EPIGENETICS, A SIGNAL SENSOR OF DIETARY COMPONENTS, IS INVOLVED IN THE HEPATIC GENE REGULATION

BY

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DISSETATION

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Abstract

Epigenetic markers are associated with a broad range of disease symptoms, including cancer, asthma, metabolic disorders, and various reproductive conditions. Changes in epigenetics status could be induced by environmental exposures such as malnutrition, stress, smoking, disease and exposure to air pollutants and organic chemicals. Dietary factors were extensively acknowledged to be one of the key environmental essences affecting chromatin structure. It can diversify the patterns of DNA methylation and histone modifications. The changes may be pathogenic and result in severe physiological consequences, particularly if the exposure occurs during critical window of development. This is potentially attributable to the property of epigenetic activities, which occurs not only in somatic and mitotic cells, but also inherited meiotically, meaning that the changes are carried over generations [1, 2]. Therefore, maternal dietary factors may profoundly characterize the phenotype of subsequent generations and consequently contribute to the early onset of chronic disease and metabolic disorder [3].

Sustained high fat feeding leads to obesity and metabolic syndrome, accompanied by low-grade inflammation, insulin resistance, and dyslipidemia. In general, the prevalence of an inflammatory response is highly associated with obesity incidence, cardiovascular diseases, non-alcoholic fatty liver disease (NAFLD) and brain damage. In liver, changes caused by low-grade inflammation may later result in progressive disease and fibrosis. Early exposure to a fat-enriched diet programs the developmental profile, thus is associated with disease susceptibility in subsequent generations. Chronic low-grade inflammation, resulting from high fat diet, is activated in the fetal environment and in many organs of offspring, including placenta, adipose, liver, vascular system and brain. It forms the biological basis for many patho-physiological changes and constitutes risk factors for disease development.

Chronic inflammatory disorders were recently identified as one of the major targets of dietary-induced epigenetic regulation [4]. Although some transgenerational effects of
high dietary fat intake have been shown in a couple of studies linked to certain types of epigenetic modulations [5-8]; these modifications and their associated physiological consequences resulting from high fat induced-chronic inflammation remain elusive. Should the MHF-resulted epigenetic code persist throughout generations, characterization in such situations may favor the prediction, early prevention, and treatment of non-communicable disease in next few generations [9].

Many studies using high fat models have consistently demonstrated the incidence of such inflammatory reactions. However, the potential contributions of epigenetic modifications toward the regulation of inflammatory genes and subsequent physiological outcomes have not been fully revealed in the high fat feeding model. Cyclooxygenase-2 (COX-2) produces prostaglandins that participate in multiple physiological and pathological processes, including the activation of inflammatory responses. In addition to many of the transcription factors known for years, it was recently suggested that COX-2 expression is also subjected to epigenetic modifications, either through histone remodeling and/or DNA methylation [10]. Therefore, our study aims to investigate the epigenetic mechanisms by which consumption of a high fat diet at different life stages influences the inflammatory marker COX-2.

Male Sprague-Dawley rats received a high fat diet at different life stages, including maternal (HF/C), post-weaning (C/HF), and lifelong (HF/HF). Liver was collected for analysis at 12 weeks of age. Results showed that the high fat diet induced the expression of COX-2 mRNA in all three high fat groups. RNA abundance of COX-2 in HF/HF was significantly higher than that of HF/C and C/HF. Meanwhile, fatty acid composition showed that the proportion of Linoleic acid and Arachidonic acid, as well as Δ6Desaturase, were significantly increased in high fat groups, potentially motivating the biochemical flow as well as providing reaction substrate for COX-2 catalysis. Genome-wide methylated DNA immunoprecipitation (MeDIP) showed that DNA hypomethylation occurred in an upstream region of the distal promoter and two coding regions of the COX-2 gene in all three high fat groups. Site-specific hypomethylation of CpG at 5’ UTR (untranslated region) of COX-2 was confirmed with bisulfite sequencing. Using in vitro
cloning and Luciferase Reporter assay, this region was identified as a novel enhancer that produces durable transcriptional activity. In conclusion, high fat intake during different life stages resulted in a varied induction level of COX-2 gene expression. Altered DNA methylation at specific gene region, including the 5'UTR enhancer sequence, may closely associate with COX-2 gene activation.

This study presented a gene-wide illustration of the diet-epigenome interaction. It also provided evidence of high fat diet-induced region-specific hypomethylation in the liver of the offspring. Since limited research has been conducted to reveal the epigenetic regulation of inflammatory markers by high fat diet, this study will not only outline the dietary outcomes on the epigenetic profile of a specific inflammatory gene, but also provide insight for future mechanistic investigation and clinical appliance in the area of epigenetics.
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CHAPTER 1. GENERAL INTRODUCTION

Environment is a major factor in disease etiology [11, 12]. A large number of environmental compounds and toxicants have been shown to promote disease, and are independent of DNA sequence alterations [13]. Nutrition is a critical environmental factor that is indispensable for an individual's well-being. Nutrients provide elements that are essential for life while sustaining basic and advanced biological functions. Macronutrients feed human energy needs and also provide elemental building blocks, while micronutrients maintain proper bodily functions on the cellular level. Under-consumption and overconsumption of certain nutrients is related to an increased health risk in both animals and humans, including a higher prevalence of obesity, cancer, diabetes, and many other non-communicable diseases. The cumulative knowledge of nutrition and other relevant research points to the importance of being aware of one’s daily dietary intake.

Non-communicable diseases are initiated through altered gene expression, and are followed by the aberrant regulation of cellular signaling pathways and physiological behaviors. The traditional understanding of DNA is that it is expressed by reading its genetic sequence, which is set in stone during our and our children’s lives. Although the genetic code is important, it is now widely accepted that DNA is not the dictator of gene expression. Environmental conditions, particularly nutrition, play a role in how DNA behaves in our children and ourselves. This is part of the relatively new scientific field of epigenetics. The so-called epigenome contains huge amounts of information, and engages in activities on top of the genome by commanding genetic activities.

Epigenetic studies offer a new way of investigating the biological roots of common diseases. The ability to assess the basis of epigenetic mechanisms has improved dramatically in the last few years. It is apparent that epigenetics may hold the key to understanding diseases that scientists have thus far struggled to comprehend, including metabolic syndrome, cancer, neurodegenerative disorders, asthma, and allergies. Age-related illnesses, such as Alzheimer’s, are a typical example of how these genes can be
turned off and on in a manner that leads to the progression of diseases. Another exciting breakthrough involves understanding cancer development. Explaining cancer by relying merely on gene mutations is rare and difficult. Researchers thus hope to develop drugs that will drive epigenetic changes that will help protect against, or stop, diseases. However, using an epigenetic drug to switch on or off certain genes may also alter other genes in the process, which makes the development of epigenetic drugs a challenging endeavor.

Epigenetic information plays an important role in modifying gene expression and can potentially affect long-term health. Programming the epigenetic profile, occurs mainly during the early stages of development, and is of critical importance to researchers. Obesity, diabetes, cardiovascular disease and hypertension can originate during the early stages of life and development, and are associated with epigenetic programming. Therefore, perinatal care is imperative for keeping epigenetic changes in check. Doing so using a combination of cutting-edge epigenetic knowledge and modern technology can be used to leverage contributions to public health, life quality, and to reduce the economic burden of disease treatment.

Based on this information and the current knowledge gap, the overall goal of the present research was to investigate the effects of early life nutrition on one’s wellbeing and disease risk in adult life using epigenetic tools. High fat diets are the predominant dietary pattern in the contemporary western world, and have triggered numerous health problems, which have drawn the attention of researchers. Therefore, this study intends to address the epigenetic regulation of inflammatory reactions, which are a major consequence, and the essential basis of, relevant pathogenic physiologies in the high fat diet scenario.
CHAPTER 2. DIET, EPIGENETICS AND INFLAMMATION

Malnutrition during Early Development

Thrifty Phenotype Hypothesis (Developmental Origins of Adult Disease)
The Developmental Origins of Health and Disease (DOHaD) hypothesis addresses the “adverse influences during early development, particularly intrauterine life that can result in permanent changes in physiology and metabolism, which increase the risk of disease in adulthood” [14]. This was initially proposed in 1992 [15] based on the speculation by Barker, et al., that low birth weight infants who survived infancy and childhood could face an increased higher risk of coronary heart disease later in adult life [16]. This hypothesis has been supported and validated by numerous subsequent studies [17] [18-22]. One piece of evidence involves the Dutch famine events during 1944 and 1945. During this year, the food supply to a densely populated area was cut off, and affected some 4.5 million people. This was a unique event that involved a brief period of intense nutritional deprivation in a previously well-nourished population whose nutrition source was promptly restored after the famine [23]. Children exposed to in utero famine were subsequently well-nourished later in childhood. Several years later, findings from the Dutch famine cohort studies provided key proofs that in utero undernutrition lead to impaired glucose tolerance and type 2 diabetes, and an increased risk of many other diseases such as heart disease, obesity, and cancer [24-26]. In addition, they showed that dietary intake during pregnancy programs metabolic profiles in the offspring, which may account for the aforementioned disease development.

The causality between early life nutrition and well-being in adulthood has become increasingly clear in recent years. Altered fetal nutrition has been shown to produce many health problems. Using a variety of animal models for maternal malnutrition, such as protein restriction and unbalanced macronutrients, it has been demonstrated that early life nutritional deprivation reduced placental weight and birth weight [27-30], increase blood pressure [31-34], and impair glucose tolerance in the offspring [35-37]. In contrast, higher intake of micronutrients during conception was positively associated
with birth size and glucose tolerance [38, 39], and negatively associated with blood pressure in the offspring [40, 41].

To date, findings from diverse studies using maternal models have led to a better understanding of pathogenic mechanisms and promoted the development of preventive approaches, as well as potential therapeutic strategies. The most widely accepted theory that underlies the developmental origins of disease is known as maternal programming. This is “a process whereby a stimulus or insult during a sensitive or critical period has irreversible long-term effects on development” [14]. There are quite a few underlying mechanisms responsible for the pathophysiology of fetal programming. Fetal nutrition is evidently an important key regulators during programming because maternal malnutrition usually fails to support fundamental tissue/organ growth and development [41]. One consequence is that essential functions associated with gluconeogenesis, lipid metabolism, insulin and cortisol secretion, endothelial function, and myogenesis are underperformed, which in turn elevates the risks of diabetes, hypertension and hyperlipidemia. [14, 41].

