in grams by two-way ANOVA indicated no genotypic effect ($F_{1,14}$= 0.33; p = 0.5754), no effect on genotype x diet interaction ($F_{1,14}$ = 0.02; p = 0.8929), but there was a dietary effect ($F_{1,14}$ = 21.05; p = 0.0004). Overall, KO mice had a lower feed efficiency average than WT mice on both diets, but there were no statistically significant differences between the KO and WT mice. On high-fat diet, WT mice had a 400% higher feed efficiency than low-fat diet and KO mice had a 640% higher feed efficiency than low-fat diet. Although these data should be interpreted cautiously because there are two statistically significant differences and one intriguing trend, these data suggest that WT mice had a higher feed efficiency than KO mice and high-fat diet intake increases feed efficiency in both genotypes.

Behavioral Changes

Behavioral data were collected. The data, however, were not included because behavioral study is out of the scope of energy balance.

ITT Analysis

Insulin Tolerance Testing is an indicator of mice's sensitivity and/or resistance to insulin. Each animal was subjected to an ITT analysis at 17 weeks of age and Figure 15 displays the results below.

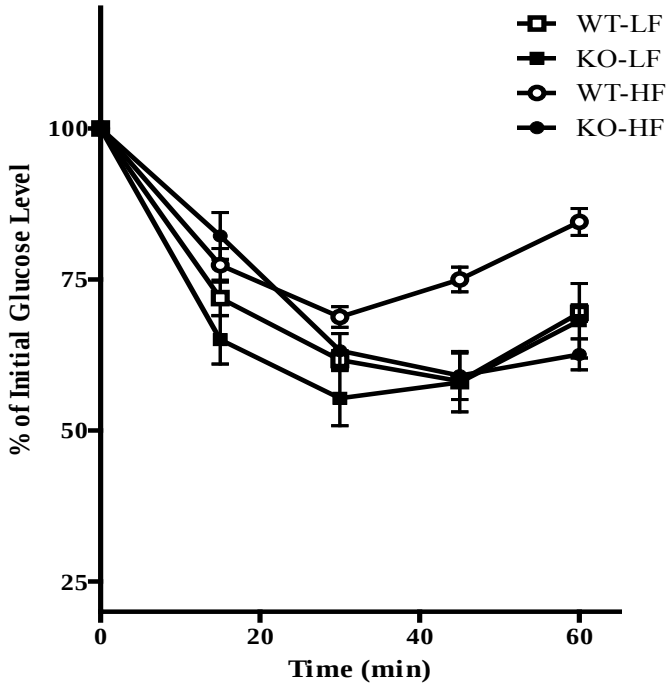


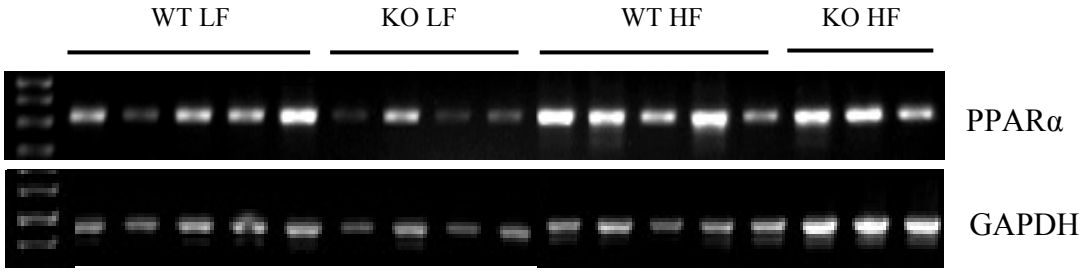
Figure 15. ITT Analysis. Findings: There was an effect of time. **Methods:** WT and MC5R KO mice (n = 4-5) injected with insulin (1U/kg) and tail vein blood was extracted at 0, 15, 30, 45, and 60 min. Glucose absorbency was measured using a glucose reagent. **Statistics:** Means are shown (± SEM).

Three-way ANOVA indicated an effect of time ($F = 21.828$; $p = 0.006$), a trend towards a dietary effect ($F = 4.85$; $p = 0.092$), but no genotypic effect ($F = 3.25$; $p = 0.146$). To normalize the data, the amount of glucose present was calculated as a percentage of the initial glucose level. The higher the percentage of glucose present in the blood suggests a decline in glucose absorbency, signifying that the body has become less sensitive to insulin. The lower the percentage of glucose present, the higher the glucose permeability, denoting an elevation in insulin sensitivity. During the ITT, both low-fat diet groups maintained similar levels of insulin sensitivity. The KO low-fat diet treatment group had a sharper initial decline in average glucose levels than the WT low-fat group, suggesting the KO mice reacted to the increase in insulin more rapidly. The low-fat KO group also experienced lower average glucose levels up until the fourth time point. Overall, it appears that on low-fat diet, KO mice may have been more prone to having higher insulin sensitivity than WT mice. In contrast, on high-fat diet, both genotypes experienced similar reactions to the increase in insulin up to the second time point. Throughout the time course, high-fat WT mice showed a significant decrease in insulin sensitivity, as was expected. Oppositely, high-fat diet KO mice showed maintenance of insulin sensitivity. KO mice on high-fat diet displayed similar glucose levels as the groups on low-fat diet, with the average glucose level at the ending time point being less than both low-fat genotypes. These findings suggest a major role of MC5R in insulin sensitivity development. When MC5R is present, obese mice develop insulin resistance, but when MC5R is deleted out of the mice' genome, obese mice maintain insulin sensitivity, and even may experience an increase in sensitivity if fed a high-fat diet long term.

RT-PCR Analysis

Various gene expressions, involved in lipid metabolism, glucose metabolism, and insulin sensitivity, were analyzed using RT-PCR.

Effects of High-fat Diet on PPARα mRNA Levels in Skeletal Muscle



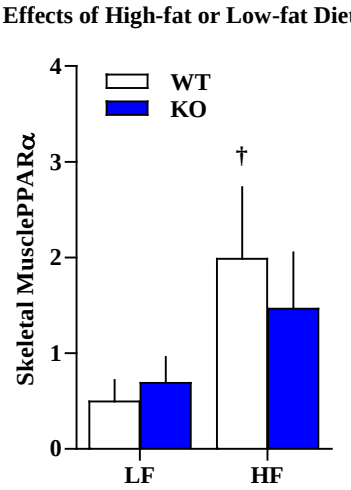
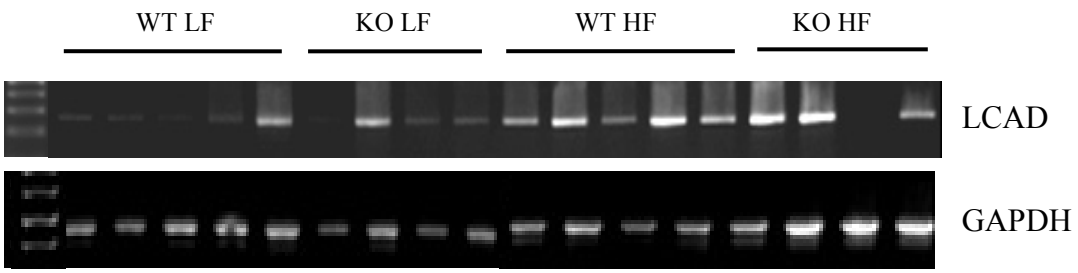


Figure 16. **Effects of HFD on PPARα mRNA Levels in Skeletal Muscle. Findings:** There was an effect of diet and a genotypic effect. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For diet: † $p < 0.05$.

Two-way ANOVA indicated a genotypic effect ($F_{1,15}$= 6.459; p = 0.02) and a dietary effect ($F_{1,15}$ = 7.12; p = 0.015), but no effect on genotype x diet interaction ($F_{1,15}$ = 1.51; p = 0.234). *Post Hoc analysis* using Bonferroni's t-test indicated that, compared to WT on low-fat diet, WT on high-fat diet had a 215% increase in PPARα mRNA levels. KO mice also experienced a 2-fold increase in PPARα mRNA levels, but there was no statistically significant difference. Although KO PPARα mRNA skeletal muscle levels tended to decrease on both diets, no significant differences were apparent, and the high-fat diet group showed a trend toward a higher degree of difference than the low-fat diet (t = 1.979; p = 0.083) (Figure 16). Although these results should be interpreted cautiously, because there was one significant difference and two intriguing trends for dietary and genotypic differences that did not reach statistical significance, these data collectively suggest that MC5R and high-fat diet intake function as mediators of fatty acid oxidation in skeletal muscle, as seen by PPARα levels (1).

Effects of High-fat Diet on LCAD mRNA Levels in Skeletal Muscle



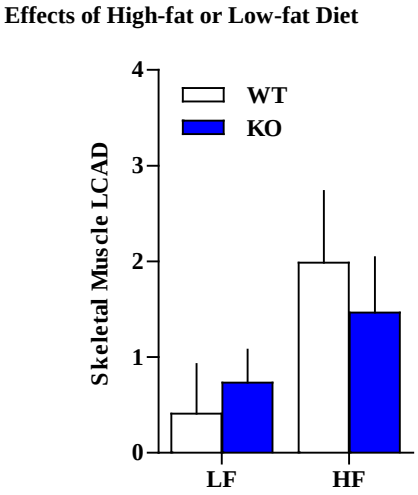
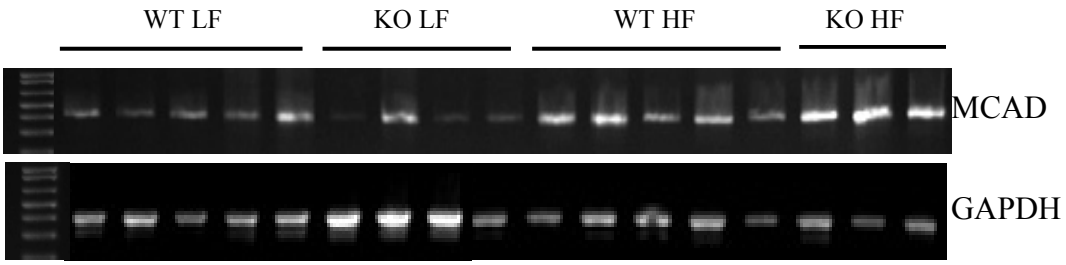


Figure 17. Effects of HFD on LCAD mRNA Levels in Skeletal Muscle. Findings: There was an effect of diet. Methods: WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks**. Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,15}$= 0.283; p = 0.602), there was a dietary effect($F_{1,15}$ = 13.501; p = 0.002), but no effect on genotype x diet interaction ($F_{1,15}$=1.346; p = 0.264). *Post Hoc* analysis using Bonferroni's t-test indicated that WT mice exhibited 400% higher LCAD mRNA levels in skeletal muscle on high-fat diet in contrast to low-fat diet. Similarly, relative to the low-fat diet, LCAD mRNA le-

vels tended to increase 2.5-fold in the KO mice on the high-fat diet, but this trend was not statistically significant (t = 1.748; p = 0.131) (Figure 17). These data mutually propose that long chain fatty acid oxidation in skeletal muscle, as demonstrated by LCAD expression, increases more drastically in WT mice relative to MC5R KO mice on high-fat diet (60).

Effects of High-fat Diet on MCAD mRNA Levels in Skeletal Muscle



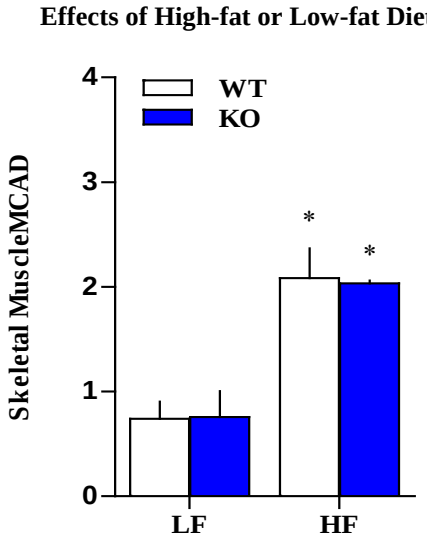


Figure 18. **Effects of HFD on MCAD mRNA Levels in Skeletal Muscle. Findings:** There was an effect of diet. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For genotype: *p < 0.05.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,15}$ = 0.00; p = 0.945), there was a dietary effect ($F_{1,15}$=31.55; p<0.001), but no effect on genotype x diet interaction ($F_{1,15}$ = 0.02; p = 0.885). *Post Hoc* analysis using Bonferroni's t-test specified that relative to low-fat diet, both WT and KO genotypes exhibited 250% higher skeletal muscle MCAD mRNA levels on high-fat diet (Figure 18). These data collectively suggest that medium chain fatty acid oxidation, as measured my MCAD expression, is stimulated by in skeletal muscle by high-fat diet, independent of genotype (60).

Effects of High-fat Diet on Akt1 mRNA Levels in Skeletal Muscle

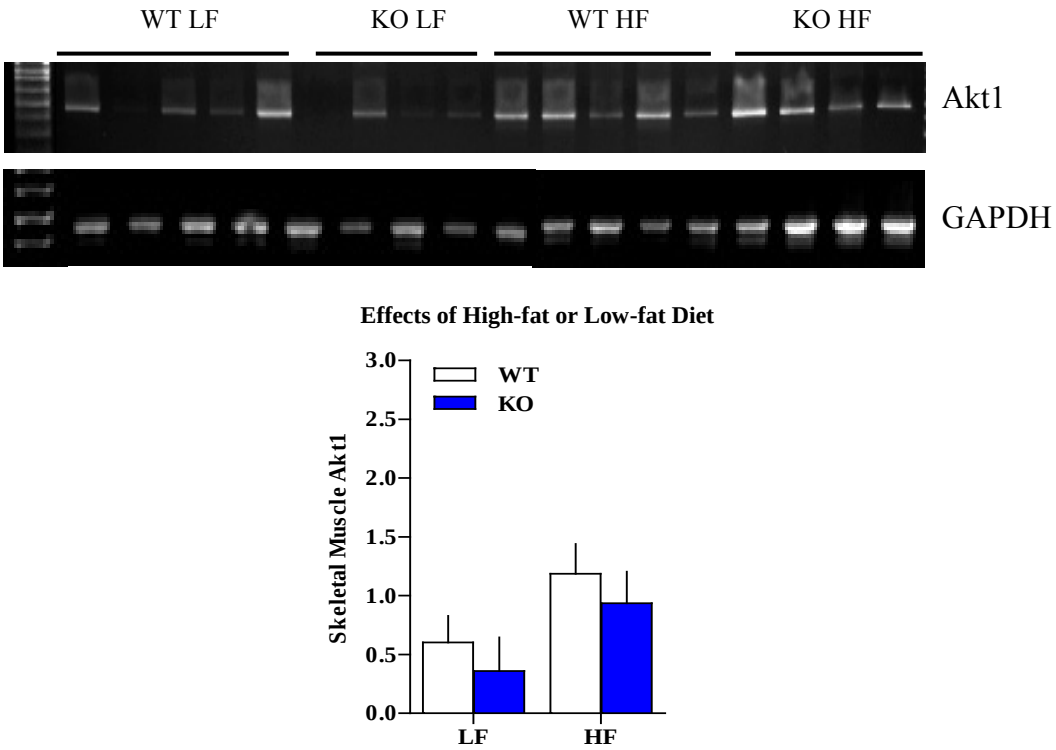
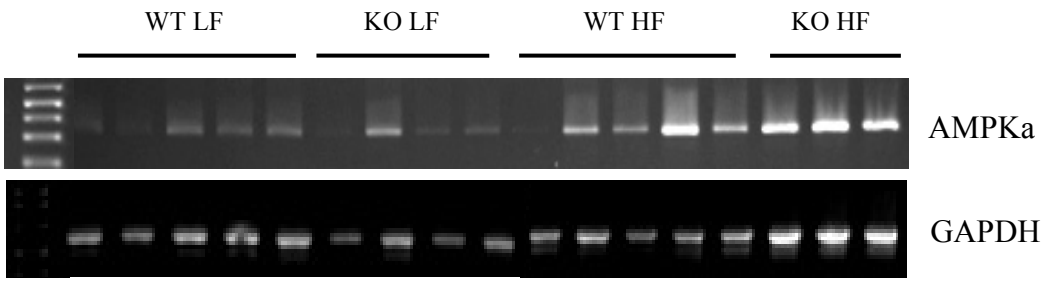


Figure 19. **Effects of HFD on Akt1 mRNA Levels in Skeletal Muscle. Findings:** There was an effect of diet. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,14}$= 1.745; p = 0.208), there was a dietary effect ($F_{1,14}$ = 7.49; p = 0.016), but no effect on genotype x diet interaction ($F_{1,14}$ = 0.043; p = 0.839). *Post Hoc* analysis using Bonferroni's t-test indicated that, although Akt1 mRNA levels tended to increase in both WT and MC5R KO mice in skeletal muscle, there were no statistically significant differences. Both genotypic trends were nearly deemed statistically significant, with the KO mice having a greater dissimilarity: WT (t = -1.869; p = 0.094) and KO (t = -2.304; p = 0.069). It is plausible that having a greater sample size would have reduced the variance, and would have revealed a statistically significant difference (Figure 19). Although these results should be interpreted cautiously, because there were two intriguing trends for dietary differences that did not reach statistical significance, these data collectively suggest that the trend in elevation of Akt1, which inhibits apoptosis and plays a role in insulin sensitivity, in skeletal muscle demonstrates a positive correlation between high-fat diet and cell growth (10, 11).

