EPIGENETIC REGULATION OF GENES RELATED TO LIPID METABOLISM BY MICRORNA IN MICE FED HIGH FAT DIET

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Food Science and Human Nutrition
with a concentration in Human Nutrition
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

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Abstract

High fat diet impacts lipid metabolism by altering the transportation, oxidation, and storage of fatty acids. Lipoprotein lipase (LPL) plays a critical role in lipid metabolism by catalyzing triglyceride hydrolysis and lipoprotein uptake in multiple tissues. A previous study reported that miR-29b negatively regulated LPL expression in mammary epithelial cells transfected with miR-29b mimics. The present study investigated changes in LPL expression and epigenetic mechanisms by the miR-29 family in different tissues in mice fed a high fat diet. Five-week old male CBA mice were fed with either a control diet (Con group, 10% kcal from fat) or a high fat diet (HF group, 45% kcal from fat) ad libitum for 11 weeks. The results showed that LPL mRNA was increased in adipose, muscle and colon in response to high fat diet. However, LPL mRNA expression decreased in the liver by high fat diet as well as hepatic lipase (HL). The results also showed the highest expression level of LPL mRNA in adipose tissue, followed by muscle, colon, and liver. Meanwhile, high fat diet reduced the expression of miR-29a/b, predicted suppressors of LPL from miR-29 family, in adipose tissue. Genomic analysis predicted several potential transcription factors of miR-29 family members that suppress the expression of miR-29s. At mRNA level, some of these transcription factors, c-Myc and EZH2, were significantly activated in response to HF diet. The present results indicated that the LPL expression could be activated by high fat diet in multiple tissues and the induction of LPL is post-transcriptionally regulated by miR-29a/b. Furthermore, the transcription of miR-29 in mice adipose was regulated by certain transcriptional factors. Overall, LPL mRNA altered in multiple tissues in response to high fat diet and is potentially regulated through transcription factors and microRNAs.

Keywords: Lipid metabolism; MicroRNAs; Transcriptional factor; Promoter region

Acknowledgements

First, I want to extend my greatest appreciation to my advisor Dr. Chen for her guidance and support of my study in the University of Illinois. For accepting me as an M.S. student, she provided me with the opportunity discovering how exciting research on nutrition is, and experiments on biochemistry provides an efficient and unique method understanding nutrition deeply. Especial appreciation to the concern and help she offered not only in study but in my daily life when I went through a hard time. Studying and working in the lab has brought me lots of precious experience and memory. I have learned to be determined and active. I have met great friend and have opportunity attending conference the with your support. It is a great honor to work with you.

I thank Dr. Pan for his advice and in all my research work. This work is a result of the suggestion and knowledge you provided to me. He taught me to think logically and work systematically like a scientist. His rigorous attitude towards science and research has encourage me to think critically and work smartly.

I also think my committee member, Dr. Arthur, for the kind and patient talks between us that have encouraged me to move on. Your great support on my research have allowed me think more and make me more confident.

I want to also express my gratitude to all the members of Dr. Chen and Dr. Pan's lab: Bianca Xu, Mingzhu Cai, Laura Moody, Diego Hernandez-Saavedra, Zheng Hua, Avery Zhang, Lei Ma. Thank you all for great support and collaboration as well as the happy hours we shared together. The accompanying by all of you has made my time studying in the university colorful and vivid.

I think all people in the Department of Food Science and Human Nutrition for your support and help when I met problems in my study and in life, which has made me complete the degree easier and more successful.

Finally, I want to thank my entire family, especially my father and mother, for your support throughout the years, which has made me become mature and independent. I want to thank to my grandparents for your unconditional and endless love.

This project was supported in part by the Campus Research Board of University of Illinois at Urbana-Champaign and the USDA Cooperative State Research, Education and Extension Service, Hatch project numbers # ILLU-698-923 and ILLU-698-379 to H.C.

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Chapter 1： Introduction

Inadequate or excessive nutritional intake and unhealthy dietary patterns impact the normal metabolic function in negative ways, while long term exposure to high fat diet causes nutrient overload and leads to metabolic disorders including dyslipidemia, type 2 diabetes, hypertension, cardiovascular, obesity and other chronic diseases.

The process of the breakdown of triacylglycerols (TAGs) from diet into glycerol and fatty acids (FAs) is called lipolysis, which is catalyzed by lipases [1]. The expression of genes encoding lipases can be post-transcriptionally regulated by microRNAs [2]. Lipoprotein lipase (LPL), a member of lipases, could be partly matched with miR-29a/b, members of miR-29 family. Many studies have reported the importance of the miR-29 family in cell proliferation, differentiation, apoptosis, and migration [3-6]. However, results of experiments conducted *in vitro* showed the negative regulatory effect of miR-29a/b on LPL, indicating miR-29a/b might be important in lipid metabolic regulation [7, 8]. However, the regulatory effects of miR-29a/b on LPL in response to dietary factors, like HF diet, *in vivo* are still poorly understood. Therefore, the responses of miR-29a/b to HF diet and the relationship between miR-29a/b and LPL were explored in this study.

In this study, miR-29 showed alternative responses to control and HF diet. The mechanisms regulating expression of miR-29 in response to dietary factors in mice are thus of interest. Based on the existing data, c-Myc was shown to contribute to widespread microRNA repression including miR-29 [9]. Although the mechanisms by which c-Myc and EZH2 represses miR-29 transcription have been extensively studied *in vitro* [10], less is known about how long-term HF diet affects miR-29 transcription with

the mediation of c-Myc and EZH2 in mice. In this study, we explored the role of c-Myc and EZH2 in miR-29 repression in mice fed HF diet.

Chapter 2： Literature Review

High fat diet and Lipid metabolism disorder

High fat (HF) diets alter the expression of genes involved in lipid metabolism and cause the increasing risk of insulin resistance, obesity, metabolic syndrome, dyslipidemia, and other metabolic disorders. Thus, it is critical to understand the regulatory mechanisms by which HF diet intervenes lipid metabolism and ultimately causes these chronic diseases. Epigenetic regulation is one of the regulatory mechanisms for lipid metabolism impacted by HF diet. It was reported that HF diet induced gene expression involved in lipolysis and fatty acid catabolism, such as LPL, Cpt1, Pparα, and Ehhadh, in different organs [11-13]. Meanwhile, genes involved in fatty acid synthesis were regulated by HF diet in main organs, such as acetyl CoA carboxylase (ACC), fatty acid synthesis (FAS), stearoyl coenzyme A desaturase 1 (Scd1), stearoyl coenzyme A desaturase 2 (Scd2), and Srebp1 [14-17]. There is no consistent conclusion about the impact of HF diet in lipid metabolism because, in some studies, the effect of HF diet is associated with the composition of fat in diet. In mice fed with HF diet (42% calories from fat) contained either saturated fat (71% 16:0 in total FAs) or n-3 fish oil (15% 16:0, 16% 20:9 in total FAs) for 12 weeks, HF diets enriched in fish oil decreased arterial LPL levels and limited LPL expression, but saturated fat diets increased LPL expression compared to control group[18, 19], indicating that the type and the portion of fats in diet may be a key factor affecting the metabolic response to HF diet.

Lipid metabolism is defined as physiological processes in the anabolism and catabolism of lipids. HF exposure for a period can program the process of lipid

metabolism, including synthesis and degradation of lipids in organs and cells. Mice fed with a long-term HF diet had problems in lipolysis or lipogenesis. For instance, induction of a transcription factor Pparα by HF diet enriched in fish oil can enhance fatty acid oxidation in mice muscle, which directed fat into oxidation rather than storage in muscle [20]. The level of Pgc1α, the cofactor of Pparα, was also higher in mice fed long-term HF diet [20, 21]. Additionally, HF diet can program FA synthesis in tissue-specific manners. ACC and Fas are critical enzymes in FA synthesis. The expression of these genes was significantly increased in adipose tissues but decreased in liver from HF-fed mice[14, 16, 17]. The dysregulation of lipolysis and lipogenesis can cause problems in lipid metabolism in main organs.

Organisms store the FFAs in adipose tissue in the form of triacylglycerols (TAGs). Overloaded fats from diet can lead to greater FFA storage and increase adipose mass [22]. Many studies showed that genes involved in FA synthesis and lipid uptake were significantly increased in the adipose tissue from HF-fed mice. In the adipose tissue, the expression of PPARs, including PPAR-α and PPAR-γ, increased in response to HF-diet [13, 14, 20, 21]. Following study illustrated that the activation of PPAR-α induced the transcription of LPL [15] and fatty acid transporters (FAT/CD36) [14]. PPAR-γ is specifically expression in adipose tissue and plays an important role in adipose tissue. It was increased in both mRNA and protein level in response to HF diet [23]. The activation of PPAR-γ leads to lower plasma glucose concentration in T2DM patients [24, 25] while the deletion of PPAR-γ gene in adipose tissue causes reduced adipocytes number accompany with elevated plasma FFA and TAG concentration [26, 27]. Major enzymes related to fat accumulation such as acetyl CoA carboxylase (ACC) and

glycerol-3-phosphate dehydrogenase (GPDH) are increased in adipose tissue in response to HF diet [23]. Once the influx of FAs exceeds the storage capacity of adipose tissues, excessive FAs will efflux to other organs including liver and muscle [28].