**Maternal High Fat (MHF) Model**

Compromised nutritional environment during early development produces both immediate health problems in newborns, and leads to continuing consequences during adulthood. Significant outcomes were revealed in earlier studies that focused on undernourished pregnancies [42-44]. These changes initially caused tissue structural modifications, which later led to impaired organ functions and biological systems [45].

In the last decade, a dietary intake greatly shifted towards the consumption of highly accessible fat-enriched foods, combined with poor eating habits. Such dietary patterns have contributed to the increased prevalence of chronic diseases. Many studies have reported higher incidences of obesity, hyperglycemia, and systemic insulin resistance, as well as other metabolic disturbances as a direct result of the prolonged consumption of high fat foods [46-48]. More importantly, this both affects individuals who consume high fat diets, and also predisposes their descendants to similar illnesses. Mounting
evidence revealed in animal studies has demonstrated that perinatal exposure to high fat diets profoundly alters the intrauterine environment, and leads to permanent phenotypic alterations with adverse outcomes that persist throughout the adulthood of the progeny. Multiple aspects of physiological outcomes were manifested, including growth problems, learning disorders, and susceptibility to chronic diseases [49] [50, 51]. Interestingly, postnatal high fat and carbohydrate challenge [52-54], or upon the addition of a drug treatment [55], can aggravate these symptoms. This phenomenon is known as the “double hit” hypothesis.

**Inflammation: a Patho-Physiological Basis for Compromised Health**

Inflammation is a part of the natural defense system that the body maintains against injury and disease. Inflammation is a response to environmental stimuli that instantly triggers a series of physiological reactions and non-specific immune responses such as increased blood flow, cellular metabolism, vasodilatation and fluid influx [56]. Moreover, chronic low-grade inflammation is often linked to disease and long-term adverse consequences, and is inevitably associated with the pathogenesis of NAFLD/NASH [57], cardiovascular diseases [58], renal failure [59], nervous system disorders [60], aging [61], diabetes [62] and several types of cancer [63]. This type of inflammatory response is commonly observed in obese individuals and in populations with sustained high fat intake. It is now know that over consumption of high fat foods before or during pregnancy also affects the inflammatory response of the next generation, in a tissue-specific and in a life stage-dependent manner.

The overconsumption of fat before or during pregnancy results in an unfavorable intrauterine milieu. This means that the concentration of energy, hormones, and blood supply are suboptimal, and this can lead to abnormal fetal growth and development [50, 64-67]. Multiple physiological outcomes related to inflammation and early life exposure to high fat diet are described below.

**Adiposity**
Earlier studies have suggested the central and enabling role of inflammation in the process of cell proliferation, also that it promotes adipogenesis, and simultaneously represses myogenesis [68, 69]. Increasing the adiposity capacity allows inflammatory signals to generate additional storage space, which constitutes a risk factor for obesity. Pro-inflammatory factors, including IL-6, MCP-1 and TNF-α, are closely associated with body fat deposits [70-73]. Certain cytokines, such as IL-6, are known to have a characteristic tissue-specific function in the process of energy mobilization. In skeletal muscle, exogenous IL-6 treatment increased fatty acid oxidation [74, 75] and glucose uptake [75]. By contrast, adipose-tissue-derived IL-6 and TNF-α promoted the development of insulin resistance in both adipose tissue [76], and skeletal muscle [74, 77]. Consequently, the above information shows that that maintaining careful control of inflammatory response in both circulatory and peripheral tissues is vital for maintaining normal physiological functions.

Some studies show that MHF diets result in increased body fat composition [66, 78], and elevated growth curve for the offspring [50, 79, 80]. MHF exposure increases adiposity [81, 82], promotes adipocyte hypertrophy [66], and is accompanied by a decrease of lean body mass [83]. The unpublished data suggests that consuming a high fat diet throughout pregnancy and lactation induces mRNA expression of IL-6 and IL-1β in adipose tissue of the offspring. The programming of these pro-inflammatory cytokines in the adipocytes can contribute to the growing adiposity capacity and excessive body weight gain. Additionally, other studies have pointed out that undesired, but normal physiological changes due to either high fat [64, 79, 84], or high sucrose challenge [50], can be enhanced when the offspring are receiving MHF. The role of systemic inflammation in adiposity might account for such phenomena. More importantly, MHF can induce inflammatory responses in tissues including, but not limited to, the adipose tissue. This will be discussed below.

**Cardiovascular Disease**

Biomarkers of Low-grade systemic inflammation, specifically CRP, IL-6 and TNF-α, are intimately associated with endothelial dysfunction, carotid artery remodeling, and
vascular stiffness [85, 86]. Furthermore, endothelial dysfunction and insulin resistance are derived from elevated inflammation [87], and may contribute to the overall progression of hypertension [88, 89], which subsequently leads to renal malfunction [90]. Chronic inflammation, in the context of atherosclerosis, also contributes to the lesion initiation, progression and ultimately clinical complications [91].

Several animal models that used a fat-rich diet throughout pregnancy and suckling have suggested that endothelial and arterial vascular function were compromised in the offspring to the extent that blood pressure increased up to a level that impaired cardiovascular and renal function [66, 92-97]. Recent evidence supports the hypothesis that MHF produces hypertension. However, it appears to develop in an age-dependent manner [98]. Chronic inflammatory reactions are potentially responsible for this effect, given that increased levels of IL-1β and TNF-α in both the circulatory system and arterial walls were detected in the offspring of high fat-fed dams [99, 100]. Nevertheless, possibilities that include other contributing factors cannot be excluded. It thus appears to be the case that MHF-induced systemic inflammation can have a cumulative and long lasting effect, if one consider the time required to eventually reach the clinical manifestation.

**Brain Damage**
In addition to the relevant metabolic disorders, excessive fat supplementation during the early stages of development also impacts cognitive function in the offspring. Studies have shown that MHF can cause hypertrophy by altering glial and microglial reactivity [79]. It is known that activation of microglia can provoke the secretion of cytokines IL-1β, IL-6 and TNF-α [101, 102]. This ultimately results in hypoxic brain injury and acute liver failure. In the MHF model, the progeny presented microglial activation and a greater reactivity to LPS, as well as induction of IL-1β [67], and IL-6 in the brain [79, 103]. However, the origin of brain cytokines in MHF offspring is still being debated. Some studies suggest that a certain level of cytokines pass through the maternal-fetal placenta barrier [104], while others indicate the local production in the fetal brain [105].
Taken altogether, these studies propose a new perspective for the underlying mechanisms that regulate brain inflammation.

It is not surprising to see heightened inflammatory reactions in the brain, but yet the subsequent outcomes deserve a closer attention, as inflammation now is believed to significantly partake in the progression of many brain diseases [106, 107]. MHF-induced cytokine production affects many compartments of the brain. It produces detrimental effects over time and has become a leading cause of undesired functional decline. IL-6 is typically released from microglia and astrocytes. It is known to accelerate the inflammatory progression by actively promoting the production of other relevant cytokines, namely IL-1β and TNF-α [108], both of which have been shown to cause severe injuries in fetal and postnatal brain [109]. The changes brought about by activated inflammation are more likely to be mechanistically associated with long-term adverse outcomes. Meanwhile, IL-6 itself is closely associated with cognitive function, hypertension, and neurogenesis. The excessive production of IL-6 in the brain was shown to impair spatial learning [110], increase hypothalamic-pituitary stimulation [111], and reduce neurogenesis in the hippocampus [112]. Consequently, one of the mechanisms by which scientists would observe undermined brain functionality in MHF offspring involves the up-regulation of cytokines such as IL-1β and IL-6 [103]. These outcomes have not yet been fully described in MHF model. Several recent studies showed multiple lines of evidences which point out that the offspring exposed to the maternal and postnatal high fat diet exhibited difficulties in retaining the previous training in a spatial learning tests [79]. Increased susceptibility to deregulated brain signaling, including the serotonergic system, can further cause perturbations on behavioral patterns [113, 114]. Additional studies should be conducted to define the specific events, but it is possible that environmental challenges, disease and aging could induce these subjects to underperform in comparison with peers.

**Hepatic Energy Mobilization**

MHF has a steatogenic and fibrogenic effect in the liver, but does not necessarily cause hepatic lipid accumulation [115, 116]. However, intrauterine exposure to high fat diets is
believed to program the hepatic metabolic patterns, and contributes to NAFLD progression in the offspring. Severe fat accumulation in liver tissue occurs when a post-weaning high fat diet is administered, compared with postnatal high fat feeding alone [84].

Several animal studies have reported that perinatal exposure to a high fat diet triggers the induction of inflammatory markers in the liver. For instance, in a lactating high fat mouse model the inflammatory markers IL-6 and TNF-α were significantly induced [115]. Another study using a mouse model observed the stimulation of CRP, Mmd2, TNFsf1 and IL-12b [84]. Some unpublished data concerning rats shows a significant induction of several other inflammatory markers, including IL-1β, IL-6 and TNF-α, in the liver of MHF offspring. Although, the exact reason for the species-specificity or feeding-timing-dependent response remains unclear, a common hepatic inflammatory effect in response to MHF was observed.