Effects of High-fat Diet on AMPKa mRNA Levels in Skeletal Muscle



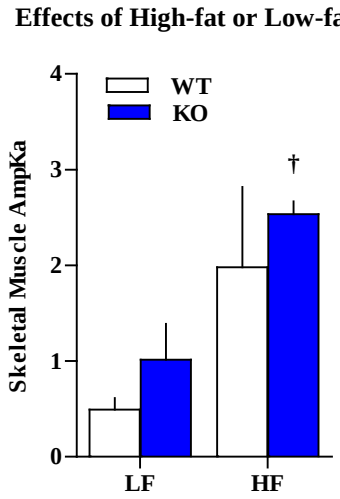


Figure 20. **Effects of HFD on AMPKa mRNA Levels in Skeletal Muscle. Findings:** There was an effect of diet. **Methods:** WT and MC5R KO mice(n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For diet: † $p < 0.05$.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,15}$= 1.11; p = 0.309), there was a dietary effect ($F_{1,15}$ = 8.63; p = 0.010), but no effect on genotype x diet interaction ($F_{1,15}$ = 0.00; p = 0.974). *Post Hoc* analysis using Bonferroni's t-test indicated that relative to WT and KO mice on low-fat diet, there was an increasing trend in AMPKa mRNA levels in skeletal muscle on high-fat diet. There was 250% higher AMPKa

mRNA levels in MC5R KO mice and comparably, AMPKa mRNA levels tended to increase 4-fold on the high-fat diet in the WT mice, but this trend was not statically significant (t = -1.932; p = 0.085) (Figure 20). These data cooperatively infer a role for MC5R in the regulation of an enzyme, AMPKa, which is a central control point for energy homeostasis, primarily fatty acid oxidation and glucose oxidation, in the cell (4, 70).

Effects of High-fat Diet on PFK-1 mRNA Levels in Skeletal Muscle

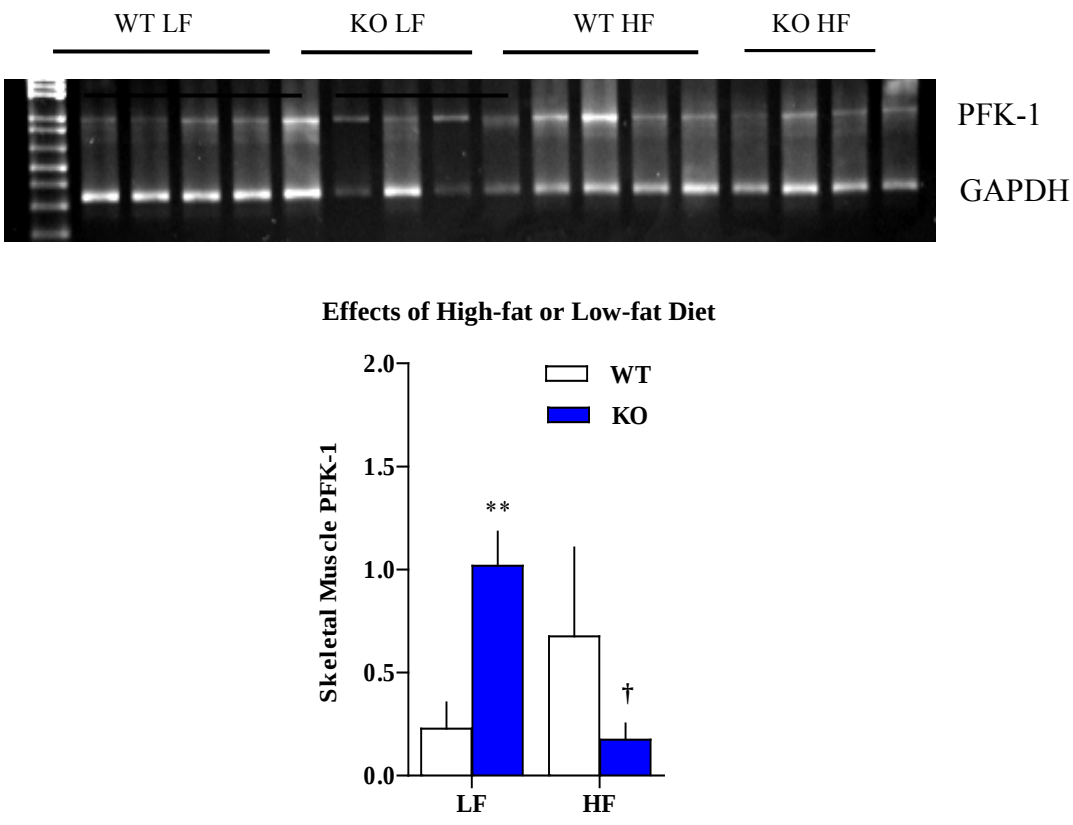
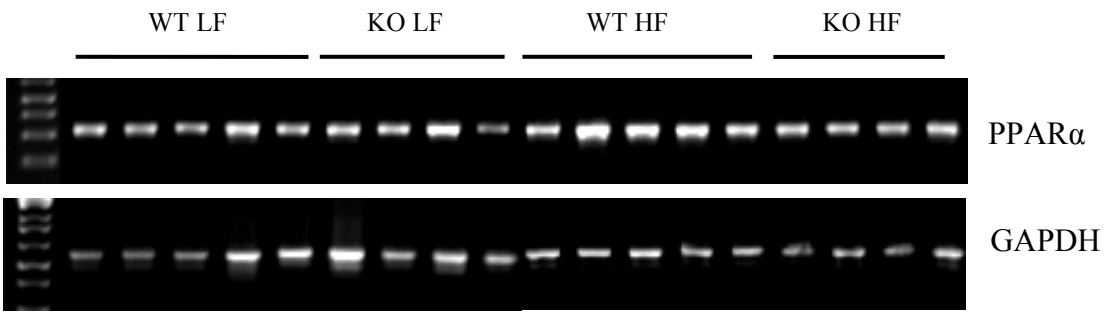


Figure 21. **Effects of HFD on PFK-1 mRNA Levels in Skeletal Muscle. Findings:** There genotype by diet interaction**. Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For genotype: **p < 0.01. For diet: † p < 0.05.

Two-way ANOVA indicated that there was no genotypic effect ($F_{1,15}$= 1.703; p = 0.213), no dietary effect ($F_{1,15}$ = 2.945; p = 0.108), but there was a genotype x diet interaction ($F_{1,15}$ = 12.603; p = 0.003). *Post Hoc* analysis using Bonferroni's t-test indicated that relative to WT mice,MC5R KO mice exhibited 450% higher skeletal muscle PFK-1 mRNA levels on the low-fat diet. By contrast, MC5R deficiency tended to reduce PFK-1 mRNA levels 400% on the high-fat diet, but this trend was not statistically significant (t = 1.49; p = 0.186). It is plausible that having a greater sample size would have reduced the variance, and would have revealed a statistically significant difference. Additionally, PFK-1 mRNA levels tended to increase 3-fold on the high-fat diet in the WT mice, when compared to KO high-fat diet levels, but this trend was not statistically significant (t = 1.389; p = 0.198). By contrast, PFK-1 mRNA levels were reduced by high-fat diet in the KO mice (Figure 21). Although these results should be interpreted cautiously, because there were two significant differences and two intriguing trends for genotypic and dietary differences that did not reach statistical significance, these data suggest a major role for MC5R in the regulation of a rate-limiting enzyme, PFK-1, for glycolysis in the skeletal muscle (72).

Effects of High-fat Diet on PPARα mRNA Levels in Liver



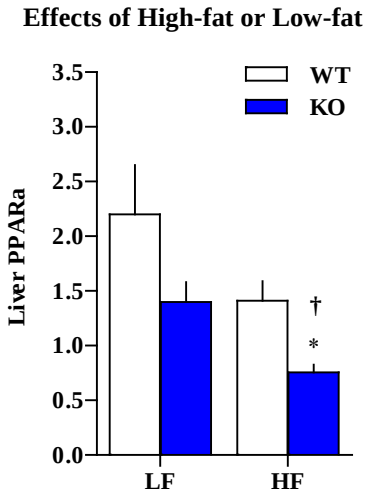
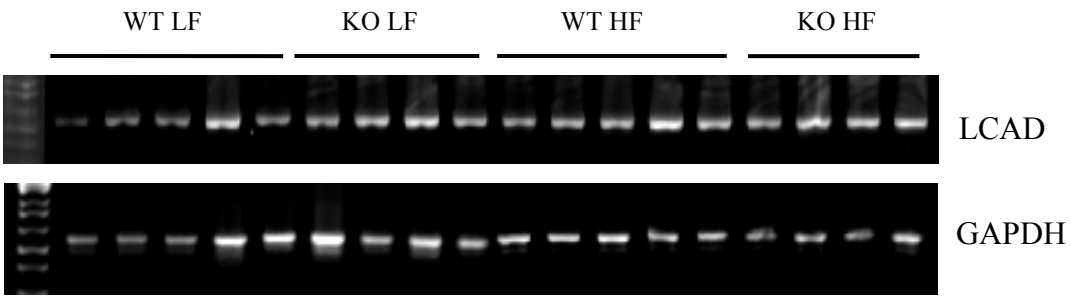


Figure 22. **Effects of HFD on PPARα mRNA Levels in Liver. Findings:** There was an effect of genotype, and an effect of diet. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For genotype: *$p < 0.05$. For diet: †$p < 0.05$.

Two-way ANOVA indicated a genotypic effect ($F_{1, 14} = 6.433$; $p = 0.024$), a dietary effect ($F_{1,14} = 6.210$; $p = 0.026$, but no genotype x diet interaction ($F_{1,14} = 0.068$; $p = 0.798$). *Post Hoc* analysis using Bonferroni's t-test indicated that, on high-fat diet, there was a 2-fold decrease in KO PPARα mRNA levels in liver compared to WT mice. On low-fat diet, KO mRNA levels similarly trended downward, but there was no statistically significant difference ($t = 1.503$; $p = 0.177$). Within genotypes, both WT and KO PPARα levels decreased on high-fat diet, but only KO mice showed a statistically significant difference, decreasing by 200%. Having a greater sample size might have reduced the variance, revealing a statistically significant difference where trends are apparent (Figure 22**)**. Although these outcomes should be interpreted carefully, because there are two significant differences and two intriguing trends for dietary and genotypic that did not reach statistically significant differences, these data collectively suggest that MC5R has a function in regulating PPARα, a receptor critically involved in controlling FAO in liver (32).

Effects of High-fat Diet on LCAD mRNA Levels in Liver



Effects of High-fat or Low-fat Diet

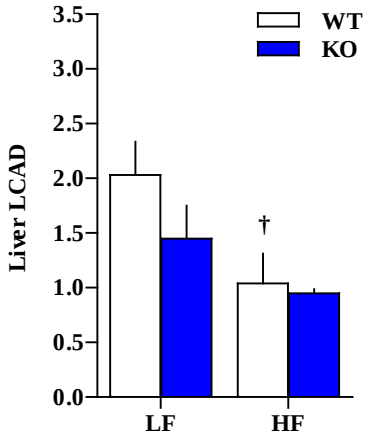


Figure 23. **Effects of HFD on LCAD mRNA Levels in Liver. Findings:** There was an effect of diet. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For diet: † $p < 0.05$.

Two-way ANOVA indicated no genotypic effect ($F_{1,16}$= 1.781; p = 0.201), no genotype x diet interaction ($F_{1,16}$ = 0.959; p = 0.342), but there was a dietary effect ($F_{1,16}$ = 8.751; p = 0.009). *Post Hoc* analysis using Bonferroni's t-test indicated that, when compared to low-fat diet, high-fat diet WT mice LCAD mRNA levels decreased 100% in liver. Although there also appears to be a decreasing trend in KO mice on high-fat diet, there was no statistical difference (Figure 23). These data collectively suggest that, contrasting to

its relative MCAD, LCAD, which is a marker for long chain fatty acid oxidation, may be regulated further by fat intake, rather than MC5R (60).

Effects of High-fat Diet on MCAD mRNA Levels in Liver

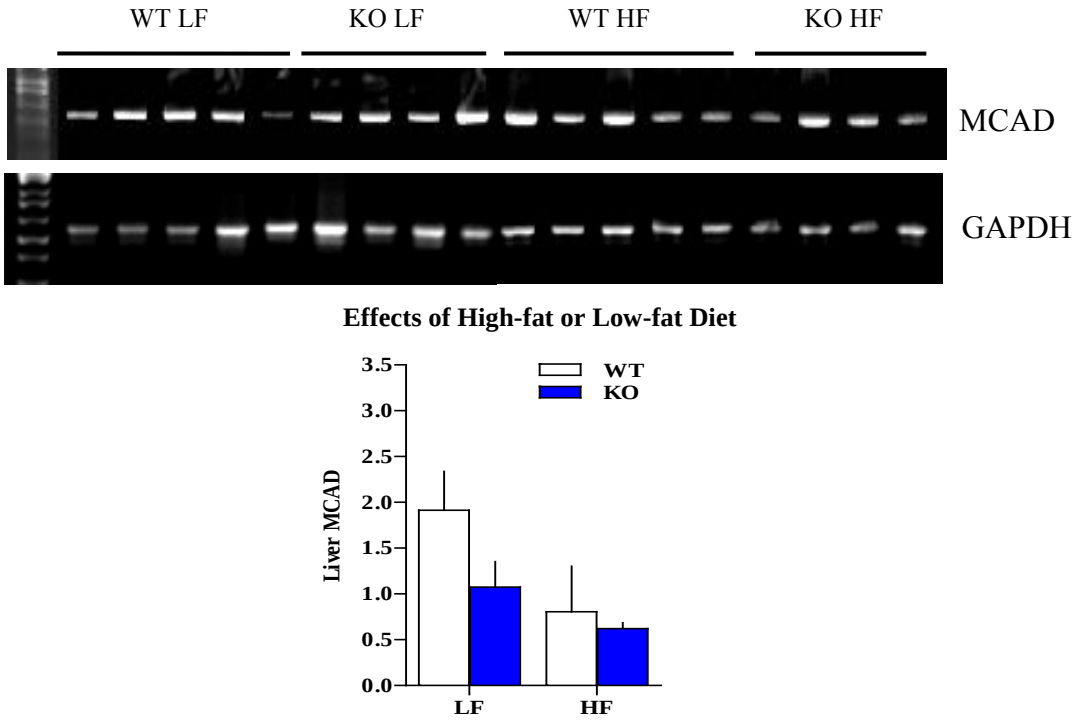
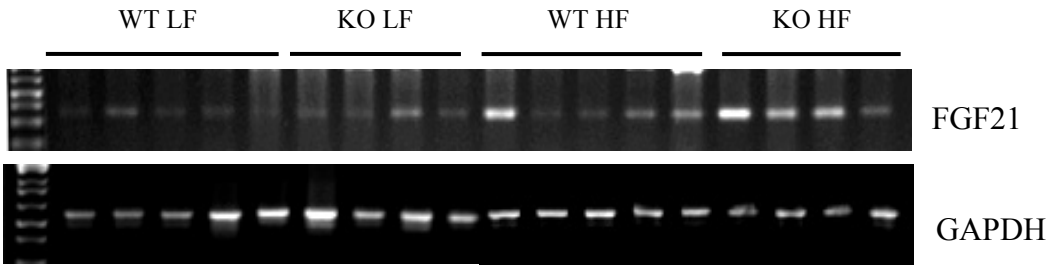


Figure 24. **Effects of HFD on MCAD mRNA Levels in Liver. Findings:** There was an effect of diet. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks**. Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR.