Liver is a critical organ involved in lipid metabolism and can be greatly affected by long-term HF diet. mRNA expression associated with hepatic lipid metabolism, including Fasn, Srebp1a, Cpt1a and Pparα [17, 21], can be altered by HF diet. The expression level of Fasn and Srebp1a were significantly decreased in liver of rats fed a HF diet (60% calories from fat) for 4 months[17], indicating that a HF diet has an inhibition impact on genes for fatty acid synthesis and lipogenesis. The expression of genes involved in hepatic lipid catabolism were affected by HF diet through epigenetic modification, such as PPAR-α, Cpt1a. It was reported higher expression levels of PPAR-α in HF-fed rat as correlated with increased expression of Cpt1a together with a rise in triacylglycerol content in the liver [21]. Several animal models of HF diet have demonstrated that HF was a major cause of chronic liver diseases, such as steatosis, as increased fatty acid flux into liver and over-accumulation of hepatic fat [29, 30].

Muscle is the major organ of FA oxidation. Some studies indicated that HF diet reduced expression of genes in FA synthesis (Fasn, Srebp) and increased expression of genes in FA uptake (CD36, LPL) and oxidation (Cpt1, PPAR-α) in muscle [17, 21, 31]. However, the effect of HF diet on FA catabolism related genes in muscle is still controversial. Some studies showed an increased expression of FA oxidation [32] while others showed a decreased expression of FA uptake (Pgc1-α) [33].

In summary, HF diet exposure has vital influence on lipid metabolism in different organs by regulating the expression of genes related to FA uptake, synthesis, and oxidation. Different organs may response to HF diet in different ways in terms of the roles they play in lipid metabolism. Moreover, the composition of fat can alter the metabolic responses to HF diets.

Lipoprotein Lipase

A variety of regulation factors has been defined in different animal models undergone long-term HF diets. To determine the importance of lipolytic and lipogenic modification in response to HF diets, clarifying the epigenetic mechanism behind the regulation of lipid metabolism in main organs takes the priority.

Lipoprotein lipase (LPL) is a member of human lipases existing in endothelium where it hydrolyzes TAGs in chylomicron and VLDL. It is essential for TAG hydrolysis in chylomicrons and VLDLs. Specifically, LPL is produced in parenchymal cells, translocated to the vascular surface of the capillary endothelium with the participation of the apolipoprotein C-II and glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1) and acts at ‘binding-lipolysis sites’ on the vascular surface of the endothelial cells [34]. Normally, LPL interacts with substrates by the N-terminal domain to finish catalysis. It was overexpressed in mice fed HF diets enriched in saturated fat for 12 weeks and overexpression of LPL accelerates FA uptake, which ultimately develops into insulin resistance in mice [18, 35].

LPL is primarily synthesized in multiple tissues such as skeletal muscle, adipose tissues, macrophages, the nervous system, and liver [36]. It hydrolyses lipids for different purposes in different tissues. In heart and skeletal muscle, LPL produces FFA

from plasma lipoproteins for beta-oxidation [37]. In adipose tissue, LPL hydrolyzes TAGs in lipoproteins and generates FFA for lipid storage, where LPL is an important factor for obesity development [38].

LPL expression can be regulated through both transcriptional and post-transcriptional mechanisms while the activity of LPL protein depends on the interaction with other proteins in specific tissues [39]. Dietary factors and nutrient states can alter LPL expression directly or indirectly through complex mechanisms at multiple levels [36]. Genomic analysis found the upstream region of LPL genes from transcription site contains several transcriptional binding elements, including the peroxisome proliferator activated receptor responsive element (PPRE) [40-42]. Peroxisome proliferator activated receptors (PPARs) can specifically bind to PPRE site in the target genes and regulates target gene expression. For LPL expression, PPARs bind to the PPRE in the LPL promoter in the form of PPAR-RXR heterodimer, a combination of PPARs and retinoic receptor (RXR), and active LPL expression in a tissue-specific manner [42]. In addition to PPARs, RXR can dependently up-regulate LPL expression in plasma, skeletal muscle, and heart, but not in adipose tissue [43]. Some other studies reported tumor necrosis factor (TNF), protein kinase A (PKA) inhibited LPL transcription while transforming growth factor-beta (TGF-beta) induced LPL transcription[44-46].

Recently, microRNAs are considered as important regulatory factors for post-transcriptional suppression of LPL by complimentarily binding with the 3’UTR of LPL mRNA. miR-29a/b can inhibit LPL expression in dendritic cells and mammary epithelial cell (MEC) respectively [47, 48]. Further research illustrated that miR-27a/b and miR-467b inhibited the LPL transcription in THP-1 macrophages and RAW 246.7

macrophages respectively [49, 50] and the inhibitory effect of miR-27 and miR-29 is synergetic [51].

Interaction proteins can also affect the biological function of LPL. For example, apoA-V promotes LPL catalytic effects by enhancing attachment of lipoproteins to GPIHBP1, the major platform form for LPL catalysis [36]. Additionally, angiopoietin-like protein 4 (Angptls 4) downregulated LPL activity through dimer-to-monomer conversion of LPL protein and competitive inhibition [52]. Other members of Angptls were subsequently found to inhibit LPL activity through Angptl3-4-8 model [53].

In summary, LPL is essential for TAG hydrolysis and FFA generation from TAG-rich lipoproteins. FFAs generated by LPL are utilized for different subsequent metabolism purpose following tissue-specific manners. Therefore, LPL plays an important role in lipid metabolism. The regulation of LPL is complex and varies in specific tissues. Dietary factors alter the expression of LPL through different mechanisms at multiple levels and ultimately impact lipid metabolism.

MicroRNAs and the function in lipid metabolism

MicroRNAs (miRNAs) are small non-coding RANs with 19-22 nucleotides that serves as post-transcriptional regulators of genes. They transcriptionally regulate gene expression by binding to 3’ untranslated region (UTR) of specific mRNAs bases on complementary sequences and cause inhibition of translation or mRNA degradation [54]. The existing of microRNAs was first observed in the cytoplasm of *C. elegans* as short antisense RNAs binding to mRNAs [55]. Subsequently, a growing number of microRNAs was identified in diverse species, including human, mouse, rat, in quick

succession. Further studies found the general negative regulatory effect of microRNAs on mRNAs and the highly conserved sequences of microRNAs across different species, identifying that microRNAs take a major place in post-transcriptional regulation [56].

The formation of mature miRNAs goes through two steps. First, the nascent miRNA transcripts (pri-miRNA), transcribing from one strand of miRNA gene by RNA polymerase II [57], are processed into ~70-nucleotide precursors (pre-miRNA) by Drosha, a RNase-III enzyme predominantly localized in the nucleus. This pre-miRNA consists of an imperfect stem-loop structure. It is subsequently cleaved into ~21-25-nucleotide mature miRNAs by Dicer, another RNase-III enzyme located in the cytoplasm [58]. After cleaved by Dicer, the asymmetrical hairpin pre-miRNA generates a small, imperfect dsRNA duplex which contains two short RNA strands. One of the strands is the mature miRNA and the other one is the complementary strand of the miRNA [59-61].

However, little is known about the transcriptional regulation of pri-miRNAs except certain miRNAs located within host genes. The host genes for miRNAs are either protein coding genes or noncoding RNA transcription units in mammalians [62]. In some cases, these miRNAs within host genes are transcribed in parallel with their host transcripts, indicating the transcription of these miRNAs is regulated by the promoter regions of their host genes [3, 63]. However, the transcription of miRNAs and host genes can also be uncoupled with separate promoters and processing procedures. Certain genome wide analyses focus on identifying the promoter regions of human miRNAs. These studies show that about 30% of intronic miRNAs transcribe with alternative promoters. These independent promoters of miRNAs can be located

thousands of nucleotides of the upstream of the miRNA sequences. Additionally, many of these promoters contains the TATA box which was found in protein coding genes [64-67].Transcription factors to miRNAs can be predicted based on genome wide survey. Interestingly, the genome wide survey reports that in some cases, the transcription factors are in turn the target of the miRNAs that they regulate, indicating the existing of feedback loops in miRNA transcriptional regulation [68].

Some miRNAs locate close in the intron of the host gene and transcribed together as one transcriptional unit called a “cluster” [4, 69]. These miRNAs usually share identical seed sequence and are defined as members of a miRNA family, like miR-29 family [70].