Inflammation triggered by MHF disturbs lipid and glucose metabolism in the offspring, and is likely to result in fatty liver disease. For instance, MHF-induced increase of circulating TNF-α constitutes a risk factors for fatty liver development [99]. Adipocyte overexpression of TNF-α has been shown to drive local lipolysis and produce free fatty acids [117], which consequently enhances fat mobilization in other tissues, including the liver [118]. This phenomenon was indeed observed in the MHF model, and increased plasma glycerol in the fetus, which suggests up-regulated lipolysis and thus increases in hepatic fat deposits [65]. Meanwhile, another study observed increased hepatic lipid synthesis and fat accumulation as results of intraperitoneal injection of TNF-α or LPS that induced TNF-α [117]. Thus, it is possible that circulating TNF-α, regardless of the stimulus, is involved to some extent in the master signaling mechanism for energy mobilization throughout the body. On the other hand, local activation of inflammatory cytokines in liver tissue, including IL-1β, IL-6 and TNF-α, often worsen insulin signaling, and also stimulate of gluconeogenesis and lipid synthesis [119]. These phenomena have been found recently in this particular MHF model. Reduced hepatic insulin sensitivity in MHF offspring was indicated by the reduced expression of IRS in the liver.
Gluconeogenesis is a pathway that helps to maintain plasma glucose, particularly during periods of starvation. This pathway was also programmed by MHF, and exhibits a significant up-regulation of hepatic Pck1 and G6Pase in the liver [65, 80]. Previous research has shown that overexpression of Pck1 is closely associated with hyperglycemia and the progression of type 2 diabetes [120]. As a consequence, increased liver weight and glycogen content were reported in a follow-up study in the offspring of this model [78]. Furthermore, hepatic lipid metabolism is affected by an MHF diet, as evidenced by the deregulation of genes involved in fatty acid transport and synthesis, as well as TAGS synthesis [84]. Given the fact that these physiological outcomes can be attributed to multiple changes throughout the entire system, additional investigations are necessary to determine whether repression of inflammation in this model is an effective approach for attenuating these symptoms.

More interestingly, fatty acid composition in neonatal liver is modified by MHF, and may partially account for the activated inflammatory response. This was exhibited together with a decrease on DHA [83] and EPA, along with an increased proportion of oleate, linoleate and arachidonate in the overall pool of both TG and FFA [121]. DHA provides protection for the cardiovascular system against coronary disease [122], and down-regulates many inflammatory mediators [123]. Arachidonic acid is a second messenger for inflammatory signaling, and actively participates in the inflammatory response and its associated physiological changes [123]. Hence, it appears that the elevated n-6:n-3 ratio in the liver can create a susceptible and enriched environment for inducing the chain of inflammatory reactions.

**Placental Dysfunction**

Placenta, the primary organ of the maternal-fetal interface, and transports nutrients from the mother to the fetus, and is the focus of attention during fetal development. Impaired functionality of the placenta is one of the major causes of fetal disease. The maintenance of placental normal function during fetal growth eliminates the predisposition to preeclampsia [124], preterm labor [125], and stillbirth [126]. Moreover, perinatal high fat intake impairs the fetal environment, triggering placental inflammation
and its associated complications [127]. More particularly, mRNA expression of pro-inflammatory cytokines IL-1β, MCP-1 and inflammatory mediator TLR4, are induced in the placenta of high fat-fed mothers [128]. Excessive expression of IL-1β and TNF-α has been shown to be associated with preeclampsia [129], and TLR4 is associated with preterm delivery [130]. Increased placental inflammation is evident, such that compromised placental functions, such as decreased blood flow on the fetal side [128, 131], may result in stillbirth. Unfortunately, a verifiable molecular mechanism for a causal relationship between placental inflammation and disturbed utero-placental hemodynamics remains elusive. Current data have suggested that these outcomes, if any, are independent of NHF feeding, but it has been confirmed that a high fat diet will certainly aggravate the chemically induced preeclampsia [132].

The fetal consequences of increases in maternal circulating cytokines and placental inflammation remain elusive. It is easy to observe the increased inflammation in fetal circulatory system, liver and brain that follow the consumption of a NHF diet [65, 133]. However, it is difficult to pinpoint the origin of these wandering cytokines, given that bidirectional transfer of pro-inflammatory cytokines is barely detectable in a normal term placenta [104, 134]. Indeed, there is evidence showing that induced cytokines in the placenta do not necessarily cross the placental barrier, which has an impact on fetal inflammatory status [105], despite the altered permeability due to placental inflammation [135]. Therefore, investigations of the transgenerational impact of up-regulated maternal cytokines and the fetal origins of inflammation in this model are expected to have clinical value and allow for the early prevention and development of therapeutic treatment.

State of the Art Research in Epigenetics

Basis of Epigenetics
Epigenetics is defined as the alterations in gene expression in the absence of underlying changes in genetic information [136]. It must be heritable from cell to cell, hence through cell lineage development or even transgenerationally from parent to
offspring to grand-offspring [1]. Epigenetic mechanisms facilitate the understanding of how environmental factors can alter the phenotypic phenomenon without changing DNA sequence, and hence the origin of some diseases that are not readily explicable in terms of conventional genetic defects.

The study of epigenetics began 70 years ago, and has continued to evolve since then. It initially explained the basic process that multicellular organisms reserved for cell differentiation, which involves a non-genetic factor that guides cell division and fate regardless of the identical genetic code shared throughout one’s system. Classic biological actions defined within the realm of epigenetics includes enzymatic methylation of cytosine bases, also known as DNA methylation, post-translational modifications of certain amino acid residues on histone tails or histone modifications, nucleosome positioning, non-coding RNAs, and transcription factor regulatory networks [137].

**DNA Methylation**

DNA is usually methylated at the carbon-5 position of cytosine that presents in 5’-CpG-3’ dinucleotides [138]. CpG islands are the regions of more than 500 base pairs in size and with GC content above 55%. It is highly conserved throughout evolution, because they are normally kept free of methylation [139]. These stretches of DNA are usually located at promoter regions and cause stable heritable transcriptional silencing. DNA methylation is primarily a stable repressive marker, yet its regulation is more dynamic than what was once believed, and it can be actively removed at specific loci as well as genome-wide during development [140].

DNA methylation usually, but not always inhibits gene transcription, usually but not always depending on the position of the methylation change relative to the position of the transcription start site [141]. Four DNA methyltransferase (DNMTs), namely DNMT1, DNMT2, DNMT3A and 3B have been found to mediate DNA methylation but perform different functions [142]. Particularly, DNMT1 maintains the basic needs of DNA methylation, copying the pattern from parental strand to the newly replicated strand
during cell division, whereas DNMT3A and 3B promote the de novo DNA methylation [142].

**Histone Modifications**

Unlike DNA methylation, histone tails can be modified in multiple ways as is the case with acetylation, methylation and phosphorylation [143]. Acetylation of histones at lysine residues is catalyzed by histone acetyltransferases (HATs), and is associated with the activation of gene transcription. Deacetylation of histones is associated with the silencing of transcription, and is catalyzed by histone deacetylases (HDACs) [144]. Overall, the acetylation of histones marks active transcriptionally competent regions, while hypoacetylated histones are found in transcriptionally inactive euchromatic or heterochromatic regions. Histone methylation regulates gene transcription depending on the site of modification. Methylation of lysine 9 on the N terminus of histone H3 (H3K9Me) is typically a hallmark of silent DNA and is globally distributed throughout heterochromatic regions such as centromeres and telomeres [145]. However, methylation of lysine 4 of histone 3 (H3K4Me) activates gene transcription and is primarily found in promoter regions [145]. Lysine can be modified by mono-, di- and trimethylation. The enormous variation is thus likely to result in a diversity of possible combinations of different modifications.

**Epigenetic Interactions**

There is emerging evidence that there are connections between DNA methylation and histone modifications [146]. Eukaryotic DNA is greatly associated with histone proteins, and forms a highly ordered and condensed DNA-protein complex termed chromatin [144]. DNA methylation influences both histone modifications and overall chromatin structure by guiding the histone deacetylation of certain residues [147], DNA methylation is intimately connected with histone modifications [148]. DNMTs as well as three protein complexes that contain methyl-CpG-binding domain [149], were found to bind HDACs, which represses gene transcription [150]. Interestingly, it was once believed that histone modification was secondary to DNA methylation. Recent studies have revealed that histone modifications can take the initiative in chromatin
conformation by assisting the process of DNA methylation [146, 151, 152]. There appears to be a close relationship between DNA methylation and histone modulations, but many questions concerning the details of such interactions remain to be answered.

Other than these modifications, RNA in various forms, including antisense transcripts, noncoding RNAs, or RNA interference (RNAi), is also capable of triggering the transcriptional silencing of genes by aiding histone modification and DNA methylation [148].

**Developmental Programming of Epigenome**

Mammalian reproduction involves a single-cell zygote giving rise to a complex living organism with a large variety of cell types. These highly diversified cellular phenotypes are produced from the identical genomic sequence by mediating the subset of genes expressed in each cell type. Thus, cellular differentiation, and tissue and organ formation, rely largely on the process of extensive erasure and the precise establishment of lineage-specific epigenetic markers, which are termed epigenetic reprogramming. The Epigenome is re-established at fertilization, beginning with the initial wide-ranging loss of epigenetic marks in the zygote and early embryo, followed by the resetting of DNA methyl marks during many stages of cell division [153].

Developmental plasticity makes the reprogramming process highly susceptible to fetal and early life environmental exposure due to developmental plasticity. Epigenetic modifications, such as DNA methylation, are substantially sensitive to perturbation at the earliest stages of development, and most likely resulting in lifelong and possibly transgenerational effects. Chromatin states that arise during this period can further affect the propensity to subsequent epigenetic change. This is evident in the predisposition of polycomb-repressive complex occupied genes in stem cells to acquire DNA methylation abnormalities in aging and cancer [154-157].

The "developmental origins of adult disease" hypothesis posits gene-environment interactions that result in long-lasting effects, and suggests epigenetic inheritance as a
prime mechanism. During the early stages of development, a folate (methyl donor) deficient diet reduces the methylation of offspring on a whole-genome scale [158]. Indeed, rodents are often used to study the subject of developmental programming. This model has gained recognition, mostly by acknowledging that in utero malnutrition not only affects gene expression through epigenetic profile editing. This is the case because the progeny responds to environmental chemicals as well as ultimate adult phenotype does [159]. At this point, limited information is available from human studies, although DNA methylation appears to be the culprit for imprinting gene regulation when early life nutrition was manipulated [160].

**Transgenerational Inheritance**

Epigenetic marks in somatic cell lineages can be transmitted to daughter cells over the lifespan of an individual, while marks established in germ cell lineage can be transmitted to subsequent generations. Determining the transgenerational effects of epigenetic mechanism requires at least three generations after the exposure. Mounting evidence has demonstrated the apparent transmission of environmental insult across generations, although this remains highly controversial [161].