Two - way ANOVA indicated no genotypic effect ($F_{1,16}$= 2.499; p = 0.133), no genotype x diet interaction ($F_{1,16}$ = 1.028; p = 0.326), but there was a dietary effect ($F_{1,16}$ = 5.834; p = 0.028). Although *Post Hoc* analysis using Bonferroni's t-test indicated that MCAD mRNA levels tended to decrease in both WT and MC5R KO mice on high-fat diet in liver, there were no statistically significant differences, the highest difference in the WT (t = 2.142; p = 0.061). Additionally, the KO mice had reduced MCAD mRNA levels on

both diets, relative to WT mice, but there were no statistically significant differences. It is plausible that having a greater sample size would have reduced the variance, and would have revealed a statistically significant difference (Figure 24). Although these results should be interpreted cautiously, because there were two intriguing trends for genotypic and dietary differences that did not reach statistical significance, these data collectively suggest that both fat intake and MC5R may play a small role in the regulation of a marker of fatty acid oxidation of medium chain fatty acids, MCAD (60).

Effects of High-fat Diet on FGF-21 mRNA Levels in Liver



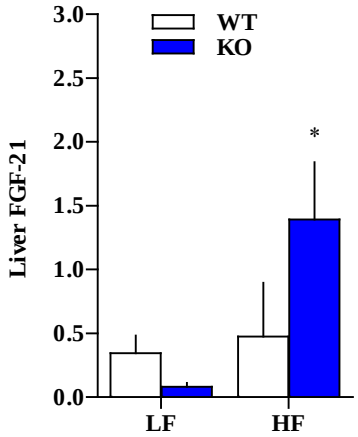


Figure 25. **Effects of HFD on FGF-21 mRNA Levels in Liver. Findings:** There was an effect of diet, and a genotype by diet interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For genotype: *p < 0.05.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,16}$= 2.178; p = 0.159), but there was a dietary effect ($F_{1,16}$ = 10.619; p = 0.005) and a genotype x diet interaction ($F_{1,16}$ = 7.108; p = 0.017). *Post Hoc* analysis using Bonferroni's t-test indicated that, relative to WT mice, Fgf-21 mRNA levels were reduced in the KO mice on low-fat diet, but this trend was not statistically significant (t = 1.169; p = 0.125). By contrast, FGF-21 mRNA levels in liver were 200% higher in KO mice on high-fat diet when compared to WT mice. Additionally, WT mice FGF-21 mRNA levels in liver were relatively unaltered when comparing diets, but, relative to low-fat diet, MC5R KO mice exhibited an 800% higher AKT1 mRNA level in liver on high-fat diet (Figure 25). Although these results should be interpreted cautiously, because there is one significant difference and one intriguing trends for genotypic and dietary differences that did not reach statistical significance, these data collectively suggest a putative role for MC5R in the regulation of a metabolic protein, FGF-21, critically involved in fatty acid oxidation and/or insulin sensitivity (71).

Effects of High-fat Diet on Akt1 mRNA Levels in Liver

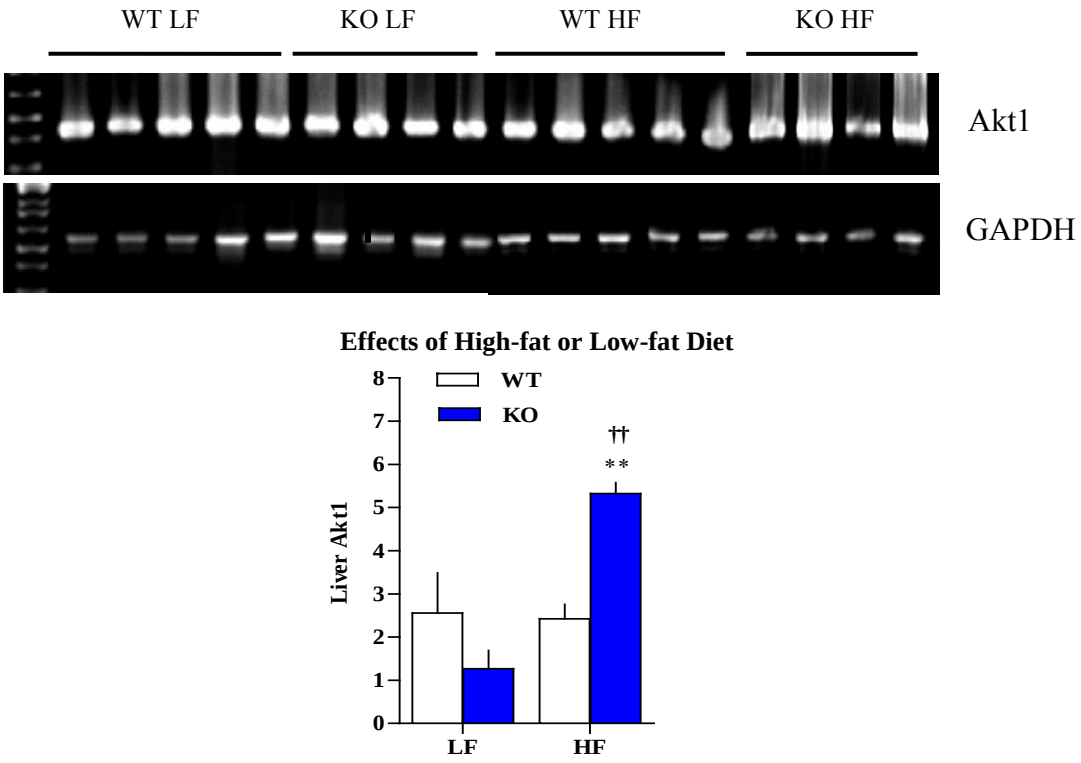


Figure 26. **Effects of HFD on Akt1 mRNA Levels in Liver. Findings:** There was an effect of diet and a genotype by diet interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For genotype: **p < 0.01. For diet: ††p < 0.01.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,16}$= 1.65; p = 0.217), but there was a dietary effect ($F_{1,16}$ = 9.83; p = 0.006) and a genotype x diet interaction ($F_{1,16}$ = 11.254; p = 0.004). *Post Hoc* analysis using Bonferroni's t-test indicated that, relative to WT mice, Akt1 mRNA levels were reduced in the KO mice on low-fat diet, but this trend was not statistically significant (t = 1.185; p = 0.267). By contrast, Akt1 mRNA levels in liver were 300% higher in KO mice on high-fat diet when compared to WT mice. Additionally, WT mice AKT1 mRNA levels in liver were unaltered when comparing diets, but, relative to low-fat diet, MC5R KO mice exhibited a 600% higher AKT1

mRNA level in liver on high-fat diet (Figure 26). There were two significant differences and one intriguing trend for genotypic and dietary differences that did not reach statistical significance. These data collectively suggest that MC5R may be a major regulator of Akt1, and glucose metabolism and insulin activity, in the liver (69).

Effects of High-fat Diet on PFK1 mRNA Levels in Liver

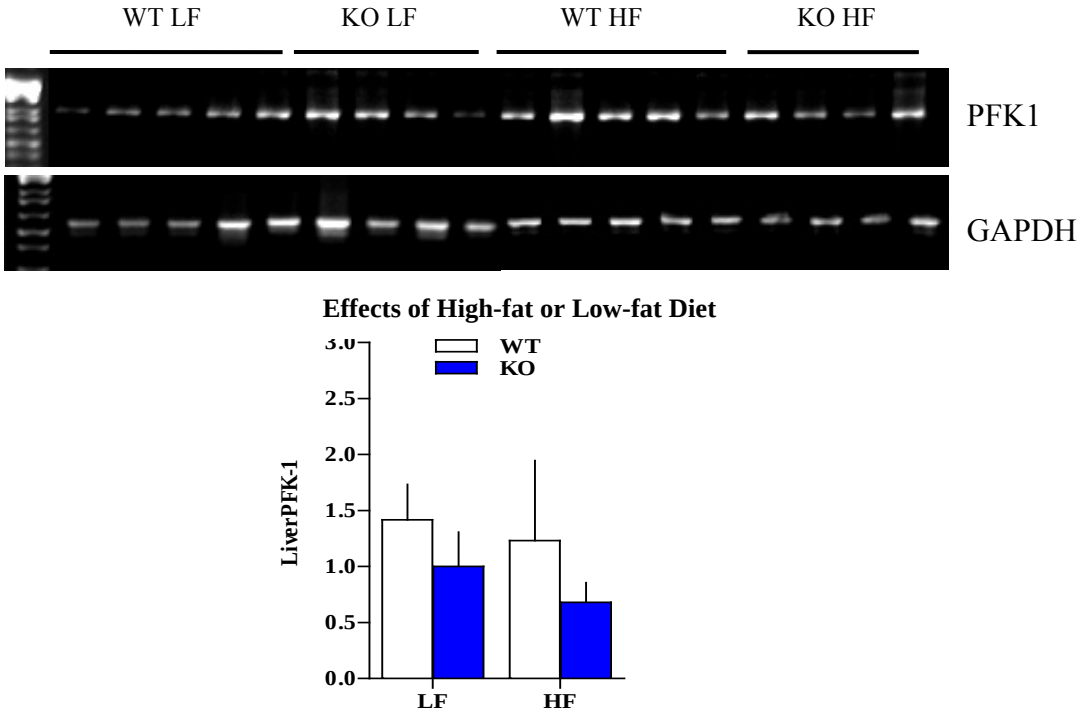


Figure 27. **Effects of HFD on PFK-1 mRNA Levels in Liver. Findings:** There were no dietary or genotypic effects. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR.

Two-way ANOVA indicated no genotypic effect ($F_{1,16}$= 2.394; p = 0.141), no dietary effect ($F_{1,16}$ = 0.661; p = 0.428), and no genotype x diet interaction ($F_{1,16}$ = 0.048; p = 0.830). *Post Hoc* analysis using Bonferroni's t-test indicated that, although PFK-1 mRNA levels in the liver tended to decrease in KO mice relative to WT mice, on low-fat

diet and high-fat diet, the trends were not statistically significant: low-fat diet ($t = 0.932$; $p = 0.376$) and high-fat diet ($t = 1.305$; $p = 0.233$). It is plausible that having a greater sample size would have reduced the variance, and would have revealed a statistically significant difference (Figure 27). Although these results should be interpreted cautiously, because there were two intriguing trends for genotypic differences that did not reach statistical significance, these data collectively suggest that MC5R may play a diminutive role in the regulation of a rate-limiting enzyme, PFK-1, for glycolysis in the liver (35).

Effects of High-fat Diet on HSL mRNA Levels in Liver

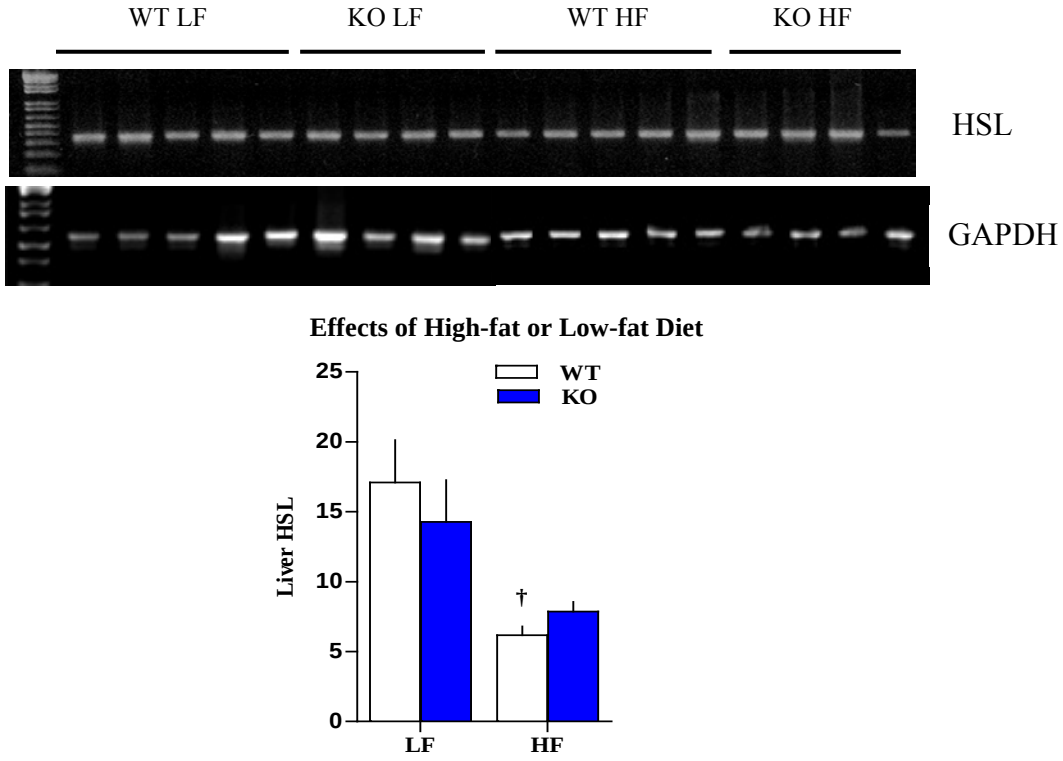
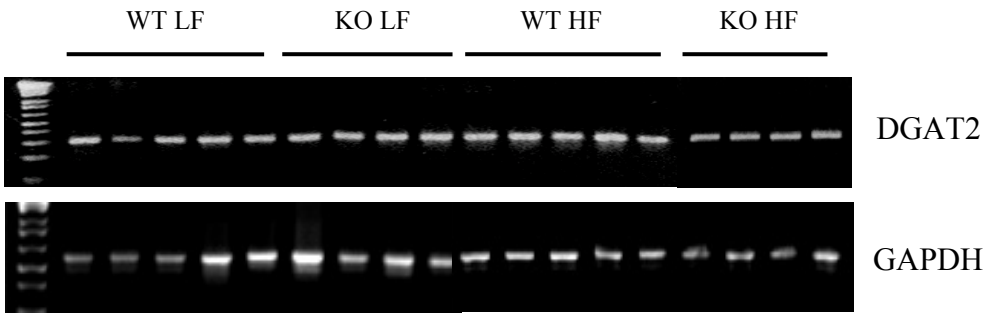


Figure 28. Effects of HFD on HSL mRNA Levels in Liver. Findings: There was an effect of diet. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For diet: † $p < 0.05$.