Numerous studies have already established the role of miRNAs in cell growth, differentiation, and apoptosis. However, recent studies are attaching great importance to the role of miRNAs in lipid metabolism. Lipid metabolism involves in several organs including adipose tissues, and each organ has unique miRNA expression profile. In adipose tissues, miR-14 is the first miRNA reported to involve in lipid metabolism in *Drosophila* [5]. Flies lacked in miR-14 had enlarged lipid droplets with elevated levels of TAGs and DAGs while the overexpression of miR-14 showed the converse effects. In mammalians, several miRNAs are reported to regulate lipid metabolism in adipose tissues such as miR-125a-5p [71], miR-155 [72], miR-103, miR-143 [73]. The expression of miRNA-125a-5p mediates lipid uptake in macrophages [71]. miR-103 and miR-143 leads to increase the expression of PPAR-γ2, and miR-103 increases the expression level of fatty acid binding protein 4 (FABP4) [73]. miR-33 family members are key regulators in lipid metabolism. miR-33a and miR-33b locate in introns of their

target genes, SREBP-2 and SREBP-1, respectively and regulate SREBP expression. SREBP regulates cholesterol synthesis through transcriptional regulation of LDL-c receptors [74].Additionally, overexpression of miR-33a/b inhibits FA oxidation [75]. miR-93 is supposed to regulate the replacement of preadipocytes and adipocytes in visceral white fat through the inhibition of Sirt7 and Tbx3 [76]. The activation of the target genes of Sirt7 increases fatty acid uptake and triglyceride synthesis or storage. Besides the miRNAs reported above, the functions of bunches of miRNAs in lipid metabolism in adipose tissue are still under research.

In addition to adipose tissue, miRNAs alter in other tissues in response to HF diet including liver, skeletal muscle, and endothelium. Studies showed that miR-467b regulated lipid metabolism by affecting the expression of hepatic lipase (HL) in C57BL/6 mice. HF diet reduced the expression of miR-467b accompanied by increased level of HL and increased risk of hepatic steatosis [11]. This group also reported that HF diet downregulated miR-21 expression in liver from mice accompanied with upregulation of fatty acid binding protein 7 (FABP7), a potential target of miR-21 [77]. Overexpression of FABP7 was observed in hepatic steatosis but the function of FABP7 in liver remain unknown [77]. miR-122 is another miRNA related to lipid metabolism through serum cholesterol regulation and is highly abundant in liver [78]. In hepatic cell lines, the overexpression of miR-33s reduced fatty acid oxidation by targeting at the mRNAs of key enzymes in fatty acid oxidation including carnitine O-acetyltransferase (CROT) , carnitine palmitoyltransferase 1a (Cpt1a), hydroxyacyl-CoA-dehydrogenase, Sirtuin-6 (SIRT6), and AMP kinase subunit-α while the inhibition of miR-33s acted conversely [79]. In skeletal muscle, there are only a few studies reported miRNAs related to lipid

metabolism to date. Several studied reported miR-1, miR-133a/b and miR-206 were downregulated and miR-144, miR-106b were upregulated in response to HF diet [80]. In endothelial tissue, miR-29b [81], miR-1 [82] and miR-155 [83] appears to play an important role in the development of cardiovascular diseases following HF diet. The expression of these miRNAs is downregulated in response to HF diet in most animal models [81-83].

In summary, miRNAs are widely related to the post-transcriptional regulation of genes related to lipid metabolism in different tissues following HF diet. The mechanisms that miRNAs regulate lipid metabolism are complex and vary across different tissues. The link between miRNAs and lipid metabolism is waiting to be investigated.

miR-29 families

The miR-29 family consists of miR-29a, miR-29b and miR-29c, sharing the same seed sequences. The genes encoding the precursors of miR-29 families locates on different chromosomes. The DNA sequences for miR-29a and miR-29b-1 locates on the reverse chain on chromosome 6 in mouse and on chromosome 7 in human, while the DNA sequence encoding miR-29c and miR-29b-2 locates on the forward chain on chromosome 1 in both mouse and human. Mature miR-29b-1 and miR-29b-2 share the same RNA sequences. miR-29s located at the same chromosome are transcribed together as a polycistronic primary transcript [9].

For miR-29a/b-1 transcripts, they can be encoded by either the last exon or last intron of the primary transcripts in human, while miR-29b-2/c can only be encoded by last exon of the primary transcripts in human [9, 84]. Different process of slicing influence the regulation of the transcription of miR-29s but yields the same mature miR-

29s [85]. The sequences of mature miR-29 family members are highly conserved with the identical seed region AGCACC across various species, including human, rat, and mouse.

Recent studies have reported several factors regulating miR-29 expression through transcriptional regulatory mechanisms. Chromatin immunoprecipitation analysis demonstrated the binding of c-Myc in the promoter regions of both miR-29a/b-1 and miR-29c/b-2 in human cell lines and the binding of c-Myc represses the expression of miR-29s [9]. The overexpression of c-Myc contributed a decreased RNA level of miR-29s in human and mouse models of B cell lymphoma [9]. Further studies reported the potential interaction between c-Myc and histone deacetylase 3 (HDAC3), histone-lysine N-methyltransferase 2 (EZH2), which cooperatively contributed to the repression of miR-29 transcription in B-cell lymphomas (BCLs) [10]. Additionally, there was a positive feedback loop between the expression of c-Myc and EZH2 where c-Myc upregulated EZH2 and EZH2 induces c-MYC in turn.

In addition to c-Myc, studies also identified several binding sites on the promoter region of miR-29s for transcriptional regulation and several signaling pathways that involved in miR-29 regulation. Mott et al. identified a Gli-binding site, and four NF-κB-binding sites on human miR-29b-1/miR-29a promoter region in cholangiocarcinoma cells, suggesting that miR-29b-1/miR-29a was suppressed by the activation of c-Myc, hedgehog, and TLR/NF-κB signaling pathways [84]. Furthermore, NF-κB can also suppress the expression of miR-29b-2/miR-29c through NF-κB-YY1-miR-29 circuitry where YY1 is a transcriptional target of NF-κB and it repressed miR-29b-2/miR-29c by binding at miR-29b-2/miR-29c promoter region in mouse myoblasts and finally affected

myogenesis [86]. Qin et al. reported that miR-29b-2 was downregulated by TGF-β (1) and the repression was mediated by Smad3 which binds to conserved sequences at the promoter of miR-29b-2 in human myeloid cells [87]. Canonical Wnt signaling pathways can also regulate miR-29a transcription. There was two potential T-cell factor/LEF-binding sites within the proximal promoter of human miR-29a, and the transcriptional factors can be activated by β-catenin during canonical Wnt signaling in hFOB1 cells [88]. Other factors, for example, CCAAT/enhancer binding protein alpha (CEBPA), may activate miR-29a/b-1. There is a CEBP binding site on miR-29a/miR-29b-1 locus in chromosome 7, and the absence of CEBPA causes silencing of miR-29a/b-1. Notably, the CEBPA only activates miR-29a/miR-29b-1 on chromosome 7 while has no effect on miR-29b-2/miR-29c on chromosome 1[89].

MiR-29 family members suppress the gene expression by binding to 3'UTR of target mRNAs as other microRNAs. The target mRNAs of miR-29 that have been investigated related to cell proliferation, differentiation, apoptosis [10, 86, 88], extracellular matrix[90-93], and lipid metabolism [7, 8, 94, 95]. In this way, miR-29s play important roles in the development of several diseases, including cancers, fibrosis, diabetes, and dyslipidemia.

MiR-29s are downregulated in several types of cancers, thereby, they serve as tumor suppressors in most studies [9, 84]. Studies reported that reduced miR-29s expression in cancers cells further deteriorated the cancer development, suggesting the restoration of miR-29s can be a novel potential therapy of several types of cancers [96, 97]. Garzon et al. conducted a study in acute myeloid leukemia and confirmed the beneficial effect of exogenous miR-29 treatment [97]. The restoration of miR-29b in

acute myelogeneous leukemia (AML) cells reduced cell growth and induced apoptosis while the overexpression of miR-29b inhibits leukemic growth in a xenograft model [97].

In addition to cell proliferation and apoptosis, miR-29s also contribute to cell differentiation. The down regulation of miR-29 during myogenesis accelerated differentiation in Rhabdomyosarcoma (RMS) cells by targeting at YY1, which was also a suppressor of miR-29 as reported in this study, indicating that miR-29 plays an important role in regulating cell differentiation [86]. Another study performed in the mesenchymal precursor cell line hFOB1.19 and human osteoblasts also showed that miR-29a is essential for mediating human osteoblast differentiation by downregulating Wnt signaling antagonist and enabling Wnt signaling [88].