Environmentally induced germline epigenetic modifications occur during the DNA demethylation and remethylation period [162]. Certain types of chemical environmental exposures have been shown to promote the epigenetic transgenerational inheritance of adult onset disease, and the transgenerational epigenetic changes can be used as biomarkers of exposure and disease [163]. Several studies of the transgenerational effects of high dietary fat intake have been shown to be linked to certain types of epigenetic modulations [5-8], direct association between these modifications and their physiological consequences that result from high fat-induced chronic inflammation remain elusive. Given the potential persistence of the transgenerational epigenetic code, characterization in such situations in MHF model may favor the prediction, early prevention, and treatment of non-communicable diseases in the next several generations [164].
Epigenetics and Disease

Epigenetic vs Genetic
Conventionally, it was believed that the inheritable material carried in the primary sequence of the DNA double helix was the predominant of the determination of variations in the susceptibility and severity of disease. The understanding of how germline genetic variations influence disease development was assisted by technological advances and was expanded in many genome-wide association studies [165-167]. Changes in the DNA sequence drastically interrupts gene expression, and can lead to the genesis and progression of several diseases [166]. However, these studies also support the idea that common genetic variants alone tend not to identify the causal loci of complex diseases, and do not dictate individual disease risk [165]. Fundamental modification of the genome, also known as epigenetics, thus becomes a determinant of disease risk and etiology [168, 169], because it allows an individual system to modify how the genome is read in response to environment cues [168, 170].

Epigenetic events are inheritable and can be long-lasting, just like genetic information. Nevertheless, epigenetics differs from genetic changes in a small number of unique characteristics. First, it does not involve alterations of the DNA sequence and is reversible in principle [171]; secondly, epigenetic events are subject to reprogramming by endogenous or exogenous factors throughout the life span in a continuous editing process [172-176]. These unique features allowed of alternative explanations of disease variability to thrive, including diseases that cannot be fully explained by genetic variations and changes.

Environmental Epigenetics and Disease Etiology
Environmental epigenetics refers to the phenomenon by which epigenetics can explain the variability in the risk and severity of environmental diseases. The external environment, which includes lifestyle factors that are linked to stress, abuse, addiction, alcoholism and metabolic changes, are comprised in such a concept [177]. Particular environmental factors, such as endocrine disruptors, infectious pathogens, outdoor
pollutants, indoor allergens, and heavy metals, have been shown to yield epigenetic changes [177].

Environmental factors can trigger complex diseases, including cancer, cardiovascular disease, stroke, pulmonary diseases, asthma, obesity, and neurodegenerative disorders [178-181]. More importantly, changes in the epigenetic status due to early-life reprogramming or experiences during adult life have shown a strong correlation with the severity and progression of these diseases [177]. Therefore, the behaviors of the epigenetic code can be expected to indicate exposure to environmental toxins, as well as risk and the progression of diseases. Current investigations are focusing on the mechanistic regulation of epigenetic events, in order to promote a better understanding of the etiology of environmental disease and future development of preventive strategies.

Disruption of either the sequence or composition of the epigenetically regulated genes that result in their aberrant expression, are considered to be the fundamental mechanism underlying abnormal differentiation and disease development. Epigenetic factors have been determined to be involved in the pathogenesis of many types of cancer and common multifactorial disorders [182]. Aberrant de novo methylation of CpG islands is a hallmark of human cancers and can be found early during carcinogenesis [183]. Functional epigenetics changes have been observed in obesity [184], diabetes [185], cardiovascular disease [186] and the constellation of abnormalities known as metabolic syndrome [187]. Neurological disorders, cancer, reproductive conditions, and the pathogenesis of asthma have been suggested by emerging evidences that are attributed to epigenetic alterations [188].

Epigenetic changes are sensitive readouts of the consequences of acute and chronic exposures to environmental factors. They greatly rely on dose, duration, and life-stage at the time of exposure. More particularly, intense effects are usually found during the susceptible window of fetal development [177], thus exposure to environmental factors during a certain developmental stage will strongly influence disease etiology. During this
time window, differentiation of cells and organ systems are susceptible to programming. Consequentially, altered programming and gene expression due to environmental factors can lead to abnormal physiology and disease in adult life [162]. Therefore, it is reasonable to assume that environmental factors predispose phenotypic changes or disease progression, and this is not limited only to the individual exposed, but also includes subsequent generations. Normally, exposure to mild environmental factors such as nutrients, toxicants, or endocrine disruptors, are insufficient in and of themselves to produce genetic mutations, but can produce alterations in the epigenome, which are known as epimutations [162]. These epimutations can potentially reside in the germline of individuals exposed and become inheritable cross generations, and lead to the early onset of certain types of diseases.

**Epigenetics and Inflammation**
Inflammatory signaling has recently been identified as an epigenetic mediator [189]. It has been suggested that histone acetylation is closely associated with inflammation [190-192]. The pro-inflammatory signaling networks of IL-1β, LPS, and TNF-α-induced pathways may impact histone modifications through the activation of HDAC1 by ubiquitination and proteosomal degradation [193, 194]. On the other hand, a growing body of evidence has shown the power of epigenetics at regulating inflammatory genes, the incidence of which may eventually result in multiple physiological consequences [195]. For example, the addition of HDAC inhibitor can reduce the expression of pro-inflammatory mediators in glial cells, and has the potential to result in improved neural cell function and survival [196]. Meanwhile, a histone acetyltransferase (HAT) inhibitor can reduce the acetylation of NF-κB, which in turn suppresses the expression of cytokines in microglial cells and prevents neuronal cell death [197]. Furthermore, a few of chromatin-remodeling proteins including Brg1, Brm [198] and TET1 [199], are thought to participate in pro-inflammatory cytokine gene regulation of IL-1, IL-6 and MCP-1, possibly through increased activation of histone modifications. However, this leads to the progression of NASH [198]. Unfortunately, evidence for epigenetic programming of the inflammatory response by intrauterine environment is scarce, even so for the MHF model. Although chromatin remodeling in this model has been confirmed for certain
functional genes [80, 200], no indication of inflammatory marker programming has been revealed in any studies involving MHF. Nevertheless, given the reciprocal interaction between epigenetics and inflammation, MHF-induced chronic inflammation has the potential to shape the epigenetic profile of other functional genes, and the expression of inflammatory genes themselves are susceptible to epigenetic modulation. More investigations are therefore needed to explore the epigenetic mechanisms and the development of cutting edge therapeutic approaches.

**Application of Epigenetics in Pharmacology**

Epigenetic changes are intertwined with a variety of diseases. Manipulation of the epigenetic status is thus likely to expand the array of its applications [201]. Indeed, epigenetic therapy is believed to be a new development in pharmacology, by virtue of attempting to correct epigenetic defects, including changes in DNA methylation and/or histone modulations. It is a medical strategy with great potential, since epigenetic defects tend to be more reversible than genetic defects when using pharmacological interventions [202-204]. So far, two clusters of drugs have been developed; one aims to inhibit DNA methyltransferases (DNMT), and results in the inhibition of DNA methylation, which blocks one type of causative mechanisms of cancer where tumor suppressor genes are hypermethylated. The other major group targets the inhibition of HDACs, which reduces histone deacetylation which may serve to mediate the anticancer effects of these drugs. Promising results have been observed in drug trials for cancer treatment. More importantly, epigenetic drugs may be capable of preventing disease, in addition to the therapeutic agents [205]. Epigenetic therapy has limitations because both DNMT and HDAC inhibitors lack specificity, which can accelerate tumor progression in certain cases [206]. A variety of molecules are involved in epigenetic mechanisms, and other potential targets are plausible for drug development purpose.

**Interaction between Nutrition and Epigenetics**

**Dietary Effects on Epigenetic Profile**
Many environmental factors can interfere with the epigenetic status and produce either transient or permanent alterations of chromatin structure. Environmental factors, smoking, chemical exposure, etc., together with eating patterns, can profoundly affect biological processes, including oxidative stress and inflammation, which will directly or indirectly shape the state of the epigenome. Epigenetic mechanisms have recently been used more frequently used to describe environmental effects on phenotypes, particularly the nutritional status.

In the 1940s, nutrient supply was initially recognized as an authoritative element, whose intensity produces long-lasting physiological phenomenon that modify the epigenetic code during a critical developmental window [160, 207]. The biological role of nutrition in the process of phenotypic differentiation has been documented in honeybees [208], and agouti mice [209], and both of them involve DNA methylation [210]. Folate is a typical nutrient that is often used when studying methylation regulation. Folate is a major source of the methyl groups for DNA and histones [211]. Moreover, imbalanced dietary intake of folate is associated with methylation changes and consequently gene deregulation, and subsequently increased disease risk [212, 213].

Food intake can also manipulate the epigenetic code by integrating miRNA into the metabolic activities of consumers, with little nutrient content relevance. It was determined that a plant-origin microRNAs, MIR168a, binds to the coding region of mammalian genes and decreased protein production [214]. A lot more investigations are required to confirm this phenomenon.

**Developmental Plasticity and Nutrition**

Developmental plasticity should ideally allow the organism to monitor its growth process in an attempt to promote phenotypic changes that better equip it to fit with the environment. However, in face of an excessive or deficient nutrient supply during the early stages of life, the progeny are more likely to be programed with greater limitations, which undermine their defensive capacity against postnatal environmental challenges. They may consequently experience abnormalities in terms of body size, growth rate,
tissue distribution, metabolic profile, and hormonal imbalance and underperformed intellectually [215, 216]. It is now generally accepted that MHF is an unfavorable intrauterine environment that is closely associated with the development of metabolic syndrome and an increased risk of an obese phenotype developing in the offspring [217]. However, researchers are only beginning to understand the basis of mechanism that leads to the initiation of those signaling cascades, which in turn produce structural and functional cellular changes, physiological damage, and ultimately chronic diseases. Evidence has shown that maternal environment affects gene transcription, partially through epigenetic modulation, including DNA methylation, multiple forms of histone modifications [218] and microRNA [219, 220]. Therefore, the concept of epigenetic regulation can serve as a critical link between maternal diet and progeny phenotype, which consequently supports the DOHaD hypothesis.