Two-way ANOVA indicated no genotypic effect ($F_{1,16}$= 0.053; p = 0.821), no genotype x diet interaction ($F_{1,16}$ = 0.844; p = 0.372), but there was a dietary effect ($F_{1,16}$ = 12.544; p = 0.003). *Post Hoc* analysis using Bonferroni's t-test indicated that while there were no differences between genotypes on low-fat diet or high-fat diet, high-fat diet administration did cause a decline in HSL levels in liver (Figure 28). On high-fat diet, WT mice experienced a 300% decrease in HSL mRNA levels when compared to WT on low-fat diet. Similarly, MC5R KO mice had a 200% decrease in HSL levels, but there was no statistical significance (t = 1.863; p = 0.105). Although this data should be interpreted carefully because there was one significant difference and one similar trend for genotypic and dietary differences that did not reach statistical significance, this data suggests that fat accumulation from excess dietary fat may regulate hormone sensitive lipase, or HSL, an enzyme responsible for breaking down fatty acids. MC5R does not appear to play a large role in controlling HSL (18).

Effects of High-fat Diet on DGAT2 mRNA Levels in Liver



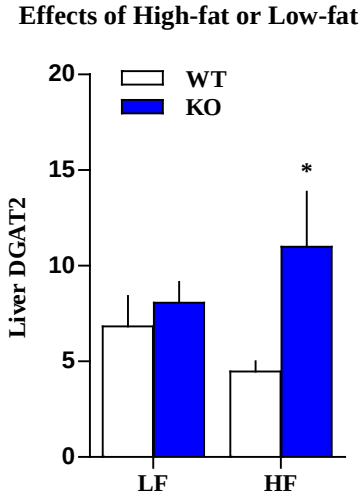
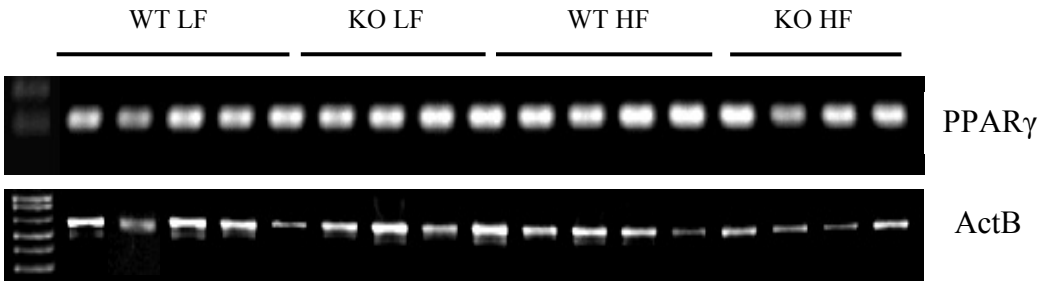


Figure 29. Effects of HFD on DGAT2 mRNA Levels in Liver. Findings: There was a genotypic effect. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For genotype: *p < 0.05.

Two-way ANOVA indicated a genotypic effect ($F_{1,16} = 5.772$; $p = 0.029$),but no dietary effect ($F_{1,16} = 0.031$; $p = 0.863$), and no genotype x diet interaction ($F_{1,16} = 2.678$; $p = 0.121$), *Post Hoc* analysis using Bonferroni's t-test indicated that on both diets, MC5R KO mice expressed higher mRNA levels of DGAT2 than WT mice (Figure 29). While no significance was shown between WT and KO low-fat diet levels, on high-fat diet, the KO mice had a 250 % higher level of DGAT2 mRNA expression than WT. Additionally, WT mice showed a decrease in DGAT2 mRNA levels from low-fat to high-fat diets, but this value was not statistically significant ($t = 21.00$; $p = 0.126$). In contrast to WT, KO mice tended to have an increase in level when administered high-fat diet. Although these effects should be translated with caution, because there was one significant difference and two intriguing trends for genotypic and dietary differences that did not reach statistical significance, these data collectively suggest that MC5R plays a role in regulating adipose storage, as seen by DGAT2 mRNA expression, in the liver (42).

Effects of High-fat Diet on PPARγ mRNA Levels in Omental Fat



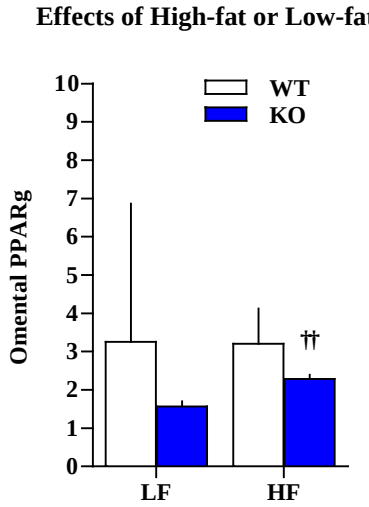


Figure 30. **Effects of HFD on PPARγ mRNA Levels in Omental Fat. Findings:** There was no effect of diet, genotype, or interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For diet: ††$p < 0.01$.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,13}$= 1.36; p = 0.265), no dietary effect ($F_{1,13}$ = 0.089; p = 0.770), and no genotype x diet interaction ($F_{1,13}$ = 0.118; p = 0.736). WT PPARγ mRNA levels appeared to be unaltered by diet, but KO mice levels were consistently lower than WT on both diets. Additionally, *Post Hoc* analysis using Bonferroni's t-test indicated that there was a statistically significant increase in PPARγ mRNA levels in KO mice on high-fat diet compared to low-fat diet (Figure 30).

These data collectively suggest that MC5R and dietary fat intake can alter PPARγ, which, when activated in adipose, serves as a stimulant of whole body insulin sensitivity and adipogenesis, and lipid storage (36, 52, 68).

Effects of High-fat Diet on LCAD mRNA Levels in Omental Fat

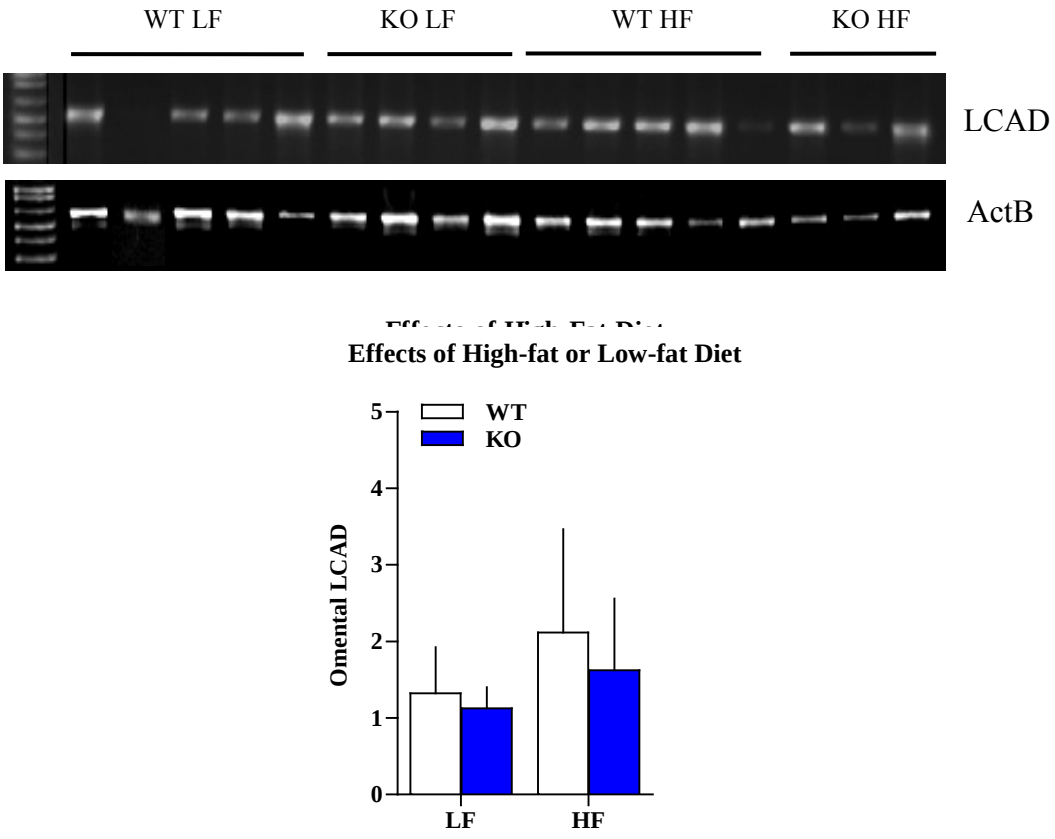
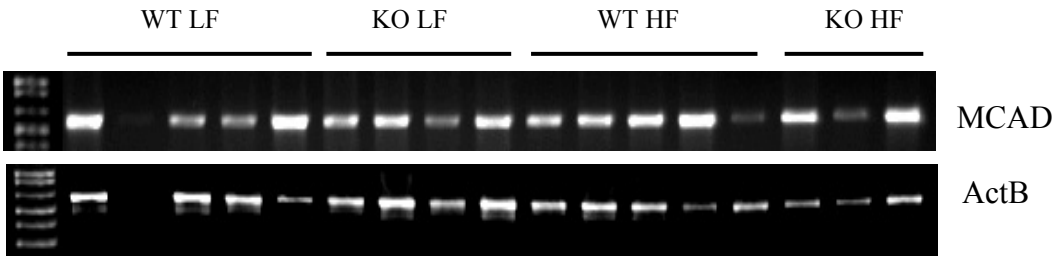


Figure 31. **Effects of HFD on LCAD mRNA Levels in Omental Fat. Findings:** There was no effect of diet, genotype, or interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,12}$= 0.413; p = 0.533), no dietary effect ($F_{1,12}$ = 1.128; p = 0.309), and no genotype x diet interaction ($F_{1,12}$ = 0.042; p = 0.841).). Although there were no statistical differences between WT and KO Akt1 mRNA levels on low-fat diet or high-fat diet, high-fat diet intake appeared to increase LCAD mRNA levels in both WT and KO mice (Figure 31). These data suggest that dietary fat intake, but not MC5R, may play a small role in regulating fatty acid oxidation by stimulating LCAD in adipose tissue (47).

Effects of High-fat Diet on MCAD mRNA Levels in Omental Fat



Effects of High-fat or Low-fat Diet

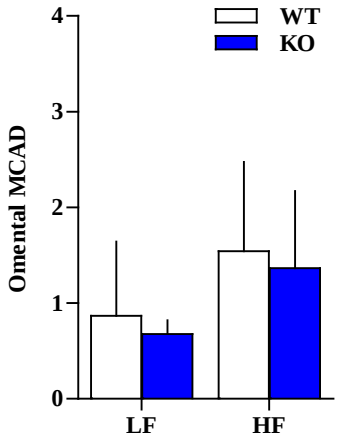
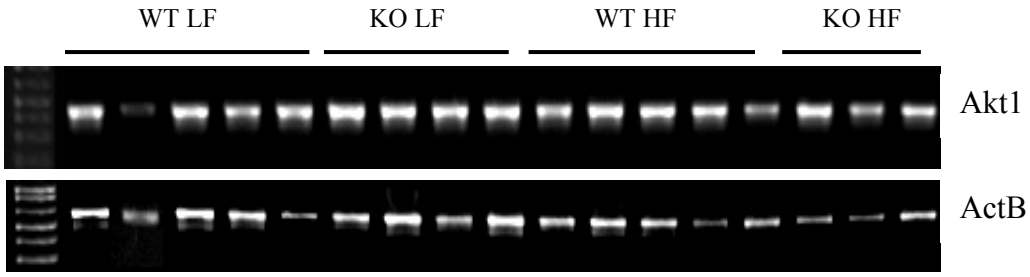


Figure 32. Effects of HFD on MCAD mRNA Levels in Omental Fat. Findings: There was no effect of diet, genotype, or interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,13}$= 0.208; p = 0.656), no dietary effect ($F_{1,13}$ = 2.858; p = 0.115), and no genotype x diet interaction ($F_{1,13}$ = 0.00; p = 0.985). High-fat diet intake appeared to increase MCAD mRNA levels in both WT and KO mice, but there were no statistical differences between WT and KO Akt1 mRNA levels on low-fat diet or high-fat diet (Figure 32). These data suggest that dietary fat intake, but not MC5R, may play a small role in regulating fatty acid oxidation by stimulating MCAD in adipose tissue (47).

Effects of High-fat Diet on Akt1 mRNA Levels in Omental Fat



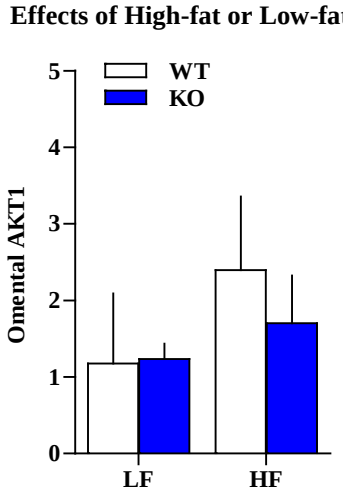


Figure 33. Effects of HFD on Akt1 mRNA Levels in Omental Fat. Findings: There was no effect of diet, genotype, or interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR.

Two - way ANOVA indicated there was no genotypic effect ($F_{1,13}$= 0.514; p = 0.486), a trend towards a dietary effect ($F_{1,13}$ = 3.792; p = 0.073), and no genotype x diet interaction ($F_{1,13}$ = 0.724; p = 0.410). There were no statistical differences between WT and KO Akt1 mRNA levels on low-fat diet and high-fat diet (Figure 33). These data suggest that Akt1, a gene involved in insulin activity, expression in adipose tissue is not affected by MC5R or dietary fat intake (31).