Although most previous researches focused on the regulation mechanism of miR-29 family members in cancer development, there is an increased number of studies revealed the role of miR-29s in lipid metabolism. Lipoprotein lipase, encoding by LPL gene, is a key enzyme in lipid metabolism for TAGs hydrolyzation in multiple tissues. Bouvy-Liivrand et al. reported that the mRNA levels of lipoprotein lipase (LPL) were inversely correlated with the expression level of miR-29a, indicating LPL is a potential target of miR-29a in 3T3-L1 adipocytes. In their study, complete LPL-3'UTR was cloned into a psiCHECK-2 plasmid and the results from luciferase assay confirmed that miR-29a inhibited LPL mRNA transcription by interacting with LPL-3'UTR [7]. Another study performed in mammary epithelial cell (MEC) supplemented the inhibition effect of miR-29b on LPL [8]. Moreover, caveolin2 (CAV2), a gene encoding protein related to lipid metabolism, was also a validated target of miR-29 family in adipose tissues [98].

In summary, miR-29 family members participate in many metabolic processes by regulating either upstream signaling pathways or downstream target genes. The metabolic changes can alter the transcription of miR-29s by activating transcription factors that have specific binding sites on miR-29 promoters. On the other hand, miR-29s can in turn downregulate their direct target genes through the conserved seed sequences in 3'UTR of the target genes. At present, most studies focus on the role that miR-29 family plays in different types of cancers, there is a growing interest from researchers in the function of miR-29 family in lipid metabolism.

c-MYC proto-oncogene

c-Myc is a transcription factor regulating expression of genes that control cell growth and proliferation [99]. It belongs to the Myc family that includes b-Myc, c-Myc, l-Myc, n-Myc and s-Myc. However, only c-Myc, l-Myc and n-Myc are oncogenes that have ability to promote tumorigenesis [99]. Most studies focus on the role of c-Myc in cell cycle progression, which is associated with cancer development.

C-Myc plays a role in glycolysis, glutaminolysis, nucleotide biosynthesis, lipid synthesis, mitochondrial biogenesis, and cell cycle progression. Increased expression of c-Myc in liver promotes liver regeneration by preceding DNA synthesis and transiting hepatocytes from G0/G1 to the S phase [100]. C-Myc is commonly elevated in most cancers. The predominant impact of high c-Myc expression on microRNA is widespread suppression of microRNAs, which in turn contribute to tumorigenesis [9]. C-Myc acts as a transcription factor of cellular glucose and glutamine metabolism by activating the transcription of genes related to aerobic glycolysis and glutamine metabolism. Genes with strong Myc binding including lactate dehydrogenase alpha (LDH-α), enolase 1

(ENO1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hexokinase 2 (HK2), lactate dehydrogenase A (LDHA), 6-phosphofructokinase (PFKM) and triosephosphate isomerase (TPI1) are upregulated by c-Myc in rat fibroblast cells, human lymphoid cells and human breast epithelial cells [101, 102]. Glutaminase (GLS) mRNA levels are elevated with c-Myc induction. C-Myc can inderectly induce GLS mRNA levels by binding to the promoter region of C9orf3, the host gene of miR-23b, and represses miR-23b transcription in human P943 cells. MiR-23b subsequently suppresses the synthesis of GLS [103]. Other glutamine transporters such as ASCT2 and SN2 are transcriptional regulated by c-Myc in vitro [104]. The regulation of glycolysis and glutamine metabolism by c-Myc is coordinated with nucleotide synthesis for cell proliferation [105, 106]. Moreover, genes related to fatty acid synthesis such as acetyl-CoA carboxylase (ACC), fatty acid synthetase (Fasn), and stearoyl-CoA desaturase (SCD) are potential targets of c-Myc as measured by global gene expression analysis [107]. Notably, Fasn and SCD are regulated by SREBP as reported in several studies [108, 109]. However, both Fasn and SCD also contained conserved binding elements of c-Myc [106], indicating the existing of c-Myc may activate genes involved in fatty acid synthesis independent from SREBP. Induction of c-Myc expression is cooperated with increased mitochondrial mass and gene expression of mitochondria biogenesis, such as PGC-1β [110-112]. These studies indicated that relationship between c-Myc and mitochondria biogenesis.

C-Myc is post-translationally regulated by mTOR, which stabilizes the c-Myc protein and promotes c-Myc translation [113]. Several studies showed that mTOR inhibitors downregulated c-Myc protein levels in different models [114, 115]. Here, mTOR is a downstream gene of PI3K-AKT pathway that can be stimulated by growth factors such

as insulin, amino acids and inhibited by low energy (high AMP/ATP ratio) and WNT signaling [116, 117].

In summary, c-Myc is a primary transcription factor that involves in numerous cellular metabolism process and cell cycle progression. It can regulate the transcription of mRNA and microRNAs at transcriptional level. At present, most studies focus on the role of c-Myc in various type of cancer models and have great interest in c-Myc regulation of glycolysis, glutamine metabolism and nucleotide synthesis. However, c-Myc provide a novel regulatory mechanism of lipid metabolism by targeting at specific sets of genes involved fatty acid synthesis and regulated these genes independent from SREBP.

Conclusion

Chronic HF diet has vital impact on lipid metabolism by regulation FA catabolism and anabolism process. Aberrant expression of genes related to lipid metabolism can cause metabolic disorders and chronic diseases such as obesity, type 2 diabetes, and cardiovascular diseases. Liver, adipose, and skeletal muscle are organs that can be affected by HF diet notably because of the roles they play in lipid metabolism. Previous studies have shown the significant and specific impacts of HF diet on lipid metabolic gene expression in these organs. It remains elusive about the mechanism that HF diet impacts metabolic function in these organs.

miRNAs regulate metabolism in most mammalian tissues including adipose tissues through complimentary pairing between ‘seed’ region of miRNAs and 3’UTR of their target genes. It was reported that miR-103, miR-143, miR-33a, and miR-29s have been

implicated as regulator of PPAR-γ, SREBP2, LPL, respectively. However, the impact of HF diet on lipid metabolic function in adipose tissues has not been completely studied.

The information about transcriptional regulation of miRNAs is limited. Certain miRNAs located within host genes may transcribe parallel with their host genes though the same promoter. The transcription factors and promoter regions of the miRNAs involved in lipid metabolism are needed to be identified under the HF condition.

To further investigate this topic, we plan to study the regulatory mechanism of lipid metabolic genes in adipose tissues from HF-fed mice. Post-transcriptional regulation of genes involved in lipid metabolism will be investigated. Furthermore, the transcriptional regulation and transcription factors of miRNAs involved in lipid metabolism will also be evaluated to further illustrated the regulation mechanism of responses from HF diet.

Chapter 3: Epigenetic Regulations of Genes Related to Lipid Metabolism by MicroRNA in Mice Fed High Fat Diet

Introduction

Environmental factors such as diet patterns, exercise, drugs, and radiation play an important role in the development of diverse chronic diseases. Diet is one of the critical factors contributing to the origin of these diseases. Long-term high fat (HF) diet causes the accumulation of fat droplets for extra triglyceride storage in multiple tissues including liver, adipose, and muscle. Fat accumulation in adipose tissue due to the increases size and number of adipocytes resulting in body weight gain and obesity. Moreover, the excessive adipocytes impacts the normal lipolytic and secretory function of adipose tissue, leading to inflammation [118, 119], insulin resistance [120] and other metabolic diseases. Abnormal lipolytic function of adipose tissue subsequently release greater amount of free fatty acids into blood causing high triglyceride levels in blood. Triglycerides in blood can flux to liver and muscle through blood circulation and cause fat accumulation in the liver and muscle [121, 122].

Lipoprotein lipase (LPL) is primarily synthesized in parenchymal cells of various tissues including skeletal muscle, adipose tissues, and liver [36]. Once synthesized, it is translocated to the vascular surface with the participation of the apolipoprotein C-II and glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1) and acts at ‘binding-lipolysis sites’ on the vascular surface of the endothelial cells in tissues [34]. Overexpression of LPL accelerates TAG hydrolysis and FA uptake. In adipose tissues, increased TAG hydrolysis causes lipid storage and enlarges

adipocytes [38]. While in muscle, FFAs generated from plasma lipoproteins by LPL are oxidized to provide energy through beta-oxidation in mitochondria [37].

LPL can be regulated through both transcriptional and post-transcriptional mechanisms. Some transcription factors regulate the LPL expression by binding to specific binding sites on the promoter of LPL gene. For example, peroxisome proliferator-activated receptors (PPARs), one of the transcription factor of LPL, up-regulated the expression of LPL by dimerizing with the retinoic acid receptor (RXR), and binding to the peroxisome proliferator activated receptor responsive element (PPRE) in the promoter of LPL gene [42]. Besides the transcriptional signaling pathways, LPL expression is also post-transcriptionally regulated by microRNAs through complementary alignment between the 3'UTR of LPL mRNA and the seed region of regulation microRNA [95]. Seed sequences of miR-29a/b, miR-27a/b and miR-467b are complimentary to the 3'UTR sequences of LPL and the inhibitory effects of these miRNAs on LPL has been observed *in vitro* [47-50].