**High Fat Diet and Epigenetics**

High fat diets impact greatly the epigenetic structure, such as the intensity of DNA methylation of genes that were highly involved in pathophysiological process. In general, metabolic and cell cycle relevant genes in liver tissue are the subject of DNA methylation regulation. For instance, methylation intensity at CpG sites of Esr1 [221], stearoyl-CoA desaturase 1 (Scd1) [222], promoter of glucokinase, pyruvate kinase [223] and MTTP [224], was differentially regulated by high fat-diets. Deregulation of these genes is primarily associated with aberrant hepatic lipid and glucose metabolism, and result in the development of various types of non-communicable diseases, including fatty liver disease and obesity. In addition, altered DNA methylation patterns of cell cycle controlled gene Cdkn1a [225], and the growth hormone secretagogue receptor [226], were reported in a MHF model, which indicates the transgenerational epigenetic effects of high fat diets. DNA methylation induced by a high fat diet elicits broad effects in other tissues besides the liver. For example, the CpG sites of FASN and Leptin in adipose tissue [227], as well as for the brain μ-opioid receptor [228], tyrosine hydroxylase and the dopamine transporter [228], leptin [229], and melanocortin-4 receptor (mcr4)[230], were reported to be differentially methylated by high fat diet. A significant correlation between DNA methylation and gene expression has been
validated in several of these studies, which indicates that consuming a high fat diet affects gene regulation by altering the DNA methylation status of gene. The effects of high fat diets on the global DNA methylation on the whole-genome level have been rarely investigated.

**Conclusion**

The understanding of the epigenetic-based mechanisms for inflammation activation in the high fat model is insufficient to explain the constellation of body responses to the environment. Previous research has shown that *in utero* exposure to high fat diets programs the increased susceptibility to chronic diseases during development and growth. There appears to be a causal relationship between low-grade chronic inflammation and such adverse outcomes. However, it is difficult to pinpoint a single causative mechanism, or even draw out a conclusion given the essential role that the inflammatory response plays in many cellular and physiological processes. Epigenetic mechanisms are associated with a myriad of developmental diseases. It is widely recognized as a new therapeutic approach nowadays, and unraveling the epigenetic regulation of inflammatory markers in a high fat model can assist disease prevention and treatment in both the current generation and in subsequent generations as well.

**Project Overview**

**Background**

Consumption of high fat increases the risk of obesity and metabolic syndrome, both of which were suggested to be closely associated with a low-grade chronic inflammation. It has been shown that high fat feeding specifically increased proinflammatory signaling, including Cycloxygenase (COX) -2 expression, in a diet-induced obesity mice model [231]. However, the molecular mechanisms regarding epigenetic modifications on COX-2 in this model remains to be elucidated. The COX family comprises two isoforms, 1 and 2, sharing significant sequence homology and catalytic activity. COX-1 is constitutively expressed to produce prostaglandins (PG) for maintaining homeostatic
functions, whereas COX-2 requires additional extracellular and/or intracellular stimuli, including exogenous and endogenous arachidonic acid [232, 233]. The COX enzymes catalyze the conversion of arachidonic acid to prostaglandins, from which the substrate for cell specific prostaglandin and thromboxane synthases are produced [234]. Prostaglandins are involved in numerous physiological and pathological processes including inflammation, and they are rapidly increased by COX-2 in the scenario of inflammation [235]. Induction of COX-2 is transient, with a rapid return to baseline within hours following treatment [236]. Therefore, expression of COX-2 is strictly regulated and it is only markedly induced during inflammation. The nutritional environment alters epigenetic profiles through DNA methylation and histone code, and thereby affects gene transcription, intracellular signaling and ultimately the development of disease. Although it is known that high fat feeding triggers an inflammatory response including COX-2, there is little knowledge of the epigenetic mechanisms involved. Our study here propose a connection between high fat diet, COX-2 transcription and epigenetic modifications, which has never been tested in previous research. By conducting the present research, it hopes to improve the understanding of epigenetic-related COX-2 gene regulation.

**Objective**
The objective of this study is to identify the molecular mechanisms involved in high fat – induced COX-2 gene expression.

**Hypothesis**
High fat diet induces inflammatory marker COX-2 expression associated with epigenetic modifications.

**Specific Aims**
1. Characterize the physiological outcomes and COX-2 response to high fat feeding at different life stage
2. Determine if COX-2 expression is associated with epigenetic modifications, including DNA methylation and histone modifications.
3. Identify if there is any regulatory element on COX-2 gene that responds to high fat signal.

Discussion

In Chapter 3, 4 and 5, this study presented several lines of evidence to demonstrate that high fat feeding (maternal, HF/C; post-weaning, C/HF and life-long, HF/HF) affects physiological and pathological phenomena in the liver of pups. The results revealed that COX-2 gene expression increased in pups' liver along with multi-regional DNA hypomethylation in high fat diet. 5'UTR hypomethylated region was further identified and characterized as an enhancer element that potentially contributes to COX-2 gene activation.

Prolonged high fat dietary feeding instigates an inflammatory response, following which a variety of proteins, including COX-2, can be significantly induced. Understanding how dietary components regulate gene expression at epigenetic level provides a novel insight that connects environment and disease predisposition. Following the completion of the present study, a link between increased hepatic COX-2 seen with high fat diet feeding with specific epigenetic modification will be elucidated.
CHAPTER 3. HIGH FAT DIET INDUCES COX-2 GENE EXPRESSION

Abstract

Sustained high fat feeding leads to obesity and metabolic syndrome, accompanied by low-grade chronic inflammation, insulin resistance, and dyslipidemia. Changes caused by low-grade inflammation in the liver can result in the progression of disease and fibrosis. COX-2 is an enzyme that produces prostaglandins, which participate in multiple physiological and pathological processes, including the activation of inflammatory responses. Male Sprague-Dawley rats received a high fat diet during different life stages, including maternal (HF/C), post-weaning (C/HF), and lifelong (HF/HF). Liver samples were collected for analysis at 12 weeks of age. Total fat accumulation and liver damage evaluation were significantly higher in HF/HF than in the rest of the treatment groups. Analysis by PCR of the expression of COX-2 mRNA showed an induction in all of the high fat diet groups, especially in the HF/HF group, which doubled the induction fold of HF/C and C/HF. Meanwhile, analysis of fatty acid composition showed that proportion of both Arachidonic acid and Δ6-Desaturase, which produces Arachidonic acid, significantly increased in the high fat groups, which may have motivated the biochemical flow as well as provided the reaction substrate for COX-2 catalysis. In conclusion, induction of COX-2 gene expression in response to a high fat diet is associated with the period of exposure.

Introduction

Modern western diets are rich in fat content. Prolonged consumption of high fat diets is the causal agent of numerous health problems, including promotion of hepatic triglyceride accumulation, insulin resistance, oxidative damage, and chronic low-grade inflammation, which leads to the progression of liver disease and fibrosis [237, 238]. A murine study showed that consumption of a high fat diet changed liver morphology, which created a permissive environment for colon cancer metastasis [239]. A high fat diet can also change cellular membrane protein composition [240], elevate the
abundance of pro-inflammatory molecules, and give rise to hepatic insulin resistance [241].

Induced pro-inflammatory signaling is usually signified by activated expression of certain genes, such as Interleukin family members and COX-2 [116, 242]. Activated COX-2, rather than COX-1, is persistently found during the activation of inflammatory responses [243]. COX-2 is a key regulatory enzyme involved in the production of prostaglandins, which are important in mediating many biological processes, including reproduction and immune function. COX-2 actively produces prostaglandins using exogenous and endogenous arachidonic acid [233]. This occurs during injuries and inflammation in the metabolic steatohepatitis scenario [244]. In addition, overexpression of COX-2 is usually associated with inflammatory responses, many types of cancers [236], and preterm labor [245].

Exaggerated COX-2 expression has significant tissue-specific consequences: accumulation of COX-2-derived prostaglandins initiates a multitude of physiological responses, which generally result in fever and pain. However, in the liver, excessive expression of COX-2 accounts for the pathogenesis of insulin resistance [246, 247], fatty liver disease [246, 248], and hepatocellular carcinoma [249]. This study is dedicated to a better comprehension of the pathogenic properties that result from the exposure to a high fat diet at different stages of life. Examination will include COX-2 gene expression and other relevant physiological features.

**Materials and Methods**

**Experimental Design**
Timed-pregnant Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were separated into two dietary groups: either control (C, 16% fat) or high fat diet (HF, 45% fat) throughout gestation and/or lactation. Rats were kept individually in standard polycarbonate cages in a humidity- and temperature-controlled room on a 12-hour light-dark cycle, with ad libitum access to food and drinking water. Body weight and food
intake were recorded every three days during gestation. Body weight of the offspring and litter size were recorded right after delivery. Twenty-four hours after birth, both groups of dams were randomly assigned 10 pups (5 male and 5 female coming from the same gestational diet) until weaning at day 21. After weaning, pups from both groups were housed individually and exposed to either C or HF diet until they were sacrificed at 12 weeks of age, generating four groups: C/C, HF/C, C/HF and HF/HF. The left lobe of the liver was snap-frozen in liquid nitrogen and stored at -70°C until use. We certify that all applicable institutional and governmental regulations regarding the ethical use of animals were followed during this research (University of Illinois Institutional Animal Care and Use Committee approval no. 09112).

**Liver Histology**

Frozen liver samples were embedded in Tissue-Tek OCT compound (VWR, Radnor, PA) and cut to a thickness of 5 µm in a cryostat at -20°C. All sections were fixed in 70% ethanol and stained with hematoxylin and eosin (H&E) or Oil red O (ORO) solution (Newcomer Supply). Histopathological examination was conducted by the certified pathologist. The grading scale used to assess microscopic damage in liver was 0: none, 1: minimal, 2: mild, 3: moderate, 4: marked and 5: severe.

**Biochemical Assays**

Frozen liver samples were ground in liquid nitrogen and homogenized in 0.3 mL saline (0.9% w/v NaCl). Homogenized samples were diluted 5 times with saline. Twenty microliter of the diluted samples were incubated with 20 µL 1% deoxycholate in 37 °C for 5 min and 10 µL of the sample were analyzed using the Thermo Infinity Triglycerides Liquid Stable Reagent (Thermo Fisher Scientific, Rockford, IL) and a standard reference kit to determine the TAG content (Verichem Laboratories, Providence, RI). The results of hepatic TAG were normalized to the amount of total protein in the sample. Serum samples were analyzed for circulating TAG using the same method.