Effects of High-fat Diet on SREBP1c mRNA Levels in Omental Fat

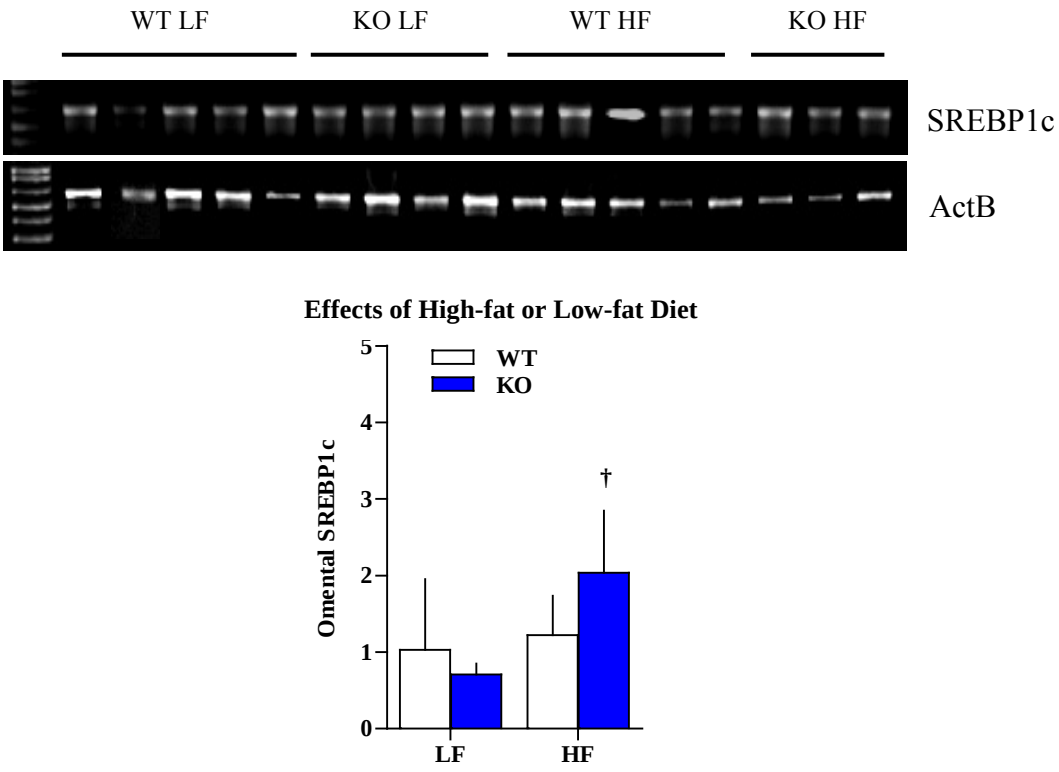
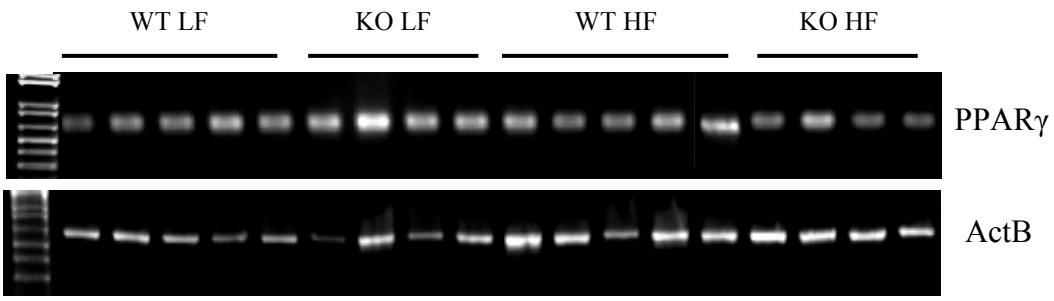


Figure 34. **Effects of HFD on SREBP1c mRNA Levels in Omental Fat. Findings:** There was no effect of diet, genotype, or interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For diet: † $p < 0.05$.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,13}$= 0.469; p = 0.505) and no genotype x diet interaction ($F_{1,13}$ = 2.45; p = 0.142), but there was a trend towards a dietary effect ($F_{1,13}$ = 4.413; p = 0.056). Although there were no statistically significant differences between WT and KO mice on either diet, on low-fat diet, KO mice SREBP1c mRNA levels tended to decrease while on high-fat diet, KO mice levels tended to increase, each compared to WT. Additionally, *Post Hoc* analysis using Bonferroni's t-test indicated that there was a 280% increase in KO SREBP1c mRNA levels on high-fat diet when compared to low-fat diet (Figure 34). Although these results should be interpreted cautiously, because there was one significant difference and one intriguing trend for genotypic and dietary differences that did not reach statistical significance, these data collectively suggest that MC5R may aid in regulating an enzyme, SREBP1c, which is stimulated by insulin and serves as the primary transcription factor for lipogenesis in adipose tissue (14, 38, 52).

Effects of High-fat Diet on PPARγ mRNA Levels in BAT



Effects of High-fat or Low-fat Diet

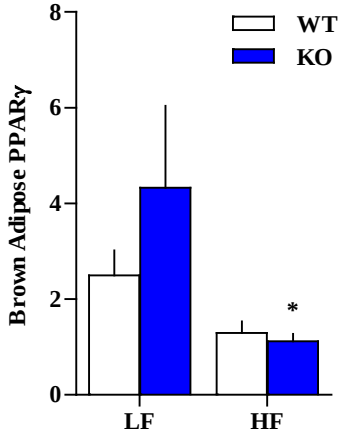


Figure 35. **Effects of HFD on PPARγ mRNA Levels in BAT. Findings:** There was an effect of diet. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For genotype: *p < 0.05.

Two-way ANOVA indicated although there was no genotypic effect ($F_{1,14}$= 1.022; p = 0.329), and no genotype x diet interaction ($F_{1,14}$ = 1.488; p = 0.243), there was a dietary effect ($F_{1,14}$ = 7.246; p = 0.018). *Post Hoc* analysis using Bonferroni's t-test indicated that there was a 200% decrease in PPARγ mRNA levels in WT mice on high-fat diet and a 400% decrease in KO mice on high-fat diet, when compared to low-fat diet, in BAT.

On low-fat diet, KO mice expressed higher levels of PPARγ than WT mice, but there was no statistically significant differences (t = -1.135; p = 0.294) (Figure 35). It is reasonable that having a greater sample size would have reduced the variance, and would have revealed a statistically significant difference. These data suggest that high-fat diet intake may possibly alter the activity of PPARγ, an enzyme stimulated by insulin and involved in adipogenesis in BAT (67, 68).

Effects of High-fat Diet on LCAD mRNA l Levels in BAT

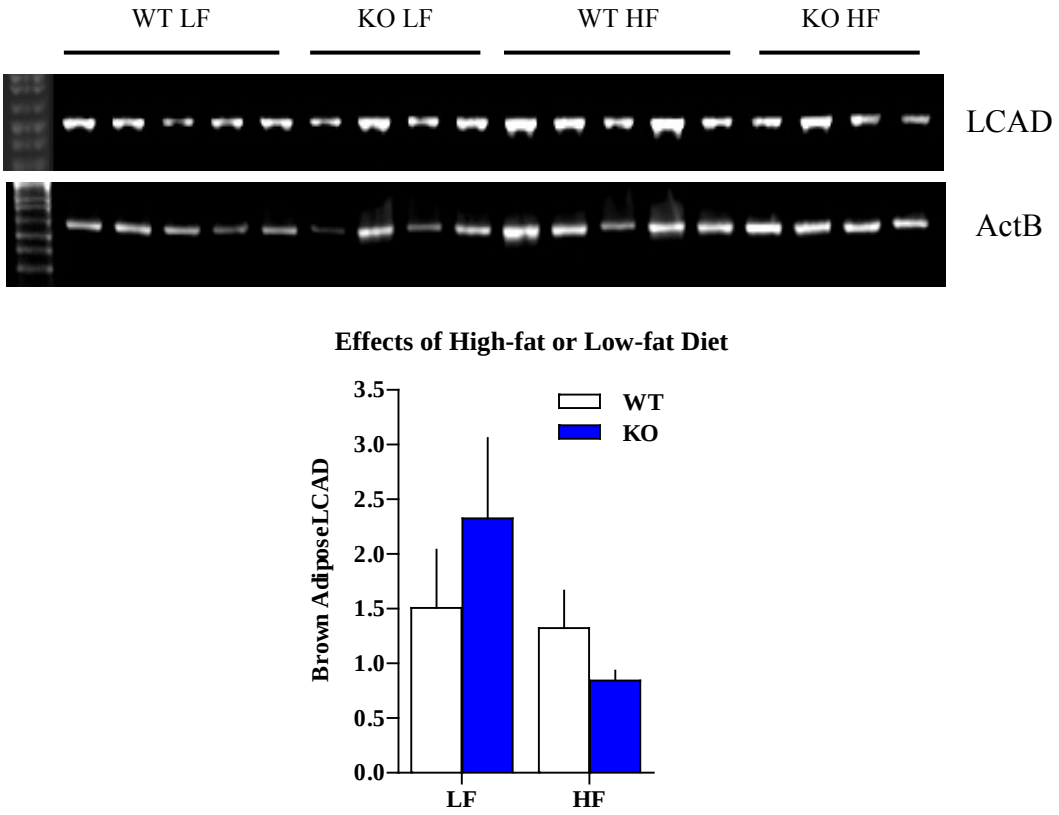
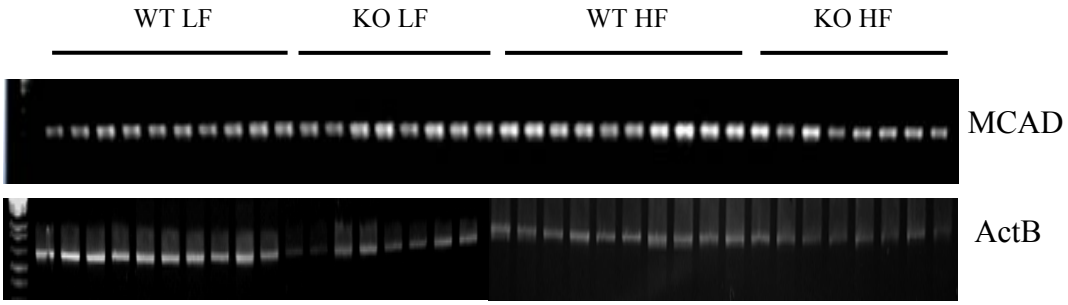


Figure 36. **Effects of HFD on LCAD mRNA Levels in BAT. Findings:** There was a dietary effect. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from 17) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,14}$=0.210; p = 0.653), there was a dietary effect ($F_{1,14}$ = 5.118; p = 0.04), but no genotype x diet interaction ($F_{1,14}$ = 3.112; p = 0.100). Within the diet groups, there were contrasting trends of LCAD mRNA levels. In low-fat diet, KO mice had higher LCAD mRNA levels than WT mice, but on high-fat diet, KO mice exhibited lower levels than WT mice, and this difference was almost deemed statistically significant (t = 2.354; p = 0.051). Additionally, KO mice on high-fat diet had a 3-fold decrease in LCAD levels when compared to low-fat diet, but there was no statistically significant difference (t = 2.004; p = 0.092) (Figure 36). It is plausible that having a greater sample size would have reduced the variance, and would have revealed a statistically significant difference. These outcomes should be interpreted carefully because there were two intriguing trends for genotypic and dietary differences that did not reach statistical significance. In conclusion, these data collectively suggest that MC5R could play a role in the control of long chain fatty acid oxidation in BAT, as seen by varying LCAD levels (73).

Effects of High-fat Diet on MCAD mRNA Levels in BAT



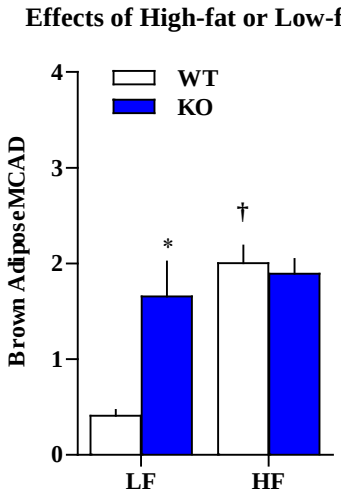


Figure 37. Effects of HFD on MCAD mRNA Levels in BAT. Findings: There was an effect of diet, genotype, and an interaction of the two. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For genotype: *$p < 0.05$. For diet: † $p < 0.05$.

Two-way ANOVA indicated a genotypic effect ($F_{1,14}$= 7.634; $p = 0.015$), there was a dietary effect ($F_{1,14}$ = 19.848; $p < 0.001$), and a genotype x diet interaction ($F_{1,14}$ = 10.91; $p = 0.005$). *Post Hoc* analysis using Bonferroni's t-test indicated that, on low-fat diet, KO mice had a 400% higher in MCAD mRNA levels in BAT than WT mice. In contrast,

WT and KO mRNA levels were similar on high-fat diet. Additionally, WT mice on high-fat diet had a 500% increase in mRNA MCAD BAT levels when compared to low-fat diet (Figure 37). These data suggest that MC5R may have a large role in the regulation of MCAD, a marker for FAO, specifically medium chain fatty acid oxidation, in BAT (67).

Effects of High-fat Diet on FGF-21 mRNA Levels in BAT

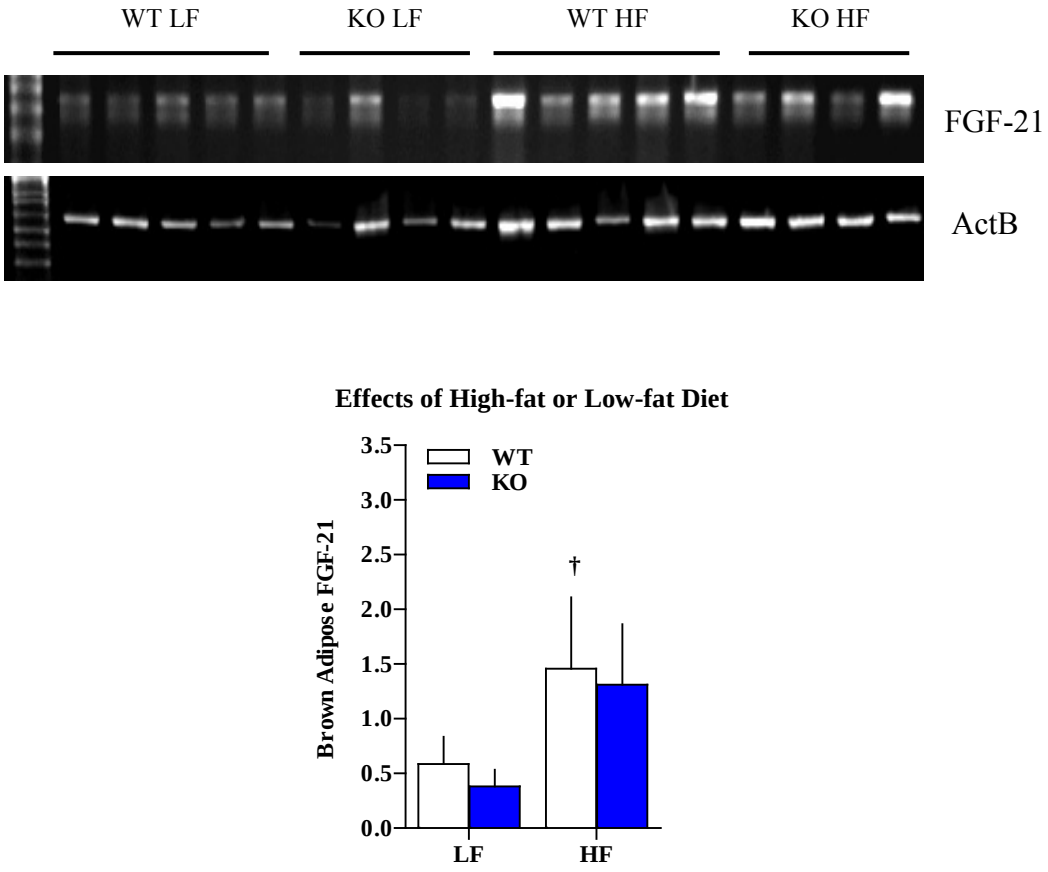


Figure 38. **Effects of HFD on FGF-21 mRNA Levels in BAT. Findings:** There was a dietary effect**. Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR. For diet: † $p < 0.05$.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,14}$= 0.309; p = 0.587), there was a dietary effect ($F_{1,14}$ = 8.088; p = 0.013), but no genotype x diet interaction ($F_{1,14}$ = 0.008; p = 0.929). *Post Hoc* analysis using Bonferroni's t-test indicated that there was a 2.5 fold increase in FGF-21 mRNA levels in WT mice on high-fat diet, when compared to low-fat diet, in BAT. Additionally, evaluated against low-fat diet, KO mice on high-fat diet expressed a 3.5 fold increase in FGF-21 mRNA levels, but there was no statistically significant differences (t = -1.620; p = 0.156) (Figure 38). It is plausible that having a greater sample size would have reduced the variance, and would have revealed a statistically significant difference. Although these results should be interpreted cautiously, because there was one significant difference and one intriguing trend for dietary differences that did not reach statistical significance, these data collectively suggest that dietary fat intake, but not MC5R, may not play a large role in regulating FGF-21, a key player in fatty acid oxidation and thermoregulation, in BAT (26).