Decreased expression of miR-29s has been observed in endothelial tissue following HF diet [123]. The mechanisms regulating expression of miR-29s are of interest. Although little is known about transcriptional regulation of miRNAs, recently, c-Myc was shown to contribute to miR-29s suppression in human cell lines [9, 10]. HDAC3 and EZH2 are also involved in transcriptional regulation of miR-29. These two transcription factors combine to form HDAC3-EZH2 complex that cooperatively regulate miR-29s with c-Myc in B-cell lymphomas (BCLs) [10]. In addition to c-Myc, other transcriptional binding sites, such as NF-κB, were found in human miR-29a/b-1 promoter region in human cholangiocarcinoma cells[84].

c-Myc is a transcription factor controlling cell growth, apoptosis, and metabolism by regulating relative genes at transcriptional levels. In various types of cancers, c-Myc binds to proximal promoter regions of genes regulating DNA replication, induces mRNA transcription and ultimately contributes to tumorigenesis [124]. However, c-Myc mainly acts as a negative transcription factor in microRNA regulation [9, 125]. MicroRNA expression levels were measured in the high and low c-Myc states in human B cells and the results showed lower microRNA levels in high c-Myc states including miR-29s while higher microRNA levels in low c-Myc states. It is reported that EZH2, an enzyme participates in histone methylation and eventually causes transcription repression, also participated in repression of miR-29s transcription. In B-cell lymphomas (BCLs), c-Myc may recruit EZH2 and repress miR-29 expression together [10]. Moreover, there are feedback loops existing between some microRNAs and c-Myc [125].

The present study was designed to investigate the regulation of lipid metabolism related genes mediated by transcription factors and microRNAs in response to high fat diet. We hypothesize that HF diet represses the transcription of miR-29 by activating the negative transcription factors and the reduced miR-29 expression leads to high mRNA level of its target gene, LPL, in adipose tissues in mice fed HF diet.

Experiment Methods

Animals and treatments

Four-week old male CBA mice (The Jackson Laboratory) were separated into two dietary groups and fed either control diet (10% kcal from fat, n=12) or high fat diet (45% kcal from fat, n=14) for 11 weeks. Mice were kept in standard polycarbonate cages (4

mice per cage) in a humidity- and temperature-controlled room on a 12-h light-dark cycle, with ad libitum access to food and drinking water. Body weight and food intake were recorded every 7 days and magnetic resonance imaging (MRI) was conducted monthly during treatment. Mice were sacred after 11 weeks of treatment. The liver, muscle, adipose and colon was snap-frozen in liquid nitrogen and stored at −70°C for future use.

RNA isolation and quantitative reverse transcription-PCR analysis

All mice tissues were ground in liquid nitrogen and lysed with Trizol Reagent (Life Technologies). Total RNA was isolated with Directzol RNA Miniprep Kit (Zymo Research), according to the manufacturer's recommendations. Total RNA concentration was quantified spectrophotometrically (NanoDrop ND-2000 Spectrophotometer; Thermo Fisher Scientific, Waltham, MA). RNA was reverse-transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed in a 10 μL reaction volume containing 5 μL POWER UP SYBR green master mix (2×, Applied Biosystems), 0.4 μL of each forward and reverse primers (5 μmol/L), and 5 ng cDNA template by cycling at 50 °C for 2 min and 95 °C for 2 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min in a StepOnePlus real-time PCR system (Applied Biosystems). After amplification, melting curves were acquired stepwise from 55 to 95 °C to ensure that a single product was amplified in the reaction. Primer sequences, synthesized by Integrated DNA Technologies, are reported in Appendix 1. Geometric mean of mRNA levels of L7a, GAPDH, and beta-actin were used as an internal control to normalize raw data.

TaqMan® MicroRNA Assays

RNA isolation follows the same steps as mRNAs. MiR-29a and miR-29b were reverse transcribed into cDNA using TaqMan Micro RNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA) and specific primers from TaqMan™ microRNA Assays (5×, Applied Biosystems). Real-time PCR was performed in a 10 μL reaction volume containing 5 uL TaqMan® Fast Advanced Master Mix (2×, Applied Biosystems), 0.5 uL each primer from TaqMan™ microRNA Assays (20×, Applied Biosystems), and 0.25 ng cDNA template by cycling at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min on a StepOnePlus real-time PCR system (Applied Biosystems). TaqMan microRNA primer sequences are reported in Appendix 2. Expression level of U6 was used as an internal control to normalize raw data.

Transcription factor prediction

Three online programs, NCBI (http://www.ncbi.nlm.nih.gov/), UCSC (http://www.genome.ucsc.edu/), and Ensembl (http://www.ensembl.org/index.html), were used for promoter search of mus-miR-29a. Also, the JASPAR (http://jaspar.genereg.net/) and Enhancer Element Locator analysis (http://www.cs.helsinki.fi/u/kpalin/EEL/) are applied to predict the transcription factor binding site.

Chromatin Immunnoprecipitation (ChIP)

For ChIP analysis, 400 mg of adipose tissues were used per sample. Six different samples were used for each treatment group. Adipose tissues were ground in liquid nitrogen and suspended in 1x PBS buffer. Protein and DNA were cross-linked by adding

formaldehyde directly to a final concentration of 1%. Cross-linking was stopped 10 min later by adding 2 mol/L glycine to a final concentration of 0.125 mol/L. Cross-linked chromatin was solubilized by sonication using a Sonic Dismembrator (Model 100, Fisher Scientific Co.) for five bursts of 40 s at 50% of maximum power output with 2 min cooling on ice between each burst. The chromatin immunoprecipitation procedure used was previously described follow a modified protocol [126, 127]. Briefly, solubilized chromatin was diluted 1:10 with dilution buffer. Normal rabbit IgG was used as a negative control for the immunoprecipitation procedure. Antibodies against c-Myc (#13987) and EZH2 (#5246) were purchased from Cell Signaling Technology (Danvers, MA). The antibody was incubated with Dynabeads Protein G (thermos fisher scientific) at 4 °C overnight to form antibody-magnetic beads complex. The amount of antibody used for incubation follows the usage information from manufacturers. 1 mL diluted sample solution containing sheared chromatin was added to magnetic beads complex and incubated at room temperature for 2 hours. Pellets containing the immunoprecipitated complexes were washed subsequently with 1 mL of low salt wash buffer, high salt wash buffer, LiCl salt wash buffer and twice Tris-EDTA (pH=8.0). The immunoprecipitated complexes with antibody, protein and DNA were eluted from magnetic beads by adding twice 250 μL of elution buffer and shaking in incubator shaker (model EXCELLA E24R, Eppendorf company) at 37 °C, 300 rpm for 15 min. The DNA fragments in the immunoprecipitated complex were released by reversing the cross-linking at 65 °C for 5 h with 20 μL 5 mol/L NaCl and 1 μg of RNase A (Qiagen, Hilden, Germany). Proteins were removed from DNA fragments by treating with proteinase K (Sigma) at 37 °C for 1 h. DNA fragments were purified using a QIAprep

Spin Miniprep Kit (Qiagen). Purified DNA was analyzed using quantitative real time PCR with primers covering potential promoter regions of miR-29a/b-1 and miR29b-2/c. The sequences of primers are displayed in Appendix 3.

Statistical analysis

A Student's t-test was used to detect the significant differences between the treated group and the control group. A P value of less than 0.05 was considered significant. All data are means +/−SEM.

Results

High Fat Diet Induces Body Weight Gain and Increases Fat Percentage in Mouse

The body weight and fat percentage of mice in either control or HF group were recorded at birth. There were no significant differences between two treatment groups. After treatment, mice consuming HF diet showed more body weight gain when compared to those consuming control diet. The difference in body weight gain between control and HF group became significant after feeding for 8 weeks. The fat percentage of mice fed HF diet was higher than those fed control diet after 1-month treatment.

High Fat Diet Induces LPL mRNA Expression in Mouse Adipose, Muscle and Colon but not in Liver

In the study, we investigated the expression of LPL in multiple tissues of mice from both Con and HF groups. Relative mRNA expression of LPL in adipose, muscle, liver and colon were measured using real time PCR (Fig. 1A). The baseline of LPL expression was highest in adipose, followed by muscle, and was lowest in liver and colon. LPL

basic expression in adipose was 2-fold higher than that in muscle and was 142-fold higher than that in liver and colon. After HF treatment, mRNA of LPL significantly increased ($P<0.05$) in mouse adipose, muscle, and colon by 1.6-fold, 1.6-fold, and 4.6-fold respectively, while decreased in liver by 0.5-fold ($P<0.05$). Furthermore, LPL expression was highest in adipose, followed by muscle, colon and lowest in liver after HF treatment. Altered LPL mRNA level in adipose was 2.8-fold higher than that in muscle, 45-fold higher than that in colon, and 458-fold higher than that in liver. As lipoproteins in liver were hydrolyzed hepatic lipoprotein lipase (HL), a liver specific lipase, we measured HL expression in liver. The expression of HL in both control and HF groups was also quantified (Fig. 1B). The results showed HL mRNA expression decreased in the liver of HF mice by 0.9-fold compared to control group, which paralleled LPL mRNA expression in liver.