**Gas Chromatography Analysis of Fatty Acid Content in the Liver**
Total lipids were extracted from frozen liver tissue (~100 mg) with the method described by Bligh and Dyer [250], using chloroform:methanol (1:2 v/v) and 0.15 N acetic acid. Each extraction solvent contained 0.05% (w/v) BHT to prevent lipid oxidation during extraction. An aliquot of total lipid was spiked with heptadecanoic acid as internal standard, dried under nitrogen and derivatized by heating at 95 °C in 3 N methanolic HCl (Supelco, Bellefonte, PA) for 40 min. After cooling to room temperature, fatty acid methyl esters were extracted with hexane. Samples were injected onto a 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector and a 30 m x 0.32 mm Omegawax 320 column with 0.25 μm film thickness (Supelco, Bellefonte, PA). Chromatography conditions used were 180 °C for 5 min, followed by a temperature increment of 6 °C per min, and ended with isothermal at 220 °C for 15 min. PeakSimple software (SRI Instruments, Torrance, CA) was used for data collection and integration. Each fatty acid was normalized to the total fat content and presented as percentage of total fatty acids.

RNA Isolation and Two-step Real Time Quantitative PCR (qPCR)
Total RNA samples from tissues and cells were extracted using TRI reagent (Sigma, St. Louis, MO). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a Thermal Cycler (Applied Biosystems) at 25°C for 10 min, 37°C for 2 hr and 85°C for 5 s. Real time PCR was then performed using SYBR Green fast master mix (Quanta Biosciences, Gaithersburg, MD) in the 7300 PCR System (Applied Biosystems, Foster City, CA) at 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. A serial dilution of a sample was used as the standard curve to quantify all the samples for relative mRNA expression level. Ribosomal protein L7a was used as an internal control. Primers used for qPCR are shown in Table 3.4.

Protein Extraction and Western Blotting
Liver tissues were lysed with 1X Laemmli Buffer [62.5 mmol/L Tris–HCl, pH 6.8, 2% SDS, 10% Glycerol v/v, 0.01% Bromophenol blue, 5% 2-mercaptoethanol, 1x protease inhibitors (Roche, Indianapolis, IN) and 1x phosphatase inhibitors (Sigma-Aldrich)]. For
longer storage, the protein samples were placed in -70 °C. Lowry assay was performed to determine protein concentration. Samples containing 30 μg of protein were resolved by 10% SDS-PAGE. A wet transfer protocol was performed to complete the transfer. A PVDF membrane (0.2 μm, Bio-Rad, Hercules, CA) was blocked in 10% milk in TBS/T (30 mM Tris base pH 7.6, 200 mM NaCl and 0.1% Tween 20) for one hour at room temperature. The membranes were incubated with antibodies against COX-2 (#4842, Cell Signaling Technology, MA) in 10% non-fat dry milk at a 1:1000 dilution at room temperature for 3 hours. SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL) was used to detect the signal and the images were captured and analyzed by a Chemi Doc XLR system (Bio-Rad). Actin was used as an internal control to normalize the protein data.

**Immunofluorescence**

To identify the localization of COX-2 in liver, immunofluorescence staining of COX-2 was conducted using an Alexa Fluor 647-labeled anti-rabbit secondary antibody (red) (Invitrogen). Frozen liver samples were embedded in Tissue-Tek OCT compound (VWR, Radnor, PA) and cut to a thickness of 5 μm in a cryostat at -20 °C. The sections were fixed in 70% ethanol, permeabilized with 0.1% Triton X-100, blocked with Image-iT FX signal enhancer (Invitrogen) for 30 min, incubated with primary antibody COX-2 (1:200, cat # 9587S, Abcam) for 2h, followed by an incubation of the secondary antibody (1:200 dilution) for 45 min in a dark chamber. The samples were counterstained with Hoechst 33342 (Invitrogen) for 15 min and then washed with PBS. Coverslip was mounted on the sections using Prolong Gold anti-fade reagent (Invitrogen). Pictures were taken using the Zeiss LSM 700 Confocal.

**Statistical analysis**

Body weight and food intake were tested using repeated-measures one-way ANOVA. The synergistic effects of maternal and postweaning high fat diet were analyzed using Two-Way ANOVA [251]. Post-hoc analysis was performed using Fisher’s least significant difference (LSD, SAS v. 9.1.2, SAS Institute, Cary, NC) when the P-value of interaction of main effects (maternal vs postweaning) was less than 0.15. Individual bars
with different letters differ (P<0.05). Significance was set at p<0.05 for all comparisons. Individual bars with different letters differ (P<0.05). Correlation between COX2 mRNA and COX-2 pre-mRNA or 6desaturase mRNA was determined by Spearman's rank correlation coefficient analysis (Spearman's rho).

Results

Growth Curve and Calorie Intake
An overall dietary effect was observed on both growth curve and calorie intake. Individual comparison between treatment groups showed that maternal diet did not significantly impact body weight gain (Figure 3.1A) nor calorie intake (Figure 3.1B) of offspring fed the same diet. However, there was a clear overall separation by post-weaning diet (Table 3.1). Offspring fed with the high fat diet exhibited a greater increase in body weight gain (Figure 3.1A) and calorie consumption (Figure 3.1B) compared to that of the control group, regardless of maternal diet.

Liver Histology and Lipid Profile
H&E and Oil Red O (Figure 3.2) staining were graded individually to evaluate the pathological changes corresponding to HF dietary intervention at different life-stages (Figure 3.2). Compared to C/C, only life-long high fat feeding (HF/HF) remarkably increased the severity of liver damage at the end of 12 weeks. Severe micro-and/or micro-hepatocellular vacuolation (Figure 3.3, top) and increased hepatic neutral lipid accumulation (Figure 3.4, top) resulted. In contrast to HF/HF, early life stage high fat treatment (HF/C) or post-weaning high fat treatment (C/HF) did not lead to severe liver damage; post-weaning high fat treatment (C/HF) elevated hepatic fat accumulation (Figure 3.4, top) whereas a NHF diet (HF/C) significantly increased circulating TAG in comparison to the remaining groups (Figure 3.4, bottom).

Liver Fatty Acid Profile and Associated Enzymes
Palmitoleic acid (PA, 16:1n-7) content was decreased in post-weaning high fat-exposed groups (C/HF, HF/HF), while the content of linoleic acid (LA, 18:2n-6) increased in the
C/HF group. Arachidonic acid content (AA, 20:4n-6) was significantly greater in high fat-treated groups, regardless of the life-period of exposure (Table 3.2). Palmitoleic and linoleic acid are fatty acids that have no direct association to COX-2 induction [252], however in many circumstances linoleic acid can be converted into arachidonic acid, which is the primordial substrate for COX-2 catalysis. To investigate the sources of arachidonic acid in the liver of all high fat groups, mRNA expression of both Δ6desaturase that converts linoleic to arachidonic acid [253] was measured. HF treatment at all life stages increased mRNA expression of Δ6desaturase significantly. Therefore, accumulation of arachidonic acid exerted by all high fat diets is due to the desaturation reaction actively associated with COX-2 induction.

**HF Diet Regulates Gene Expression**

COX-2 mRNA expression was notably increased in all high fat-fed groups (HF/C, C/HF and HF/HF) (Figure 3.5). Statistical analysis revealed that compared to C/C, exposure to a high fat diet during an early developmental (fetal and suckling) or postnatal period significantly induced COX-2 mRNA expression by ~10 fold; Life-long feeding of the high fat diet (HF/HF), however, stimulated COX-2 transcription to a remarkably high level, which was significantly different than COX-2 levels in both HF/C and C/HF; furthermore, COX-2 pre-mRNA, as well as Δ6desaturase mRNA in HF/HF, did not differ from HF/C or C/HF, which may be due to the high variation within the groups. Nevertheless, both COX-2 pre-mRNA and Δ6desaturase mRNA were positively correlated to COX2 mRNA level (Figure 3.7), suggesting a synergistic effect of maternal and post-weaning high fat diet on these two genes.

**Protein Abundance of COX-2 Increased by High Fat Treatment**

To confirm the protein content of COX-2 in the four treatment groups, both Western blot (Figure 3.6) and Immunofluorescence (Figure 3.8) were conducted. Western blot provided an average value of COX-2 protein content in each group and showed that only the HF/HF had a significantly increased amount of COX-2 compared to the C/C group. Neither HF/C nor C/HF are statistically different than C/C. Nevertheless, protein amount was positively and significantly correlated to COX2 mRNA level (Figure 3.7).
Immunofluorescence presented both protein localization and quantification of COX-2. A representative image from each group was presented and showed the relative quantity of COX-2 on the tissue section. Almost no visualized COX-2 staining could be seen in C/C. The most abundant COX-2 positive signal is received from HF/HF, followed by HF/C and C/HF.

**Discussion**

The results demonstrate for the first time a double-hit effect of a high fat diet on COX-2 gene expression. Exposure to a high fat diet throughout the lifespan leads to a more severe activation of COX-2 than a simple maternal or post-weaning introduction. Furthermore, as opposed to maternal or post-weaning diet, alone, life-long high fat feeding exerts a synergistic effect on COX-2, resulting in a significantly greater induction of COX-2 in liver tissue.

A previous study that used a mouse model stated that early-life exposure to a high fat diet programmed susceptibility to NAFLD development in the offspring [84]. Fetal programming thus may create an environment that is less resistant to the progression of pathogenic events. The likelihood of developing liver disease dramatically increased in offspring exposed to high fat diets during fetal growth. This hypothesis was validated by both physiological and pathological evidence, which showed that the most definitive progression towards NASH occurred in the HF/HF group, rather than C/HF or HF/C [84]. This study agreed with this previous observation by showing the evaluation of hepatic damage and lipid accumulation, as well as the increased gene expression of the pro-inflammatory marker COX-2, a central reflection of both chronic inflammatory status as well as liver injury. These measurements reinforced the knowledge that the severity of liver damage is exaggerated when the offspring are exposed to a high fat diet during early development. The synergistic effect of maternal and post-weaning high fat diets on COX-2 gene expression was statistically significant and may biologically account for greater detrimental outcomes in adulthood.
Only certain types of cells can express COX-2 upon being stimulated by inflammatory signals, and these are mostly non-parenchymal cells (NPC) such as Kupffer and endothelial cells [254, 255]. For example, it has been suggested that peroxidization of fatty acids was responsible for activated COX-2 expression in NPC, but not hepatocytes [254]. Our immunofluorescent images showed that COX-2 protein abundance varied according to the time of high fat exposure, in agreement with the mRNA level. COX-2 positive staining was seen sporadically at certain sites in HF/C and C/HF. In contrast, HF/HF treatment produced more COX-2 signal. This once again strongly suggested the maternal fetal programming effect, meaning that exposure to a high fat diet during early development primes the liver tissue of male offspring with a more intense response to post-weaning high fat contact. However, it was difficult to differentiate the cell types in the liver of HF/HF pups which produced excessive amounts of COX-2. Still, this outcome could be attributed to a wider range of NPC activation, or to hepatocyte activation. Further investigation is needed to answer this particular question.