Effects of High-fat Diet on Akt1 mRNA Levels in BAT

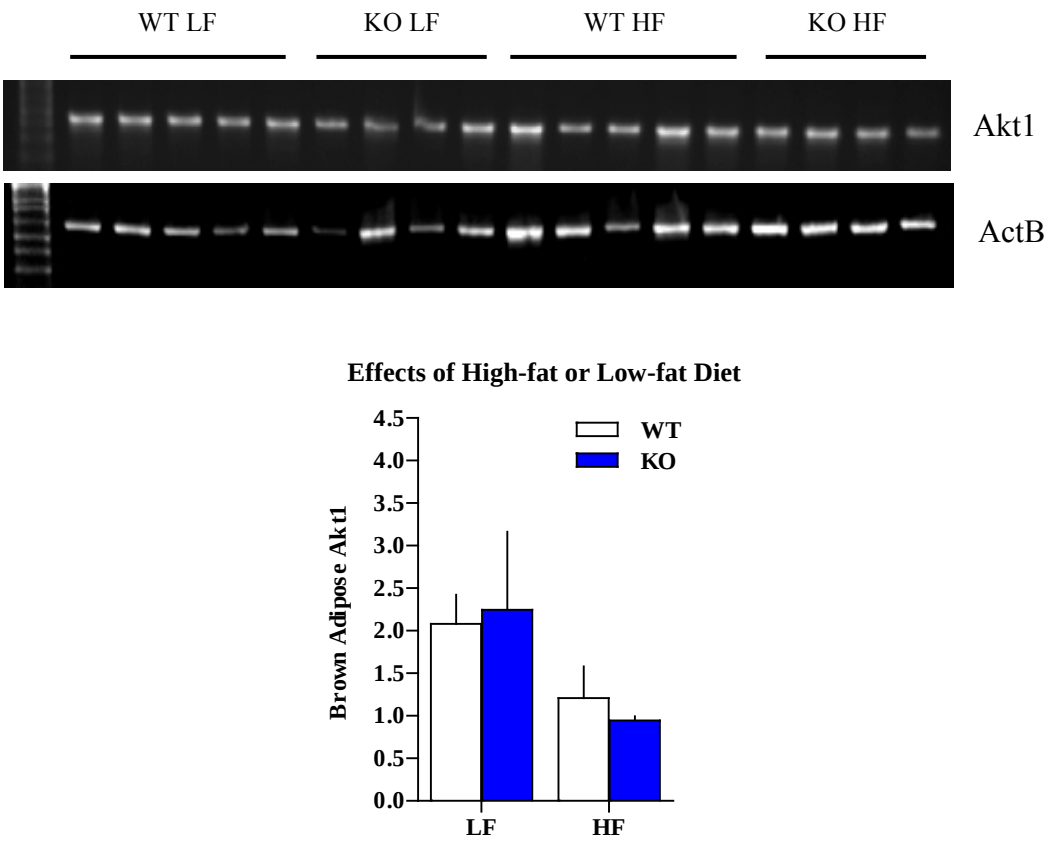


Figure 39. **Effects of HFD on Akt1 mRNA Levels in BAT. Findings:** There was no effect of diet, genotype, or interaction. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR.

Two-way ANOVA indicated no genotypic effect ($F_{1,14}$= 0.12; p = 0.734), no genotype x diet interaction ($F_{1,14}$ = 0.551; p = 0.470), but a trend towards a dietary effect ($F_{1,14}$ = 3.841; p = 0.071). Although there appeared to be a tendency for a decrease in Akt1 mRNA levels in BAT in both genotypes on high-fat diet, there were no statistically significant differences (WT: t = 2.008; p = 0.08 and KO: t = 1.259; p = 0.255) (Figure 39). Although these results should be interpreted cautiously, because there were two intri-

guing trend for dietary differences that did not reach statistical significance, these data collectively suggest that dietary fat intake, and not MC5R, may play a role in regulating the gene Akt1, which may be required for insulin regulation of glucose uptake and lipogenesis in BAT (65).

Effects of High-fat Diet on SREBP1c mRNA Levels in BAT

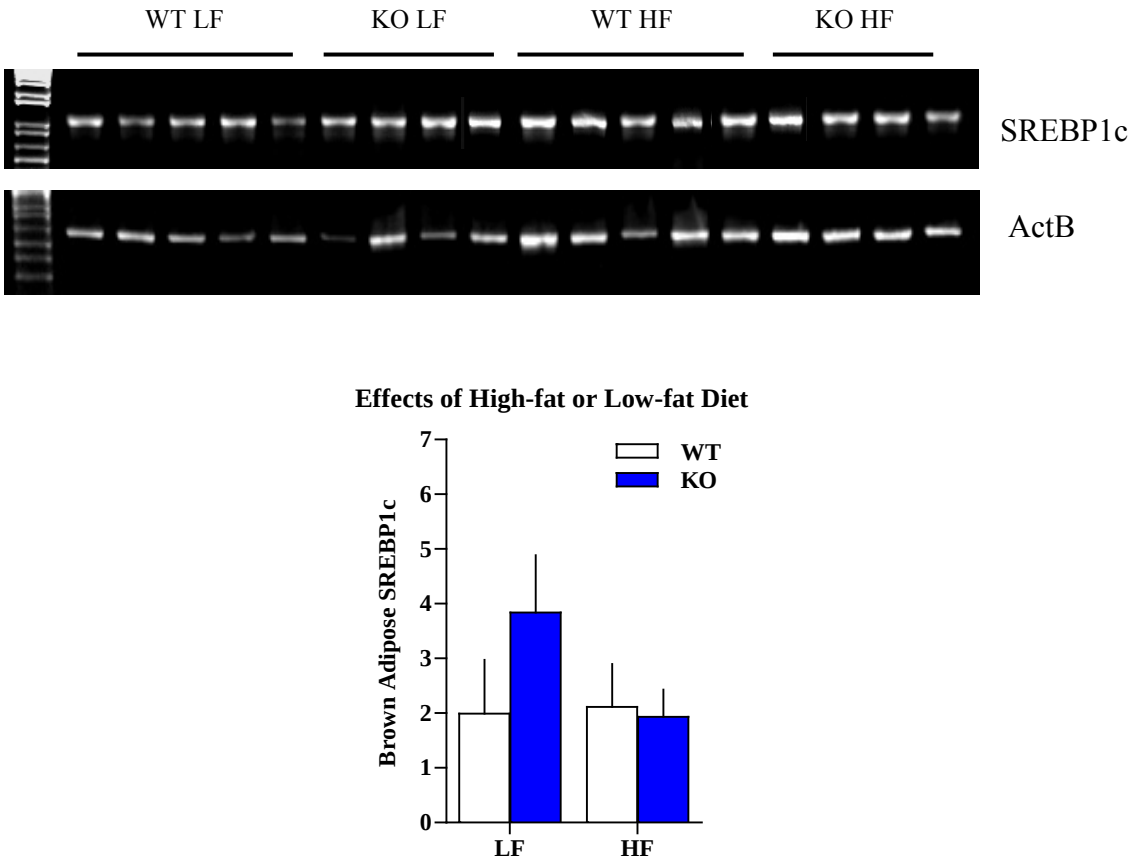


Figure 40. **Effects of HFD on SREBP1c mRNA Levels in BAT. Findings:** There was no effect of diet, genotype, or an interaction of the two. **Methods:** WT and MC5R KO mice (n = 4-5) were placed on LFD (14% kcal from fat) or HFD (45% kcal from fat) for 9 weeks. **Statistics:** Means are shown (± SEM) of normalized PFK-1 to GAPDH obtained in semi-quantitative RT-PCR.

Two-way ANOVA indicated there was no genotypic effect ($F_{1,14}$= 1.836; p = 0.197), no dietary effect ($F_{1,14}$ = 2.117; p = 0.168), and no genotype x diet interaction ($F_{1,14}$ = 2.721; p = 0.121). Although there were no statistical differences between WT and KO SREBP1c mRNA levels on low-fat diet or high-fat diet, KO mice on low-fat diet tended to express a 200% higher level of SREBP1c mRNA than WT mice on low-fat diet (Figure 40). These data suggest that MC5R may play a role in regulating the enzyme SREBP1c, which is involved in adipocyte differentiation and lipogenesis (62). Multiple gene expressions in the four tissues analyzed were significantly altered by MC5R, dietary fat, or both. Table 7 displays genes whose expression was significantly changed.

Table 7. Significantly Altered Genetic Expressions

Genes Analyzed	Skeletal Muscle (-)MC5R	Skeletal Muscle HF Diet	Liver (-)MC5R	Liver HF Diet	Omental Fat (-)MC5R	Omental Fat HF Diet	BAT (-)MC5R	BAT HF Diet
PPARα		↑	↓	↓				
LCAD				↓				
MCAD		↑					↑	↑
DGAT2				↑				
HSL				↓				
FGF-21				↑				↑
PPARγ						↑		↓
SREBP1c						↑		
Akt1			↑	↑				
AMPKa		↑						
PFK-1	↑	↓						

↑↓: increase/decrease in WT expression ↑↓ : increase/decrease in MC5R KO expression

DISCUSSION

Tissue Weights

Each tissue was weighed directly after extraction. Interestingly, liver weights differed between genotypes and diets. The liver plays an imperative role in fatty acid oxidation and glucose metabolism. On both diets, KO mice had a smaller liver than WT mice, and KO mice experienced a significant growth in liver mass on high-fat diet, when WT mice did not. Similar to liver, kidneys hold responsibility in lipid metabolism. On both diets, KO mice had smaller average kidney sizes than WT mice, especially on the low-fat diet. When administered high-fat diet, both genotypes experienced an increase in kidney size, but only the KO mice showed a statistically significant elevation. The differences in sizes of livers and kidneys between WT and KO mice suggest alterations in fat metabolism between diets and genotypes. The deletion of MC5R might cause the KO mice to either metabolize fat at a higher rate or not store fat at as high a capacity as WT mice. When given a high-fat diet, only the KO mice experienced a significant increase in liver and kidney size. While the WT's continued to burn fat at a similar rate to the mice on low-fat diet, the KO mice might have been required to alter the method by which they metabolize fat in order to maintain body lipid homeostasis. In contrast to liver size patterns, the pancreas weight was similar in KO mice and WT mice on low-fat diet, but showed a significant enlargement in both genotypes on high-fat diet. Additionally, the pancreas of the KO on high-fat diet was larger than the pancreas of the WT on an identical diet. The

pancreas is responsible for releasing digestive enzymes to break down carbohydrates and glucose metabolism hormones such as glucagon and insulin. The preputial glands, which are also responsible for releasing hormones into the body, were also affected by MC5R. Overall, the preputial glands of the KO mice were smaller in size than the preputial glands of the WT mice. When on high-fat diet, the glands of the WT mice tended to increase, and KO mice glands did not, creating a significant difference between genotypes on high-fat diet. Skeletal muscle weight did not differ between genotypes on either diet, but KO mice had an average larger skeletal muscle weight on high-fat diet when compared to low-fat diet. WT mice experienced no difference. The higher weight in skeletal muscle might be due to the decrease in FAO occurring in the muscle when MC5R is not present.

All fat pads in both genotypes were significantly different on high-fat diet. The elevation in each fat pad weight suggests that both MC5R KO mice and WT mice developed an obese phenotype by the completion of the study. The only genotypic difference that occurred was in the omental fat region. While the KO and WT mice had similar omental fat weights on low-fat diet, the WT mice experienced a greater increase in omental fat on high-fat diet. This created a greater significance between WT low-fat diet and WT high-fat diet groups, and also suggested a significant decrease between WT mice on high-fat diet and KO mice on high-fat diet. Interestingly, the omental fat region is most highly associated with obesity and cardiometabolic diseases. The decrease in adipose tissue in the omental region when MC5R is absent is another way MC5R may affect metabolism.

Overall, the presence of MC5R appears to have an effect on the metabolism, and therefore the tissue weights, of the mice.

Body Weight, Food Intake, and Caloric Efficiency

Body weight was measured twice a week for the duration of the experiment. After analyzing the body weight data, significant differences appeared not only in overall body weight gain, but in the body weight gain patterns of the four treatment groups. When fed a low-fat diet, both genotypes had similar weight gain, even though on low-fat diet, KO mice had significantly lower average initial weights than WT mice. In contrast, when the mice were fed high-fat diet, WT mice exhibited an overall higher weight gain than KO mice. When analyzing the data, a difference in trends appeared. There was a distinct time point in which the KO mice began gaining more weigh on high-fat diet than WT mice. During weeks nine through eleven, WT mice gained more weight than the KO mice on high-fat diet. In contrast, during weeks twelve through eighteen, which is the majority of the experiment, KO mice gained more weight than WT mice on high-fat diet. The data suggest that in the short-term, WT mice have a higher propensity for weight gain than KO mice, on either diet. In contrast, when placed on high-fat diet, long-term, KO mice might gain weight than WT mice. Another aspect taken into account when looking at body weight gain is the decline in weight gains from week 16 to 18 in the high-fat diet groups, particular the MC5R KO mice. The alteration in the direction of weight gain could be attributed to the behavioral and ITT done on the mice. First, week

sixteen's behavioral testing might have been the cause of the slight drop in weight gain for both genotypes. Second, week 17's ITT might have continued the negative affect on weight gain for both genotypes, with KO weight gain decreasing more severely than WT weight gain. This suggests that different stimuli affects KO mice, in both diet groups, more largely than WT mice. The difference in rates of weight gain between KO and WT groups suggest that MC5R again plays a role in lipid metabolism and/or glucose metabolism.

Food intake analysis displayed multiple significant differences as well. Over the course of the experiment, on low-fat diet, MC5R KO mice ate significantly less than WT mice, but when fed high-fat diet the two groups intakes were similar. As expected, both genotypes had a significant decline in overall food intake compared to the low-fat diet groups when given the higher calorie high-fat diet. The smaller body size of the KO mice might have been the cause of the lesser food intake or vice versa. Similar to body weight, food intake for each treatment group declined in response to the behavior testing during week sixteen. Low-fat diet group's food intake levels increased to previous levels after the testing and continued to increase during ITT on week seventeen. In contrast, both genotypes on high-fat diet had a decrease in food intake after behavior testing, and had a continued decline during week seventeen after ITT. Dietary fat content affected \food consumption after the insulin increase in their system.

Caloric intake and overall feed efficiency was the highest for the high-fat diet groups. Caloric intake analysis suggests that MC5R plays a role in regulating the quantity of calories the mouse consumes. On low-fat diet, the KO mice had significantly lower caloric intake than WT, and when administered high-fat diet, the WT mice experienced a non-significant increase in caloric intake while KO mice had a significant increase of 20%. While the data also suggests that WT mice may have a higher feed efficiency than KO mice and high-fat diet intake increases feed efficiency in both genotypes, inspecting the feed efficiency weekly displays an interesting alternative. For the majority of the experiment, the KO high-fat diet group appears to have had a higher feed efficiency than the WT group on high-fat diet. If weeks sixteen and seventeen were excluded, KO mice may have had the highest feed efficiency. The data suggests that while MC5R KO mice showed a greater response to behavioral and metabolic stimulation, they may also exhibited a higher rate of feed efficiency over a long term experiment.

ITT Analysis

The ITT analysis results suggest a major role of MC5R in insulin sensitivity and resistance. While there was a slight difference in the way MC5R KO mice and WT mice handle excess insulin, the difference was exaggerated when the mice are placed on high-fat diet. When MC5R was present, obese mice develop insulin resistance as expected, but when MC5R was absent, the KO obese mice maintained insulin sensitivity. Further, if the trend were to continue, a long term study in which MC5R KO mice are placed on

high-fat diet may show an increase in insulin sensitivity in the KO mouse. This finding is significant in that MC5R may play a role in the dissociation between obesity and insulin resistance.