High Fat Diet Suppresses miR-29a/b Expression in Mouse Adipose

MiR-29s potentially suppress the expression of LPL as there are conserved complimentary pairing between miR-29a-3p, miR-29b-3p and LPL 3'UTR (Fig. 2A and B). As adipose is a critical organ in lipid metabolism and expresses highest mRNA level of LPL, we explored alteration of miR-29a/b expression and relationship between miR-29a/b and LPL expression in response to HF diet in adipose. The transcription of miR-29 family members including miR-29a and miR-29b was measures in adipose tissues. miR-29a ($P=0.06$) and miR-29b ($P<0.05$) RNA level was decreased in adipose from HF group (Fig. 2C). HF group with higher LPL expression had lower miR-29a and miR-29b expression.

High Fat Diet induces c-Myc binding at promoter region of miR-29s and Inhibits the Transcription of miR-29s cooperated with EZH2 in Adipose Tissue

To identify potential negative transcription factors of miR-29s induced by HF diet, mRNA abundance of these transcription factors of miR-29s was analyzed in adipose tissues (Table. 2). mRNA levels of c-Myc, EZH2, NFkB1 were significantly elevated in the adipose of the HF group compared with the control group ($P<0.05$), which was inversely correlated with miR-29a and miR-29b expression in adipose tissue. It was reported that some of these transcription factors may act together to be involved in miR-29 expression [10]. Then, we analyzed the promoter regions of miR-29a/b1 and miR-29b2/c for binding sites and the results showed that there were four potential c-Myc binding sites in ~6 kb upstream of miR-29a/b1 (S1, S2, S3, and S4) and in ~6 kb upstream of the host gene of miR29b2/c (S1, S2, S3, and S4) in mouse (Fig. 3A). We investigated whether c-Myc and EZH2 binds to promoter regions of miR-29s and cooperatively repressed miR-29 transcription using chromatin immunoprecipitation (ChIP). The results showed that c-Myc and EZH2 bound to promoter regions of miR-29a/b1 and miR-29b2/c (Fig. 3B-E). Moreover, both c-Myc and EZH2 bound to S2 of miR-29a/b1 and S1, S4 of miR-29b2/c and these bindings increased in response to HF diet (Fig. 3B-E). However, bindings of EZH2 were also increases in HF group at S3 of miR-29a/b1 and S3 of miR-29b2/c (Fig. 3D-E). Overall, c-Myc binding was significantly induced by HF diet at the promoter regions of miR-29s, including miR-29a/b1 (S2, $P<0.05$) and miR-29b-2/c (S1, S4, $P<0.05$) (Fig. 5B-C). Meanwhile, EZH2 also binds to promoter regions of miR-29s and the bindings increased in mice from HF group (Fig. 5D-E). Part of EZH2 binding sites corresponded to c-Myc binding sites. The results

indicated that c-Myc and EZH2 cooperatively inhibited the transcription of miR-29s in the adipose tissues of mice fed HF diet.

Discussion

The present study reports a novel finding that a high fat diet activates the transcription of lipoprotein lipase in mouse adipose tissues through several epigenetic mechanisms. First, a high fat diet activates the transcription of transcription factors, c-Myc and EZH2, leading to increased binding of these two transcription factors at the promoter region of miR-29s. C-Myc and EZH2 are negative transcriptional regulators of miR-29s and induced binding at promoter regions of miR-29s inhibits miR-29 transcription. Reduced transcription of miR-29s causes elevation of its target gene, LPL, through post-transcriptional regulation in adipose tissues in response to high fat diet. Increased lipoprotein lipase expression lead to accelerated TAG hydrolysis and FA uptake in adipose, which may be one of the reasons for HF-induced weight gain and fat percentage induction.

Lipoprotein lipases (LPL) is a critical enzyme associated with FA uptake in multiple tissues. LPL expresses in tissue-specific manners [40]. HF diet induces LPL expression in adipose tissue with the mediation of PPARγ [128]. LPL mRNA induction is more significant in female rats compared to male in adipose tissue [21]. The mRNA expression of LPL also increased in muscle in response to HF diet [21]. A study in obese mice showed that HF diet down-regulated LPL mRNA level in colon, which was consistent with the occurrence of obesity [129]. The alterations of LPL mRNA expression in adipose and muscle are consistent with our results showing a significant increase of LPL mRNA in adipose and muscle in mice fed HF diet. However, the

expression of LPL mRNA increased in colon in the present study. The results indicate that peripheral tissues including adipose, muscle and colon compensate excessive fat from diet by inducing lipoprotein lipase expression. Hepatic lipase (HL) is a liver specific-enzyme involved in liver lipid metabolism. There is a negative correlation between the liver HL level and the development of hyperlipidemia [130]. HF diet elevates TC, TAG and LDL-c in serum and significantly reduces the mRNA expression of HL in rats [131]. In the present study, we observed significant decreases of HL and LPL mRNA levels in liver even through the expression level of LPL in liver is much lower than that in other tissues. HL catalyzes TAG hydrolysis in chylomicron remnants and IDLs and facilities reverse cholesterol transport by providing HDL with apolipoproteins and phospholipids [132, 133]. Therefore, we speculate about the contribution of reduced HL levels to the accumulation of IDL in liver and the impairment of reverse cholesterol transport.

LPL activity has been reported to be regulated by several biochemical mechanisms, including transcriptional and post-transcriptional regulation as well as protein interaction. MicroRNAs are post-transcriptional negative regulators by binding to their target sequences on 3'UTR. Luciferase expression with LPL 3'UTR allele combinations demonstrated the binding of miR-29, miR-1277 and miR-410 in HEK-293T cells [95]. Genomic screen in mice identified that miR-29a is a repressor of LPL in liver and the decreasing of hepatic miR-29a levels causes lipid accumulation in mice liver [134]. Another study showed that the luciferase expression decreased in mammary epithelial cells after miR-29b co-transfection with LPL gene report vectors, indicating miR-29b was also a suppressor of LPL [8]. In the present study, we observed a negative

correlation between LPL and miR-29a/b when comparing HF group to control group, indicating miR-29s are involved in LPL post-transcriptional regulation in adipose tissues from mice fed HF diet. Expression reduction in miR-29b was more significant than that in miR-29a, indicating miR-29b may play the domain role in LPL down-regulation.

C-Myc is a master transcriptional regulator and potentially regulates more than 1500 genes as compiled in Myc target gene database (www.myccancergene.org), including genes for normal mRNAs and microRNAs [9, 124, 135]. The activation of c-Myc is common in many cancers, which may contribute to cancer development by reprogramming the transcription of miRNAs related to tumorigenesis [9]. HF diet can also induce c-Myc in various animal models [136, 137]. The predominant consequences of elevated c-Myc level in human and mouse lymphoma cells are widespread suppression of miRNAs, including miR-15a, miR-16, miR-22, miR-26s, miR-29s and miR-30s [9]. Normally, c-Myc protein expression in adipose is low, but HF diet increases the protein expression of c-Myc in adipose tissues [138]. The data present here suggest that dietary fat abundance enhances c-Myc mRNA expression and engages repression of miR-29s by binding to the promoter regions of miR-29 clusters. Although members of the miR-29 family located at two different chromosomes, the data indicated that c-Myc bound to both chromosomes in response to HF diet. The results suggest that the induction of c-Myc and the binding of c-Myc at the promoter regions of miR-29s may be the mechanism that repress the transcription of miR-29a/b in adipose tissues from mice fed HF diet. Additionally, other transcription factor such as EZH2, NF-κB, HDACs, can also regulate the expression of miR-29s as other studies reported [10, 84, 139]. While it is reported that EZH2 could be recruited by c-Myc. EZH2 is an enzyme catalyzing

histone methylation. It is upregulated in multiple cancers and inhibits genes responsible to repress tumor development [140, 141]. In the present study, EZH2 is also upregulated and binds to the promoter regions of miR-29s in adipose from HF treated mice, indicating miR-29 repression is caused by histone methylation at the promoter region. Previous studies have showed that the downregulation of miR-122, miR-125b and miR-106 was associated with DNA methylation in liver injury [142], lower miR-200b, miR-152 and miR-10a expressions was related to higher DNA methylation in bladder cancer [143], and miR-10a was silenced because of abnormal DNA methylation in gastric cancer [144]. These publications indicate that DNA methylation may be an important factor regulating microRNA expression. Therefore, c-Myc and EZH2 may act together repressing the transcription of miR-29s through DNA methylation in HF-fed mice adipose.