Interestingly, the hepatic fatty acid profile was also modified by high fat dietary treatment. Neither the control nor high fat diets contain significant amounts of arachidonic acid, which could account for the animals experiencing such an increase. However, the proportion of arachidonic acid increased in the liver of all HF-treatment groups in comparison with the control. Arachidonic acid is mainly distributed throughout the lipid bilayer, and functions as a major substrate for cyclooxygenase enzymatic reactions. In the presence of adequate amounts of linoleic acid or fully functional Δ6desaturase, the conversion of linoleic acid to arachidonic acid is enabled [256]. In our study, Δ6desaturase mRNA abundance positively correlated with COX-2 gene expression across high fat treatment groups, which suggests a direct crosstalk between the enzymes. Previous dietary studies have suggested that hepatic Δ6desaturase can be induced by low level of dietary intake of essential fatty acids [257]. Control diet (AIN-93) was used in this study because it provides complete nutrients that optimally meet animal requirements. Therefore, the induction of Δ6desaturase could be triggered by other cellular signaling molecules that participate in COX-2 reactions.
Conclusion

Based on the results of the present study, it is not surprising to find a synergistic effect on hepatic physiology as a result of combined maternal and post-weaning high fat feeding. The most intriguing part resides on the gene programming of COX-2. This is the first study to date to demonstrate that a “double hit” impact occurred on the gene expression level. In addition, it was revealed that in high fat dietary models the body adapted to environmental changes, meaning that it produced more arachidonic acid as a substrate for the support of COX-2 catalysis. Pre-mRNA expression correlated with an mRNA level of COX-2, which suggests a transcriptional regulation of hepatic COX-2 by high fat diets. As regards the current knowledge gap concerning the epigenetic regulation of COX-2, current study will further investigate DNA methylation and histone modifications in the next chapter.
CHAPTER 4. HIGH FAT DIET INDUCES EPIGENETIC ALTERATIONS OF COX-2 GENE

Abstract

High fat diet is associated with pathological development and the progression of liver diseases. It is hypothesized that high fat diet changes the genomic landscape of DNA methylation and histone code, leading to differential expression of COX-2 in hepatic tissue. Methyl-DNA immunoprecipitation assay in combination with high-throughput sequencing (MeDIP-seq) and methylation-sensitive restriction enzyme sequencing (MRE-seq) were applied to illustrate the genomic landscape of DNA methylation modified by a high fat diet. The results showed that in all three high fat dietary groups, multiple regions of the COX-2 gene exhibited DNA hypomethylation, including the 5'UTR and multiple coding regions. Chromatin Immunoprecipitation analysis (ChIP) showed that high fat diets programmed histone methylation and acetylation by increasing H4Ac, H3Ac and H3K4Me2, which characterize an open chromatin structure and active gene transcription. In conclusion, activation of COX-2 mRNA in the livers of high fat treated pups is associated with DNA hypomethylation and histone modifications.

Introduction

High fat dietary feeding results in various long-term health problems, and overproduction of COX-2 plays a central role. More particularly, COX-2 induction is known to actively participate in body weight and fat mass gains, blood pressure elevation, glucose and lipid level increases, glucose tolerance impairments and steatohepatitis development [258-260]. COX-2 performs critical biological functions, so its expression should be closely monitored.

Epigenetic modification dynamically orchestrates gene expression. It actively participates in the processes of cell differentiation, proliferation, and apoptosis [261] primarily through transcriptional and post-transcriptional manipulation of gene activity.
[262]. The epigenetic profile is subject to re-modulation through inter- or intracellular signaling[263]. Therefore, the management of epigenetics is a novel contemporary therapeutic approach for the treatment of certain diseases.

It has recently been recognized that dietary fat molecules have the potential to stimulate cellular epigenetic signals, profoundly affecting chromatin structure as well as gene expression, signaling pathways, and subsequent biological processes [264]. Consuming a high fat diet increases the risk of obesity and metabolic syndrome, which are often accompanied by low-grade chronic inflammation [265]. Moreover, high fat diets have been previously shown to program the epigenetic profile across numerous tissues [225, 266]. Reprogramming the histone code [200], together with altered DNA methylation [225], have been documented for differentially expressed genes in the high fat model. Although gene deregulation can be attributed to aberrant DNA methylation and/or histone modifications, it has been considered to play a role in the production of pathophysiological outcomes [267]. However, it remains unclear whether, first, the alteration of DNA methylation and/or histone modifications are solely accountable for high fat-induced COX-2, and secondly, whether the timing of exposure to a high fat diet differentially shapes the epigenetic profile.

A wide range of stimuli, including pathogens, cytokines, nitric oxide, and growth factors, are known to quickly but transiently activate COX-2 transcription [268]. Depending upon the cell type and stimulus, transcriptional control of COX-2 depends on a combination of transcription factors that bind to specific sites on the 5′-UTR (untranslated region) of the gene. More particularly, binding motifs of NF-κb (nuclear factor κb), AP-1 (activator protein 1), and CREs (cAMP-response elements), were found at gene 5′UTRs [269]. Transcription of COX-2 is also influenced by the acetylation status of histone and non-histone proteins. Upregulation of p300, a histone acetyltransferase (HAT), activates COX-2 by acetylating NF-kB components [270]. By contrast, HDAC inhibitors suppress the activation of COX-2 expression in human primary myometrial cells [271] and cancer cell lines [272] by preventing the binding of transcription factor c-Jun to the COX-2 promoter.
DNA methylation status may likewise affect COX-2 expression because two CpG islands are located at the core promoter of COX-2, the hypermethylation of which usually results in transcriptional silencing [273]. On the other hand, post-transcriptional regulation of COX-2 can occur through changes in its mRNA stability [274]; the 3’-UTR of COX-2 contains numerous AU-rich elements (AREs), which are bound by specific trans-acting ARE-binding factors. Many of these AREs are located within the first 150 nucleotides of the COX-2 3’-UTR. CUGBP2 is a RNA binding protein. When it is bound to specific AREs within the first 60 nucleotides of the 3’-UTR, that stabilizes COX-2 mRNA while inhibiting its translation. Consequently, there are many known checkpoints for the study of COX-2 gene expression. However, there is a lack of up-to-date understanding of the epigenetic-based molecular mechanisms by which high fat diet activates COX-2 gene expression. Our study aims to investigate the epigenetic regulation of COX-2 using a high fat diet, and seeks to determine whether the timing of high fat exposure generates a differential DNA methylation pattern and histone modifications.

Materials and Methods

Genomic DNA Isolation
Ten milligram of liver tissue was ground in liquid nitrogen and genomic DNA was extracted in 600µL of Extraction Buffer (50mM Tris, pH 8.0, 1mM EDTA, pH 8.0, 0.5% SDS, 1mg/ml Proteinase K) at 55 °C overnight. Lysates were centrifuged and supernatant was transferred to 2mL phase lock gel (PLG) (5’-Prime, Fisher Scientific Company, LLC). Phenol/chloroform extraction (PCI) was performed to purify DNA. Briefly, equal amount of aqueous sample and organic extraction solvent were gently mixed in PLG tube and centrifuged at 16,000 g for 5 min to separate the phases. Upper phase was transferred and 1µL of RNase (Roche, 10mg/mL) was added with, followed by 1 hour of incubation at 37 °C. Another PCI and 1Xchloroform extraction was performed. Purified DNA was precipitated with 1/10 volume of 3M Sodium Acetate (pH
5.2), and 2.5 volumes 100% EtOH. DNA pellet was washed with 70% EtOH and resuspended in TE.

**Methyl-DNA Immunoprecipitation Assay (MeDIP) in Combination with High-Throughput Sequencing (HTS)**

DNA methylation status in liver samples of rats was examined using sequencing-coupled methylated DNA immunoprecipitation (MeDIP-seq). Genomic DNA from each liver sample was purified and subjected to sonication to ~100–500 bp. Sonicated DNA was end-repaired, A-tailed and ligated to adapters following the standard Illumina protocol. Agarose size-selection was used to remove unligated adapters. A mouse monoclonal anti-methylcytidine antibody (Eurogentec) was then applied to adaptor-ligated DNA for each immunoprecipitation. Fifteen cycles of PCR were performed on the immunoprecipitated DNA using the single-end Illumina PCR primers. Final size selection (220–420 bp) was performed in a 2% agarose electrophoresis, and the resulting reactions were purified with Qiagen MinElute columns. Quality was checked by spectrophotometry and Agilent DNA Bioanalyzer, which indicated an average fragment size of 150 bp. An aliquot of each library was diluted and used as template in four independent PCR reactions to confirm enrichment for methylated and de-enrichment for unmethylated sequences, compared to 5 ng of input (sonicated DNA). Each library was diluted to 8 nM for sequencing on an Illumina Genome Analyzer following the recommended manufacturer's protocol at the core facility of the Center for Comparative and Functional Genomics [275, 276].