RT-PCR Results

Skeletal muscle is a primary figure in metabolic activity (1). One metabolic gene, MCAD, which stimulates medium chain fatty acid oxidation, was stimulated by high-fat diet in skeletal muscle. Instead of being stored in tissue, medium chain fatty acids are transported throughout the body and swiftly oxidized into ketones, increasing energy expenditure (60). MC5R exhibited no effect on the activity of MCAD and therefore had no effect on the amount of circulating and oxidized fat in the skeletal muscle. LCAD, which stimulates the oxidation of long chain fatty acids, was slightly affected by the presence of MC5R (60). High-fat diet stimulated LCAD in both genotypes, with WT mice having had a greater increase than KO mice, but neither showed a statistically significant difference. The KO mice exhibited a tendency to express LCAD, and therefore FAO, at lower levels on high-fat diet. Long chain fatty acids are stored as adipose, and the trend of LCAD mRNA expression to increase in the both groups on high-fat diet suggest that the presence of excess fat in the diet increased the oxidation of fat in the skeletal muscle.

Not only does MCAD and LCAD show that fat oxidation increased in skeletal muscle with the presence of MC5R, PPARα expression provides further evidence. PPARα plays a central role in the regulation of FAO and levels in the skeletal muscle tended to increase in both genotypes on high-fat diet, but WT mice displayed significance in elevation, whereas KO mice did not (23). MC5R presence in WT mice increased the amount of FAO occurring when compared to KO mice. In addition, AMPKa is a fuel detecting enzyme that serves as a central point for energy homeostasis, primarily in the stimulation of fatty acid oxidation and glucose uptake (1). For WT and KO groups, high-fat diet caused AmpKa expression to increase, but only significantly in the KO genotype. The deficiency of MC5R may have caused a decrease of FAO in the muscle, and consequently a lack in energy supply. When fuel is low, AmpKa is activated to attempt to create energy by oxidizing fat or taking in excess glucose. It is suggested that the mechanism by which AmpKa activation increases glucose uptake is through insulin sensitization in the skeletal muscle. The significant increase in AmpKa expression in KO mice between low-fat and high-fat diet may indicate that the absence of MC5R increased insulin sensitivity in the skeletal muscle (40). Glycolysis activity in the skeletal muscle was shown by the rate-limiting enzyme for glycolysis, PFK-1 (30). On low-fat diet, the deletion of MC5R caused drastic stimulation of glycolysis in the skeletal muscle. When high-fat diet was administered, the rate of glycolysis increased in the WT genotype, but significantly decreased in the KO group, when compared to low-fat groups. MC5R plays an important role in regulating glucose oxidation in the skeletal muscle. Akt1 inhibits apoptosis and plays a role in insulin sensitivity in skeletal muscle (63). In both WT and KO groups,

Akt1 expression was elevated on high-fat diet. While both genotypes exhibited higher Akt1 levels on high-fat diet, on each diet, KO mice tended to show lower levels than WT mice. The tendency of Akt1 to show lower expression in MC5R KO mice suggests that MC5R may have affected the rate of apoptosis in skeletal muscle. Additionally, the increase of Akt1 in response to high-fat diet administration demonstrated a positive correlation between high-fat diet and cell growth. Overall, FAO in skeletal muscle was significantly affected by the combination of the deletion of MC5R and high-fat consumption. The lack of MC5R reduced FAO in skeletal muscle when excess fat was present. Similarly, glycolysis is affected by MC5R. Under normal diet circumstances, MC5R absence caused an elevation in the rate of glycolysis. When a high-fat diet was given to the KO mice, glycolysis drastically decreased. Again, the existence of surplus dietary fat altered the effect of MC5R in skeletal muscle. Interestingly and in contrast to glycolysis, insulin sensitivity increased in KO mice on high-fat diet. The increase in insulin sensitivity in KO high-fat diet skeletal muscle could be partially responsible for the outcome seen in the ITT analysis.

The liver is a central site for FAO in the body (32). FGF-21 is a metabolic protein which can regulate the rate of fatty acid oxidation and/or be responsible for insulin activity (12, 71). Levels of FGF-21 in the low-fat diet mice were not significantly different, although the KO group trended towards lower levels when compared to the WT group. However, when placed on high-fat diet, FGF-21 expression slightly increased in the WT genotype, but drastically increased in the KO genotype. The data suggests that, as opposed to the

WT mice, the deletion of MC5R in the KO mice causes a either a significant increase in fatty acid oxidation or an increase in insulin sensitivity controlled by FGF-21 when the animal is on high-fat diet. In contrast, FAO stimulated by PPARα in the liver is oppositely affected by MC5R when compared to FGF-21 (32). High-fat diet feeding decreased FAO in both genotypes, but only KO mice experienced a significant decrease in PPARα expression. Between high-fat diet groups, KO mice had significantly lower PPARα levels than WT mice. This data suggests that PPARα might regulate the oxidation of fatty acids, FGF-21 is involved in insulin sensitivity, and MC5R plays a major role in regulating both metabolic pathways in the liver. Although there is a difference in FAO between groups, as seen by PPARα expression, MC5R expression did not significantly affect MCAD expression in the liver. Similar to PPARα, there was a decreasing trend in MCAD expression from low-fat diet groups to high-fat diet groups, as well as a decrease from WT mice to KO mice in both diet groups. The data suggests a decrease in FAO of transported medium chain fatty acids in mice on high-fat diet, as well as in KO mice. MC5R deletion might have decreased FAO of transported fats in the liver, but there were no significant differences. Contrasting to its relative MCAD, LCAD, which is a marker for long chain fatty acid oxidation, may be regulated further by fat intake, rather than MC5R. High-fat diet fed mice exhibited lower LCAD levels, but only WT mice showed a significant decrease from low-fat to high-fat. HSL is another gene which is involved in lipid metabolism, specifically in the breakdown of lipids in preparation for oxidation, storage, etc. (18). While a high-fat diet tended to decrease HSL mRNA levels in both genotypes, WT mice had a significant decrease while KO mice did not. Similarly

to the expressions of genes involved in fat breakdown, high-fat diet decreased adipose breakdown in the liver. Since the livers of the WT mice were burning fat at a higher rate than KO mice, a gene involved in fat storage, DGAT2, was investigated to further explain the lipid metabolism in the liver. Diacylglycerol O- acyltransferase 2, or DGAT2, is involved in lipid storage in the tissue (42). While low-fat diet did not affect DGAT2 mRNA levels in the liver of either genotype, high-fat diet feeding altered expression, but in contrasting directions. Excessive fat intact caused the WT mice to experience a decrease in DGAT2 levels, while the KO mice had a significant increase in DGAT2 levels. The data suggests that MC5R might reduce lipid storage in the liver. While the presence of MC5R and an elevation in fat intake caused a decrease in fat storage in the liver, the deletion of MC5R under similar circumstances caused adipose to accumulate in the liver, as opposed to being oxidized. The excess storage of lipids in the liver may be induced by the reduction of FAO in KO mice skeletal muscle and liver.

Glucose oxidation, or glycolysis, in the liver was measured by the expression of PFK-1 (30). For both diets, MC5R KO mice trended towards lower PFK-1 levels than WT mice. Dietary differences were not evident between genotypes. The absence of MC5R caused a decrease in the rate of glycolysis, independent of diet. Insulin sensitivity and lipid metabolism in the liver is also regulated, in part, by Akt1. Akt1 stimulates insulin sensitivity in liver cells and is a regulator of glucose homeostasis (61). Additionally, Akt1 expression may decrease fatty acid oxidation. It has been suggested that Akt1 plays a role in regulating lipid metabolism in skeletal muscle (3). Evidence suggests that Akt1

expression creates a tonic decrease in FAO in skeletal muscle (3). Akt1 and lipid metabolism in the liver has not been extensively investigated or reported, but studies in similar tissues, such as skeletal muscle, have suggested an association. Compared to WT mice, KO mice had a tendency to be lower on low-fat diet, but were significantly higher on high-fat diet. Additionally, KO mice had a 500% increase in Akt1 levels on high-fat diet as opposed to low-fat diet. This suggests that MC5R deletion may have increased insulin sensitivity and/or decreased FAO in the liver of KO obese mice. As seen by all genes analyzed representing lipid metabolism, the absence of MC5R caused a decrease in FAO in the liver, independent of diet. Instead of being oxidized, the surplus lipids in the liver accumulated and was stored as adipose. While there was a decrease in FAO in the liver of KO mice, MC5R stimulated an increase in the rate of glycolysis. Not only might the KO mice have had a hindered ability to metabolize fat and instead stored excessive fat, their ability to metabolize glucose was disabled as well. Further, in response to high-fat diet, the lack of MC5R might have increased insulin sensitivity, seen by Akt1 and FGF-21 levels. While both genes expressed similar levels in WT and KO mice on low-fat diet, expression is significantly increased in KO mice when fed high-fat diet. The elevation in dietary fat caused the KO mice to increase their insulin sensitivity.

Omental adipose tissue is a region of fat that has the potential to negatively affect one's overall health. The omentum is relatively close in proximity to the liver, and excess of fat tissue in this region can cause health risks such as high LDL cholesterol, high blood pressure, and high blood sugar, and cardiometabolic diseases (34). FAO can be a consi-

derable aid in reducing the size of the omentum. MCAD, which again measures fatty acids transported into the tissue and oxidized, was not significantly affected by MC5R. On low-fat diet and high-fat diet, MC5R KO mice tended to exhibit slightly lower levels of MCAD expression than WT mice. While on high-fat diet, average fatty acid oxidation was elevated in both genotypes. The data shows that dietary fat intake and/or MC5R may have played a small role in regulating fatty acid oxidation of transported fatty acids by stimulating MCAD in adipose tissue. Similarly, LCAD expression may be affected by both dietary fat intake and MC5R. While LCAD expression represents the oxidation of fatty acids which are stored in the tissue, the results were parallel to MCAD expression in omental fat. Dietary fat intake and MC5R may regulate FAO by stimulating LCAD in adipose tissue (48). Like FAO, the rate of glycolysis can be altered due to various stimulants or antagonists. In omental fat, Akt1 acts as an activator of glycolysis (31). Even though high-fat diet tended to increase average Akt1 levels, WT at a higher rate than KO, expression, and therefore glycolysis, in adipose tissue was not significantly affected by MC5R or dietary fat intake. Omental fat is involved in lipid metabolism and in insulin sensitivity. PPARγ has various duties and its expression is one way to not only understand omental fat's role adipogenesis, but in insulin sensitivity as well. When given a low-fat diet or a high-fat diet, MC5R KO mice expressed a lower average of PPARγ than WT mice. Additionally, while WT mice had consistent levels on each diet, a high-fat diet caused KO mice to increase PPARγ levels. MC5R and dietary fat intake can alter PPARγ, which, when activated in adipose, serves as a stimulant of whole body insulin sensitivity and/or stimulates adipogenesis (52). Another gene which is involved

in insulin activity, SREBP1c, was also affected by both MC5R existence and dietary fat. SREBP1c in omental adipose tissue is stimulated by insulin and serves as the primary transcription factor for lipogenesis in adipose tissue (14, 38, 49, 52). The deletion of MC5R did not affect SREBP1c under normal circumstances, but the consumption of a high-fat diet caused SREBP1c levels to significantly increase from the KO low-fat diet group and non-significantly increase when compared to the WT high-fat group. While the volume of omental fat, as shown by the lipogenic effect of SREBP1c, increased in the absence of MC5R, insulin sensitivity elevated as well. In omental fat, FAO was somewhat altered by the presence of MC5R. On both diets, KO mice experienced insignificantly lower expressions of genes related to FAO. Comparable to skeletal muscle, the lack of MC5R caused a reduction in FAO. Additionally, MC5R is significantly involved in insulin sensitivity in the omental fat. As seen by PPARγ and SREBP1c expression, insulin sensitivity increased in KO on high-fat diet, but not in WT mice. Concerning overall FAO in omental fat, no values were significant, but there were trends that the absence of MC5R may lower FAO in KO mice. PPARγ expression alternately suggests that adipogenesis was increased in KO mice when fed a high-fat diet. This suggests that omental fat did not play a large role in FAO in the body. On the other hand, insulin sensitivity appeared to be affected by omental fat. WT mice experienced no affect by dietary fat on insulin activity, but KO mice showed a significant contrast between diets. Instead of having an increase in insulin resistance when their fat intake increased, MC5R mice showed an increase in insulin sensitivity. The increase suggests dissociation between fat intake and insulin resistance in the omental fat.

BAT is a tissue which is involved in FAO and thermoregulation (53, 73). MC5R is present in this tissue and has an effect on regulating BAT metabolic functions. For example, when measuring FAO by the expression of MCAD, we found that MC5R played a large role in regulating MCAD, or the oxidation of medium chain fatty acids, in BAT. When fed a low-fat diet KO mice burned a significantly higher amount of fatty acids than WT mice. On high-fat diet both genotypes expressed similar rates of FAO; WT mice experienced a significant increase in FAO when compared to low-fat diet while KO mice maintained their elevated expression. In contrast to MCAD expression, LCAD expression was not altered significantly between treatment groups. LCAD expression on low-fat diet was similar to MCAD expression in that MC5R KO mice had a higher level of oxidation of stored fatty acids than WT mice. When placed on high-fat diet, WT mice had no differing effects, while KO mice experienced a decline in LCAD expression, and therefore long chain, or stored fatty acid oxidation (73). These trends suggest that MC5R affected the regulation of FAO of stored fatty acids on both diets. Under low-fat diet feeding in KO mice, FAO increases in BAT, but when excess fat is presented into the body, FAO does not increase further but is maintained. FGF-21 is a key regulator of FAO in BAT (12). The data shows that on either diet, MC5R deficiency did not have an influence on FAO in BAT. While both genotypes experienced an increase in FGF-21 expression on high-fat diet, only WT mice displayed a significantly higher increase. Additionally, MC5R may play a role in regulating the enzyme SREBP1c, which is involved in adipocyte differentiation and lipogenesis (14, 50, 62). SREBP1c expression was not significantly altered between treatment groups, but there was a contrast in expression

between low-fat diet genotypes. The deletion of MC5R caused an increase in adipogenesis under low-fat diet circumstances. When administered a high-fat diet, MC5R KO mice's rate of adipogenesis declined to rival those of WT mice on either diet. This suggests that the lack of MC5R affected the rate of lipogenesis in BAT, but only on low-fat diet. PPARγ's presence in BAT caused alterations in the tissue's metabolism of adipocytes and insulin activity (53). In the area of lipid metabolism, the activation of PPARγ in BAT has been shown to stimulate adipocyte differentiation. PPARγ's role in insulin sensitivity is not clear seeing as some studies indicate that PPARγ stimulation causes insulin sensitivity in the body while others suggest PPARγ stimulation leads to insulin resistance (54). In this study, PPARγ expression was significantly decreased in both genotypes on high-fat diet. While on low-fat diet, KO mice tended to have a higher average of PPARγ expression when compared to WT mice, but there was no significant difference. This data suggests that high-fat diet intake may alter the activity of PPARγ and therefore, adipogenesis in BAT. The effect of MC5R on insulin activity through PPARγ is unclear. Akt1 is active in regulating insulin motivated glucose uptake in BAT (65). While there were no substantial changes in Akt1 expression between groups, there were trends between low-fat and high-fat diets. Both genotypes displayed a decline in Akt1 expression when placed on high-fat diet. While the presence of MC5R may not play a role in regulating Akt1, dietary fat intake may have an effect on the regulation of the gene and decrease insulin regulation of glucose uptake in BAT. Data proposes that MC5R did play a significant role in FAO in BAT. The deletion of MC5R caused an increase in FAO under low-fat diet circumstances. When given high-fat diet, KO mice

maintained elevated FAO, while WT mice experienced a significant elevation in response to the excess dietary fat. Additionally, adipogenesis was altered similarly to FAO, as seen by Akt1 expression levels. BAT may have experienced an elevation of FAO in response to the decrease of FAO in skeletal muscle. Furthermore, insulin sensitivity was affected by MC5R. While insulin sensitivity decreased in BAT as expected in both genotypes when fed high-fat diet, sensitivity was maintained in KO mice fed a low-fat diet. This suggests that under basal circumstances, the absence of MC5R caused the animal to be more insulin sensitive.