In summary, we have demonstrated that HF diet causes the activation of transcription factors, c-Myc and EZH2, and enhanced binding of transcription factors at the promoter regions of miR-29s in adipose tissue, which repressed transcription of miR-29a/b. Our study also demonstrated that miR-29a/b might be a post-transcriptional repressor of LPL in adipose tissue with HF treatment. Our study showed a potential model where HF diet induced LPL expression with c-Myc/EZH2 - miR-29 regulation in adipose tissue, which provides a potential connection between oncogenes and lipid metabolism.

Future Study

The results of the present study suggest that HF diet induces expression of LPL through c-Myc/EZH2 – miR-29 – LPL axis. As LPL hydrolyzes triglycerides in lipoprotein

and generates free fatty acids [40, 145], the induction of LPL in adipose, muscle and colon may cause an increased fatty acid influx in these tissues. Increased influx in these peripheral tissues will probably affect TAG synthesis and FA oxidation, which may be one of the causes for HF-induced dyslipidemia. Therefore, the current study provides a potential regulatory mechanism behind HF-induced dyslipidemia, which can be applied to study the therapy for dyslipidemia that intervenes the regulator function in this axis.

For further study, the storage and oxidation of excessive fatty acid generated by overexpressed LPL in different tissues can be investigated by measuring TAG synthesis genes such as DGAT and β-oxidation genes such as Cpt1. Additionally, as the regulatory mechanisms by which miR-29b regulated LPL were studied in mammary epithelial cells [8]. Meanwhile, in present study, we observed inverse relationship between miR-29a/b and LPL in adipose from mice. Thereby, further study can be performed *in vitro* using adipocytes to test the binding of miR-29s at 3'UTR of LPL and confirm the post-transcriptional regulatory effect of miR-29s on LPL. Furthermore, this study reported c-Myc/EZH2 – miR-29 – LPL regulatory axis. Future study can focus on treatments for dyslipidemia caused by HF diet by targeting at this regulatory axis. For example, researchers can explore other transcription factors, histone proteins and DNA methylation involved in miR-29s regulation cooperated with c-Myc in adipose tissue under HF treatment. The reagents and drugs affecting the function of EZH2 or c-Myc can also be studied. In addition to adipose tissue, this regulatory axis can also be applied to other tissues and to determine the effects and mechanisms of therapies for HF-induced dyslipidemia.

Tables and Figures

Table 1. Macro- and micronutrient composition of the diet in this study

Diet Formula	**Control**		**High Fat**	
	gm%	kcal%	gm%	kcal%
Protein	19.2	20	24	20
Carbohydrate	67.3	70	41	35
Fat	4.3	10	24	45
Total		100		100
kcal/gm	3.85		4.73	
Ingredient	gm	kcal	gm	kcal
Casein, 30 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	452.2	1808.8	72.8	291
Maltodextrin 10	75	300	100	400
Sucrose	172.8	691.2	172.8	691
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	177.5	1598
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0

Table 1 (cont.)

Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye#5	0.04	0	N/A	N/A
FD&C Red Dye#40	0.01	0	0.05	0
Total	1055.05	4057	858.15	4057

Table 2. Relative mRNA expression of potential negative transcription factors of miR-29s in mice from Con and HF group[1,2]

	Con	**HF**
c-Myc	0.50 ± 0.07	1.12 ± 0/11 *
EZH2	0.94 ± 0.06	1.23 ± 0.11 *
HDAC3	1.38 ± 0.06	1.00 ± 0.08 *
NFkB1	0.74 ± 0.05	0.98 ± 0.09 *
CTNNB1	0.55 ± 0.04	0.60 ± 0.06
Gli1	1.00 ± 0.11	0.30 ± 0.05 *
YAP1	1.18 ± 0.05	0.92 ± 0.07 *

1 All values are means ± SEM, n=12 (Con) or 14 (HF). *P<0.05

2 Expression of mRNA is the ratio to the geometric mean of L7a, GAPDH and Actb.

A

B

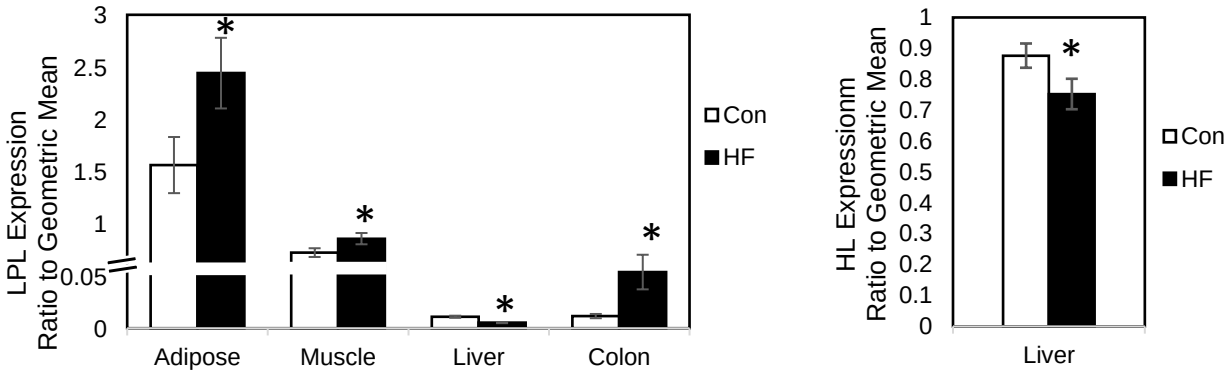

Figure 1. mRNA expression of lipoprotein lipase and hepatic lipase in multiple tissues. A) LPL mRNA expression in mouse adipose, muscle and liver. B) HL mRNA expression in mouse liver. All values are means ± SEM, n=12 (Con) or 14 (HF). *Different from Con, $P<0.05$.

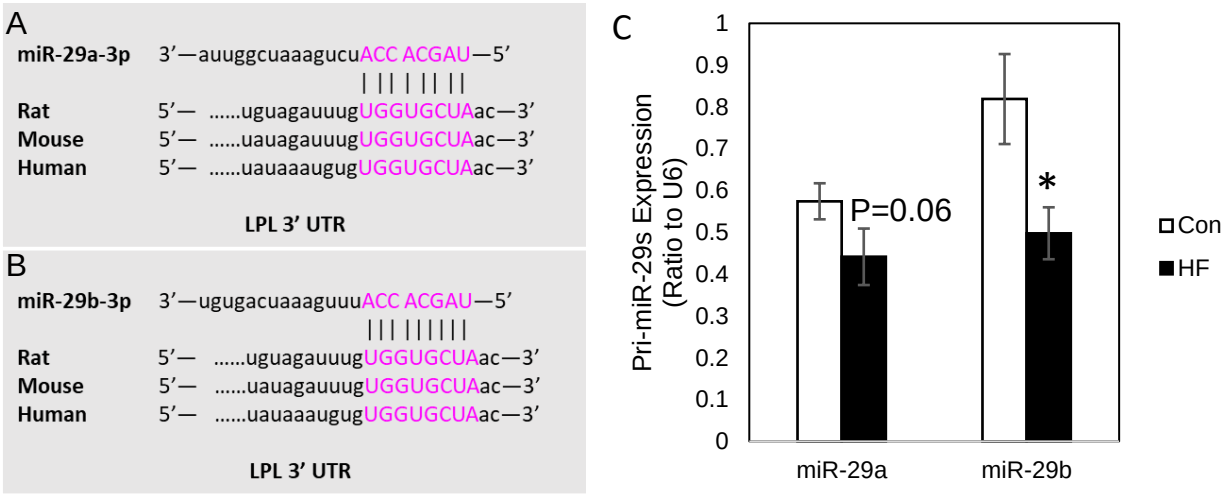

Figure 2. A) Conserved complimentary pairing between miR-29a-3p and LPL 3'UTR. B) Conserved complimentary pairing between miR-29b-3p and LPL 3'UTR. C) miR-29a/b expression in mouse adipose. All values are means ± SEM, n=12 (Con) or 14 (HF). *Different from Con, P<0.05.

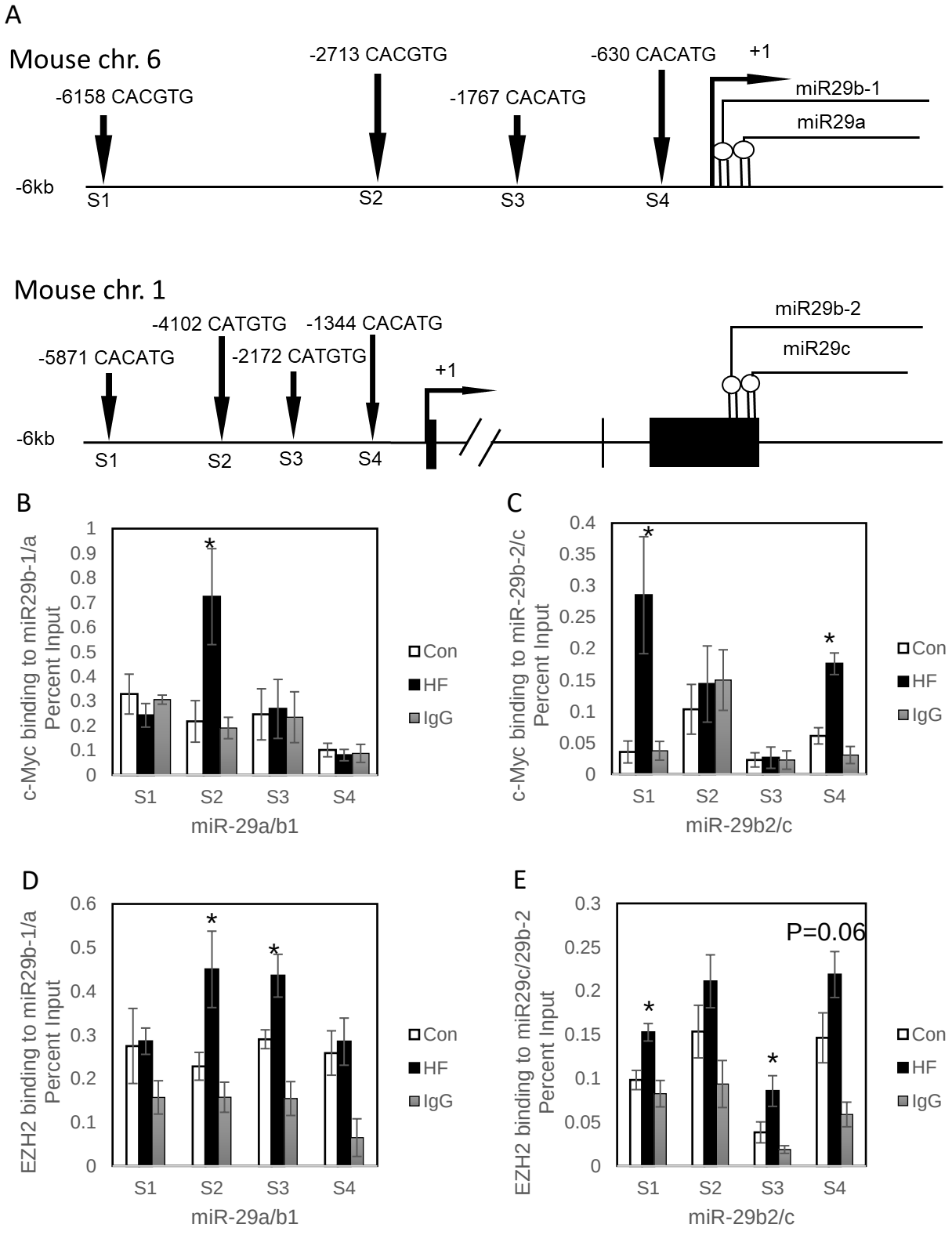

Figure 3. A) Schematic diagram showing location of Myc-binding sites of pri-miR-29a/b1

Figure 3 (cont.)

and pri-miR-29b2/c regulatory region. S1, S2, S3 and S4 represent Myc-binding site, which has E-box sequence. Both pri-miR-29s are highly conserved in their putative promoter region on chr.6 (pre-miR-29a/b1) and on chr.1 (pre-miR-29b2/c) respectively. B-C) ChIP assay showing c-Myc binding on pri-miR-29a/b1 (B) and pri-miR-29b2/c (C) promoters. D-E) ChIP assay showing EZH2 binding on pri-miR-29a/b1 (D) and pri-miR-29b2/c (E) promoters.

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Appendix A: Supplementary Tables

Table 3. List of Primers for Real-time Quantitative PCR

Gene Name	Location	Sequence	Ensembl/NCBI ID
LPL	mLPL+471F	ACCACAGCAGCAAGACCTTC	ENSMUST00000015712.14;
	mLPL+522R	GGGCACCCAACTCTCATACA	
HL	mHL+698F	ATGGCTGGAGGAATCTGC	ENSMUST00000034731.9;
	mHL+761R	TCCCAGGCTGTACCCAAT	
CPT1a	mCpt1a +160 F	CATGAAGCCCTCAAACAGAT	ENSMUST00000025835.4
	mCpt1a +235 R	TGCCATTCTTGAATCGGA	
Cpt1b	mCpt1b+963F	AGAGACAGACTTGCTACAGC	ENSMUST00000109313.8;
	mCpt1b+1039R	AGACCTTGAAGAAGCGAC	
C/EBPα	mCEBPa+1372F	TCGGTGCGTCTAAGATGA	ENSMUST00000042985.10;
	mCEBPa+1433R	AAAGTCTCTCGGTCTCAAGG	
NF-κB	mNFkB1+1009F	CATTCTGACCTTGCCTATC	ENSMUST00000029812.13;
	mNFkB1+1074R	CTTCTCTCTGTCTGTGAGTTG	

Table 3 (cont.)

Gli1	mGli1+1054F	AATGATGCTTCACCCCCAGT	ENSMUST00000026474.4;
	mGli1+1130R	TGCCAACCATCATATCCAGC	
HDAC3	mHDAC3+318F	CACCAAGAGCCTTAATGC	ENSMUST00000043498.7;
	mHDAC3+397R	TGTAACGGGAGCAGAACT	
Yap1	mYap1+911F	CCATAAGAACAAGACCACATCC	ENSMUST00000065353.12;
	mYap1+987R	TCTGAGTGATCCTCTGGTTCAT	
EZH2	mEZH2+847F	GGCACCGCAGAAGAACTG	ENSMUST00000081721.12;
	mEZH2+921R	TTCAGGAGGCAGAGCACC	
c-Myc	mCmyc +1369F	CCAGCAGCGACTCTGAAGAAGAGC	ENSMUST00000188482;
	mCmyc +1459R	TCCGACCTCTTGGCAGGGGTTT	
ß-catenin	mCTNNB1+980F	CTTTAAGTCTGGTGGCATCC	ENSMUST00000007130.14;
	mCTNNB1+1058R	CGTGATGGCGTAGAACAGTA	

Table 3 (cont.)

L7a	mRpl7a +176F	AACTTCGGCATTGGACAGGACA	ENSMUST00000102898;
	mRpl7a +289R	TTTGAGCCGCTTGTAGAGGATAGC	
GAPDH	mGAPDH +149F	CCA GCT TGT TCC TTC AGACC	ENSMUSE00001143324;
	mGAPDH +234R	GCC CTC AAG GAC AAAGAC AG	
ß-actin	mActB+153F	GAT GCT CCC CGG GCTGTA TT	ENSMUST00000100497.10;
	mActB+219R	TTC CCA CCA TCA CAC CCTGG	

Table 4. List of Primers for TaqMan MicroRNA Assays

Assay Name	Assay ID	Catalog	Mature miRNA Sequence	miRBase Accession Number/ NCBI Accession Number
hsa-miR-29a	002112	4427975	UAGCACCAUCUGAAAUCG GUUA	MI0000087
hsa-miR-29b	000413	4427975	UAGCACCAUUUGAAAUCA GUGUU	MI0000105
U6 snRNA	001973	4427975	GTGCTCGCTTCGGCAGCA CATATACTAAAATTGGAAC GATACAGAGAAGATTAGC ATGGCCCCTGCGCAAGGA TGACACGCAAATTCGTGA AGCGTTCCATATTTT (Control Sequence)	NR_004394

Table 5. List of Primers for Chromatin Immunoprecipitation

Gene Name	Location	Sequence	Ensembl /NCBI ID
miR-29a/29b-1	miR29b-1-588F	GAAAGTCTCAGCACTCAG	ENSMUST00000083670.2;
	miR29b-1-515R	TCTCACTATGTAGCCCTG	
	miR29b-1-1739F	AAAGAGGGCATCACATCC	
	miR29b-1-1678R	TTCAGTTCCCAGCACACA	
	miR29b-1-2952F	GGTGCTCATTCAAGTGTGC	
	miR29b-1-2873R	AGTGAGAGGCATACACGGA	
	miR29b-1-5928F	ATTCCCCCCTGTCTTGACAT	
	miR29b-1-5859R	GCTGCTGGTAGTTCTGGAAGTT	
miR-29b-2/c	A330023F24Rik-1436F	ATGAGTTCTCCCCACACCTTCT	ENSMUST00000194584.1;
	A330023F24Rik-1369R	AGCCTAGAACCCAGAGTTTCCA	

Table 5 (cont.)

A330023F24 Rik-2406F	TGATGCTGTTGAGTCCTG
A330023F24 Rik-2343R	CAGTATTGGGGATAAGGG
A330023F24 Rik-3772F	CTGTTCTTTAGTGAGGCTCC
A330023F24 Rik-3693R	TGAGCAGGCAGACAAGAT
A330023F24 Rik-5417F	CAATCTGCCACACCTCAGTG TT
A330023F24 Rik-5350R	ACCTTGCCCGATAGTTTCCA