CHAPTER IV

SUMMARY AND CONCLUSIONS

SUMMARY

The results of this project suggest that MC5R plays a role in lipid metabolism and insulin sensitivity. Body weight and food intake data shows that MC5R KO mice might have the ability to gain more weight than WT mice do when fed a high-fat diet over a period longer than 9 weeks. ITT analysis showed that the absence of MC5R may cause obese mice not only to maintain their insulin sensitivity, having glucose absorbance values rivaling those of WT and KO on low-fat diet, but begin to experience an increase in insulin sensitivity greater than the low-fat diet mice. In this study, MC5R expression serves as a means by which obesity and insulin resistance were dissociated. RT-PCR results further support the idea that MC5R had an effect on metabolic activities. In skeletal muscle and liver, FAO decreases in KO mice on high-fat diet. To compensate for the excess fat accumulated from the reduction of FAO in skeletal muscle, the liver stored surplus fat and the BAT exhibited an increase in FAO of medium chain fatty acids, or readily available energy. We interpret the body weight gain data to suggest that high-fat feeding for a longer period than 9 weeks would cause the liver will continue to store fat and cause body weight to increase. Furthermore, glycolysis was only affected by MC5R in the skeletal muscle. While low-fat feeding elevated the rate of glycolysis in the skeletal muscle of KO mice, high-fat feeding decreased glycolysis in this tissue. Lastly, the ITT evidence for increased insulin sensitivity in KO mice on high-fat diet might be attri-

buted to improved insulin sensitivity in multiple tissues. Specifically, in skeletal muscle, liver, and BAT of MC5R KO mice, high-fat feeding increased the expression of molecular markers of insulin sensitivity. KO mice also displayed an increase in insulin sensitivity in BAT on low-fat diet. Overall, these data suggest that insulin resistance and obesity were no longer associated when MC5R was absent.

Figure 41 displays an overall picture of the effects MC5R has on each specific tissue's metabolic activity.

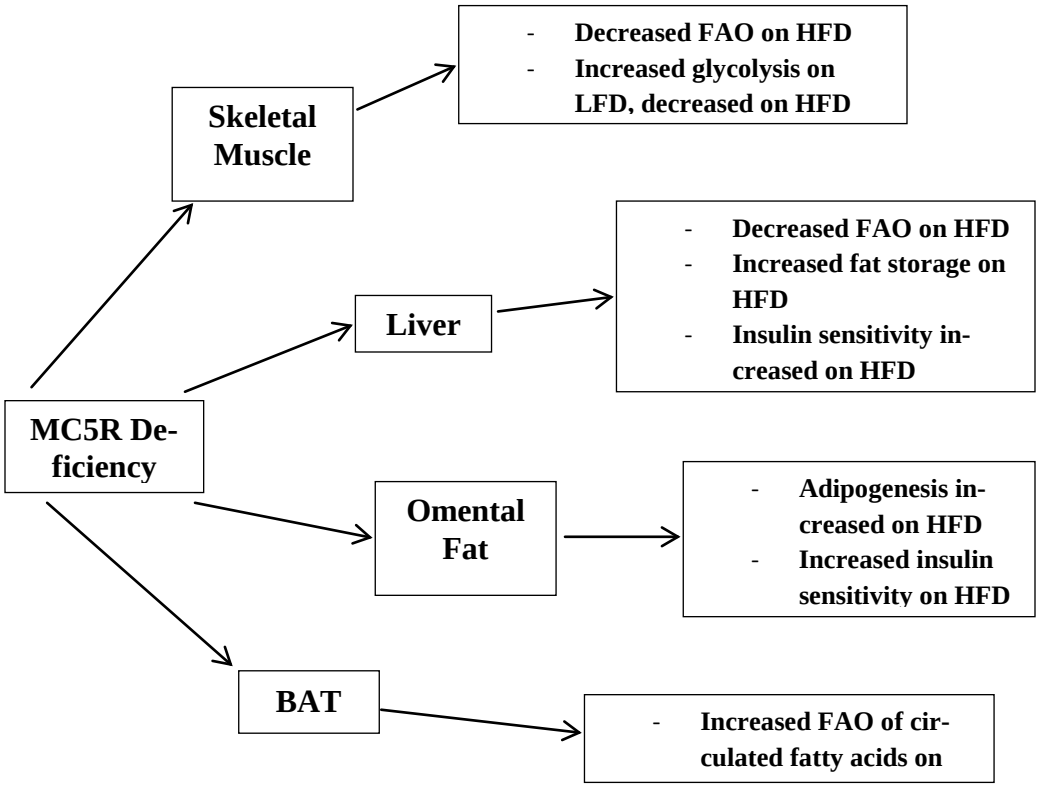


Figure 41. **Summary of MC5R Effects on Tissue's Metabolic Activity.**

CONCLUSIONS

The understanding we have gained of the role of MC5R in lipid metabolism and insulin sensitivity will potentially aid the understanding of the mechanism by which the two are related. This mechanism pertaining to MC5R's involvement in the dissociation of excessive weight gain and insulin resistance could be used in the future to develop therapeutic drugs that could prevent the onset of early type 2 diabetes in a person who is becoming overweight and/or obese. The fear of becoming diabetic as a result of developing obesity would be lessened. The two no longer would necessarily go hand in hand.

Further studies that should be conducted include:

- Feeding a high-fat diet to MC5R KO mice for 12 weeks or longer.
- Further RT-PCR and western blotting of various metabolic genes involved in lipid metabolism and insulin activity in tissues of MC5R KO mice on low-fat and high-fat diets.
- Investigation of tissues such as heart, kidneys, and brain of MC5R KO mice fed a high-fat diet.
- Behavioral evaluation of mice to investigate depression related behavior of MC5R KO mice.

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APPENDIX A

EXPERIMENTAL PROTOCOLS

INSULIN TOLERANCE TESTING (ITT)

(Dr. Chaodong Wu)

Supplies:

Gloves of various sizes
Sharpie
Allergy-free syringe
Heparin tubes
20 μl pipette and tips
Ice bucket
Welled plate
Surgical scissors
Kim wipes
Insulin
Glucose reagent

Procedure:

1. Organize mice for easy and fast selection
2. At 0 timepoint, inject mice with insulin (1 U/kg)
3. Collect blood sample (5 μl) from tail vein at 15, 30, 45, and 60 min after bolus insulin injection
4. Place samples in heparin tube, then on ice
5. Centrifuge samples and extract plasma
6. Place plasma sample in well with glucose reagent, alongside glucose standards
7. Measure absorbency over timepoints

KILL DAY SET-UP & CHECKLIST

General

- Tape bench papers on lab benches and line with extra bench papers; replace bench papers as necessary.
- Place gloves, boxes of Kim wipes, sufficient napkins, utensils, and sharpies at kill station.
- Tape 2 biohazard bags on benches near kill station
- Make RNAse-free & regular 1X PBS solution, ensure plenty and chill in 4° C.
- Complete rat's 48-hr diet intakes
- Place foil packets at kill station for tissue collection

Kill Station

- Ziploc bags for rat bodies
- PBS in glass -RNAse Free
- Surgical tools
- Straight scissors (black handle)
- Forceps
- Extra bench papers
- 2 ml tubes for plasma collection
- Ice bucket for plasma
- EDTA for plasma collection
- 200 ul pipette with tips
- Liquid Nitrogen Container
- Balance
- Sharpie
- Gloves

Blood Station

- Centrifuge
- 2 ml tubes for plasma collection
- Funnel
- Ice bucket for plasma
- EDTA for plasma collection
- 200 ul pipette with tips

Morning of Kill

1. 2. Scoop lots of ice and fill necessary containers
2. Make 1X PBS solution and chill
3. Remove denaturation solution from fridge; keep in ice.
4. Turn on centrifuge
5. 7. Prepare and move mice from basement to kill station; keep food in cages

6. Pre-weigh foil packets and make sure they are labeled properly and placed at kill station

After Kill

1. Store samples in appropriate place
2. Clean up
3. Autoclave biohazard trash and bring to dumpster
4. Bring mice bodies to freezer in basement
5. Wash tools and glassware; put to dry in oven

RNA EXTRACTION PROTOCOL
(Dr. Caurnel Morgan)

Supplies:

2 ml tubes
Glass mortar and pestle and/or 18-gauge needle
50 ml conical
Centrifuge
Vortex
Water bath
Homogenization buffer
Beta-Mercaptoethanol
1X Protein digestion Buffer
Proteinase K
100% Ethanol
Phenol Chloroform
Acidic Phenol Chloroform
5 M NaCl
Water
Ice Bucket

Procedure:

1. Prepare tissue homogenate:
    a. You will need 3mL of homogenization buffer for each sample, plus 2mL extra buffer (E.g., 6 samples requires 20mL buffer).
    b. Add 7uL beta-mercaptoethanol (BME) to homogenization buffer to get 0.1M BME. (For 6 samples, 20mL homogenization buffer, this would require 140uL BME)
    c. Add 30mg-150mg tissue to 3mL homogenization buffer. Homogenize the tissue using a glass mortar and pestle and/or passing the tissue through an 18-gauge needle.

2. Precipitate RNA:

    a. Add at least 3 volumes of 4M lithium chloride to the homogenate (for a 3mL homogenate, this would require at least 9mL 4M LiCl). Mix by inverting until there are no separate layers of liquid.
    b. Precipitate the RNA at 4^C overnight.
    c. Sediment the RNA at 10,000g at 4^C for 1.5 hours.
    d. Decant the supernatant.

3. Protein Removal:

    a. Prepare a 45^C water bath. Add proteinase K to 0.2mg/mL in 1X protein digestion buffer. You will need 0.5mL buffer per sample.
    b. Decant the supernatant off the RNA-containing pellet. Save the supernatant for protein and/or DNA extraction.
    c. Re-suspend the pellet in 0.5mL protein digestion buffer with proteinase K. The pellet will be hard at first. Incubate the pellet at 45^C for 2-5 minutes and break up the pellet by vortexing and/or using an 18-gauge needle.
    d. Incubate the sample in protein digestion buffer for 1 hour at 45^C.
    e. Adjust volume to 0.8-1.0mL with water. Phenol/chloroform extract the sample until the interphase is completely clear. Always do at least 2 extractions.
    f. Add 2-2.5 volumes ethanol to the sample and mix by inverting several times. Alternatively, you may add 0.6-1.0 volumes of isopropanol instead of ethanol. Add $1/24^{th}$ volume 5M NaCl to the sample and mix by briefly vortexing. Store the RNA on ice for at least 15 minutes.
    g. Sediment the RNA by spinning at 15,000g for 15 minutes at 4^C. Decant off the supernatant and remove the residual supernatant with a pipette.
    h. Re-suspend the pellet in 400uL water. Extract twice with acidic phenol.
    i. Repeat steps 8 and 9.
    j. Add 0.3mL-1.0mL 70% ethanol the each sample and loosen the pellet by vortexing.
    k. Sediment the RNA at 12,000g for 5 minutes at 4^C. Decant off the supernatant and remove the residual ethanol by pipette.
    l. Re-suspend the pellet in water or formamide.

Notes:

1. For RNAse-rich tissues you should homogenize 20-30mg of tissue. You may want to pre-grind the tissue in liquid nitrogen.

    a. Very tough tissues such as muscle or heart can either be frozen and crushed in liquid nitrogen beforehand or (quickly!) cut into smaller pieces with scalpels while submerged in homogenization buffer.
    b. If extracting from fat tissue, place on ice, directly after homogenizing, for 5 minutes. Centrifuge and extract out the middle aqueous layer which contains the RNA.

Solutions:

Homogenization Buffer
Mix in a 50mL tube: (final concentrations in parentheses)
26.7mL GuanidiniumThiocyante, 6M (4M)
2.0mL Tris, pH 7.5, 1M (50mM)
2.0mL EDTA, 0.2M (10mM)
1.0mL 20% Sodium N-Lauroyl Sarcosine (0.05%)
Adjust final volume to 40mL with ddH_2O

10X Protein Digestion Buffer
Mix in a 50mL tube: (final concentrations in parentheses)
4.0mL Tris, pH 7.9, 1M (100mM)
10.0mL EDTA, 0.2M (50mM)
20.0mL 20% Sodium N-Lauroyl Sarcosine (10%)
Adjust final volume to 40mL with ddH_2O

Acidic Phenol-Chloroform:

Component	By Number of Samples	For 6 Samples
Buffered Phenol organic phase pH 4.5	0.83mL * Sample #	4.98mL (5mL)
Chloroform	0.17mL * Sample #	1.02mL (1mL)

MMLV REVERSE TRANSCRIPTASE PROTOCOL

Supplies:

Thermocycler
0.65 ml tubes
20 µl pipette with tips
200 µl pipette with tips
Heat block
dNTPs
Random hexamers
10X M-MLV Buffer
M-MLV
RNAse inhibitor
Water

Procedure:

1. Thermal Cycler and Heat Block Preparation
    a. Set the heat block to 65^C.
    b. Program the thermal cycler: set the heated lid to 70^C. Program the cycler to hold at 25^C until released, 25^C for 10 min, 37^C for 50 min and 70^C for 15 min.
    c. Allow sufficient time for the block and thermal cycler to reach start temperatures.

2. Master Mix A preparation
    a. Calculate the amount of master mix according to the "master mix A" table and add the appropriate amount of each component.
    b. Mix master mix A by pipetting.

Component	Calculation
dNTPs (10mM each)	1.1uL * sample number
50ng/uL random hexamers	1.1uL * sample number

3. Sample Preparation

    a. Dilute 1500ng of each RNA to a final volume of 10uL in a 0.5mL microcentrifuge tube.
    b. Add 2uL Master Mix A to each sample
    c. Incubate samples at 65^C for 5-10min
    d. Transfer samples *immediately* to ice. After at least 2 min, quick spin the samples and place them back on ice.

4. Master Mix B preparation

    a. Calculate the final volume of master mix B by multiplying (number of samples + 0.5) * 8uL.
    b. Thaw the 10X M-MLV buffer and invert the tube until all the white precipitate dissolves. Add the appropriate amount of each component to the master mix (see table below).
    c. Mix by pipetting

Component	Calculation
10X M-MLV Buffer	2 * (sample # +0.5)
M-MLV	1.2 * (sample # +0.5)
RNAse Inhi-bitor	1uL
Water	To final volume
Final Volume	8* (sample # +0.5)

5. RT Reaction

    a. Add 8uL master mix B to each sample and mix by pipetting.
    b. Put samples into the thermal cycler and release the hold.

RT-PCR PROTOCOL

Supplies:

Thermocycler
0.65 ml tubes
20 µl pipette with tips
200 µl pipette with tips
2X Ready Mix
RNA from sample tissue
Primer mix
Water

Procedure:

1. Prepare the thermal cycler
    a. Calculate the extension time by multiplying amplicon length in kb * 60s (minimum 30s).
    b. Determine the hybridization temperature by the lower Tm (in 50mM NaCl) of the two primers.
    c. Determine cycle number based on the abundance of the mRNA.
    d. Program the thermal cycler: Set the heated lid to 101^C. Program the machine to hold at 94^C until released, 94^C for 50s, Repeat [cycle number] of times: (94^C 20s, [hybridization temperature] 20s, 72^C for [extension time]), 4^C hold.

2. Master Mix preparation

Component	Calculation
2X Ready Mix	8uL * (sample number + 0.5)
Primer Mix (10uM each)	0.8uL * (sample number + 0.5)
Water	5.2uL * (sample number + 0.5)

3. Sample Preparation

    a. For each sample, add 2uL cDNA + 14uL master mix to a tube on ice.
    b. Start thermal cycler. When the lid and the sample block have both reach temperature, add the samples to the block.
    c. Close the lid and release the 94^C hold.

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