**Chromatin Immunoprecipitation [275]**

ChIP analysis was employed according to a modified protocol [277]. Briefly, 200 mg frozen liver samples from each pup were ground and resuspended in phosphate buffered saline (PBS). Cross-linking was performed in 37% formaldehyde for 10 min on a rotator at room temperature. The pellet was resuspended in nuclei swelling buffer and lysed in SDS lysis buffer, both of which contained protease inhibitors. The chromatin was sonicated (Fisher Scientific, model 100 Sonic Dismembrator) on ice with 7 bursts for 40 s and 2 min cooling interval between each burst with power set at 5. After removing cell
debris, sheared chromatin was diluted in ChIP dilution buffer. Diluted lysate (1 ml) was incubated with 2 μg of primary antibody of interest (Table 4.1) overnight at 4 °C on a hematology mixer (Model 346, Fisher Scientific). Pre-blocked salmon sperm DNA/protein G agarose beads (60 μL, 33% slurry; Millipore) were then incubated with each chromatin sample for 2 hours, followed by centrifugation for 1 min at 4 °C. Supernatant from incubation with normal rabbit IgG was saved as input. The pellets containing immunoprecipitated complexes were washed by a series of salt buffers, and antibody/protein/DNA complexes were eluted from Protein G agarose beads. After incubation at 65 °C for 5 hours with 20 μL 5 M NaCl and 1 μg of RNase A to reverse the cross-linking, protein was removed by Proteinase K, and DNA fragments were separated from chromatin structure. DNA was purified by a Wizard SV Gel and PCR Clean up System (Promega). Purified DNA with a dilution series as a standard curve was quantified by real time PCR reaction with primers designed for COX-2 promoter (Table 3.4).

**Statistical Analysis**

ChIP results are expressed as mean±SEM. Mean differences in promoter protein binding, and histone modification between C/C and HF/HF groups were determined by SAS statistical analysis using one-way ANOVA. Significance level was set at P<0.05 for all comparisons.

**Results**

**HF Diet Induced Hypomethylation of COX-2 Gene**

MeDIP-seq results showed a decreased DNA methylation intensity at the COX-2 5’UTR upstream and three coding regions (Figure 4.1, 4.2), whereas little difference was found at any β-actin regions (Figure 4.3, 4.4), suggesting that the exposure to high fat diet induced region-specific hypomethylation of COX-2 in rat liver. No DNA methylation differences were observed at the two CpG islands located at the proximal promoter region of COX-2. Decreased methylation intensity consistently occurred in all HF groups at 1KB, 2KB and 4KB of the coding region and 1.2 KB of 5’-flanking region.
**Histone Modifications**

Chromatin Immunoprecipitation [275] analysis showed that histone acetylation (histone H3 and H4) and methylation at lysine residues (K4, 9 and 27) of histone H3 in HF/C and C/HF were not different from that of C/C group (data not shown). However, life-long high fat feeding (HF/HF) modulated histone tails of COX-2. A wide range of reduced association of HDAC3 to the DNA sequence was observed, which may correlate to the increased acetylated histone H3 (H3Ac) and H4 (H4Ac) at coding regions (Figure 4.5). Also, increased di-methylation of histone H3 lysine 4 (H3K4Me2) occurred across multiple sites at both upstream and coding regions. Increased tri-methylation of histone H3 lysine 9 (H3K9Me3) was detected at -1.2kb and +3kb. The overall binding levels of H4Ac, HDAC3 and H3K4Me2 are remarkably higher than IgG, whereas H3Ac, H3K9Me3 and H3K27Me3 are relatively low. Increased acetylation of histones H3 and H4 often suggests an open chromatin structure and thus greater accessibility of DNA transcription regulators [278]. Hence, life-long feeding of a high fat diet programs histone modifications of the COX-2 gene in the liver, which in turn matches the activated transcription patterns.

**Discussion**

This is the first report concerning how high fat diet-induced COX-2 gene expression is associated with persistent decreased DNA methylation at 5’ flanking and multiple coding regions. However, histone reprogramming, unlike DNA methylation, can be detected only in HF/HF pups, meaning that the modifications were dependent on the stage of life during which exposure to a high fat diet occurred.

The interaction between nutritional and epigenetic influences on gene regulation has been of great interest to scientists who deal with chronic diseases and the improvement of human health. For decades, the limited experimental technology for the detection of DNA methylation constrained most studies to focus exclusively upon the impact of the diet on the methylation profile within a limited region of a single gene, and its
association with gene expression. The combination of MeDIP-seq and MRE-seq provide a detection method that provides a comprehensive high-resolution genome-wide coverage. This allows researchers to map out a slight shift in DNA methylation in the human genome [279].

High fat diets have been shown to mediate DNA methylation on the gene promoter and affect gene expression, which thereby leads to physiological alterations. The genome is composed of gene and non-gene associated regions. This study investigated the effects of high fat diets on the DNA methylation pattern within the associated genetic region, including the promoter, coding, and downstream regions. A study reported that high fat diets induced greater DNA methylation in the leptin promoter, which parallels the decrease in the expression of leptin [229]. Using a genome-wide promoter to analyze DNA methylation has shown that fatty acids increase the methylation of PPARγ coactivator-1 (PGC-1α) in skeletal muscle cells [280]. A recent study, which used Illumina's Infinium Bead Array, showed that a short period of high fat overfeeding induced genome-wide DNA methylation changes [281]. Still, there is no evidence on how maternal and post-weaning diet, alone or combined, can impact DNA methylation profile of COX-2 in the livers of the offspring. Combining two advanced sequencing methods, namely MeDIP-seq and MRE-seq, allowed us to be the first to present evidence that high fat diets induce region-specific hypomethylation of COX-2 in the liver of rats.

DNA methylation that occurs at genomic locations other than the promoter region can affect gene expression as well. It have been previously showed that a high fat diet was able to induce COX-2 gene transcription, independent of the feeding period. The present study observed that high fat feeding resulted in DNA hypomethylation at the 5' upstream and coding region of COX-2. Generally speaking, DNA hypomethylation is closely associated with gene repression or silencing. Our results thus suggest that high fat dietary intake delivers a strong signal to the epigenome, generating epigenetic behaviors, which in turn cause DNA hypomethylation in regions other than the promoter: this might be related to activation of COX-2. In addition, the existence of
positive correlations between DNA methylation in regions other than the promoter sequence and COX-2 gene enhancement might lead to the discovery of novel regulatory regions.

High fat diets enabled alterations of DNA methylation profiles, which led to further investigation of histone modifications. This study investigated several histone variations of interest, and found that the histone patterns for HF/C and C/HF were quite different than HF/HF. Comparing to the C/C group, no sound evidence of altered histone modifications was found in HF/C or C/HF. It thus remains difficult to define any possible participation of these histone modifications by the activation of COX-2, or DNA hypomethylation at any genetic region in HF/C and C/HF groups. Nevertheless, there appears to be a direct link to the HF/HF group. In most cases, histone acetylation, namely H3Ac and H4Ac, and H3K4Me2, are associated with transcriptional activation. Unlike DNA methylation, a particular configuration of histones depicts a very unique pattern in response to exposure of a high fat during different stages of life. It is worth noting that the effects of double-hit theory were reflected on the gene expression level of COX-2, as well as some physiological measurements, but neither for DNA methylation nor for histone modifications. Although changes in histone modifications were only detected in HF/HF, we cannot rule out the possibility that other histone modifications were taking place in these treatment groups, given the limited modifications tested and current knowledge of the histone code.

Fatty acids have been described as signal transducers that can be sensed by the epigenome [264]. Our results revealed a full landscape of DNA methylation profiles of COX-2, and showed that high fat diets removed methyl groups from CpG sites in multiple regions. The length of the traditional basic promoter of COX-2 is limited to 1kb, which comprises two detectable CpG islands. In our study, none of the changes, or only minimal changes, were identified by MeDIP-seq within this promoter region or the CpG islands, while a substantial reduction in methylation intensity was observed in the promoter upstream and coding regions, which correspond to the activation of COX-2. Interestingly, the location and grade of reduction is similar among all HF groups,
regardless of the stage of life during which exposure to a high fat diet occurs. Thus, DNA hypomethylation of the COX-2 gene is less likely to be connected to a double-hit effect on COX-2 mRNA abundance in this particular model.

**Conclusion**

Our findings provide insight into nutritional modulation of the epigenome, which is involved in liver health and function. For the first time anywhere, the impact of a high fat diet on the full landscape of DNA methylation of COX-2 was showed in the livers of rats. In our high fat feeding models, hepatic COX-2 activation is persistently accompanied by DNA hypomethylation, but is not accompanied by histone modifications. These region-specific hypomethylations induced by high fat diet are positively associated with changes in gene expression. Future site-specific validation research should be conducted in order to confirm DNA methylation change and to identify the transcriptional functions of these regions.
CHAPTER 5. AN ENHANCER WAS CHARACTERIZED AT COX-2 UPSTREAM REGION

Abstract

High fat diets, regardless of the feeding period, significantly induced COX-2 gene expression in the livers of male offspring pups. Genome-wide methylated DNA immunoprecipitation (MeDIP) showed that multiple regions of COX-2 gene sequences were hypomethylated, which corresponds to the activation of gene expression. Bisulfite sequencing was therefore performed to confirm the MeDIP results, and showed that hypomethylation occurred on a specific CpG site within the 5’UTR of COX-2. This region was further cloned to test the transcriptional activity using a luciferase reporter assay. The results show that this hypomethylated region produced strong enhancer activity. Further characterization showed that the binding elements HOXD8, RAF and CTCF contribute to the enhancer’s activity at different levels. In addition, the transcriptional activity of this enhancer cannot be further induced by the presence of arachidonic acid. In conclusion, high fat induced hypomethylation of this enhancer may be responsible for triggering gene activation of COX-2.

Introduction

COX-2 is present in cells in minute amounts under normal conditions [282], which justifies why induction of the gene is necessary and under exquisite regulation. Many experiments have been conducted to study COX-2 gene regulation, such as the Luciferase reporter assay, a widely used lab tool that is used to determine promoter strength and enhancer activity. Various cis-elements in the human COX-2 promoter were revealed that operate to control luciferase reporter activity. These core promoter elements, including nuclear factor kappaB (NF-kB), CCAAT/enhancer-binding protein (C/EBP) and cyclic AMP-response element (CRE) sites, were shown to be modulated in response to environmental stimuli, such as sulforaphane [283] and 6-(Methylsulfinyl)hexyl isothiocyanate [284]. These critical binding elements are exclusively located within 300 base pairs upstream of the transcription starting site.
(TSS). Highly conservative binding domains for CREB, NF-Kb, AP-1, and C/EBP can also be found within the promoter region of murine COX-2 [285]. Despite the fact that a number of transcription factors are commonly found in humans, murine, and rats, each of them may exhibit unique responses toward different environmental stimuli, and these require further inspection and specification. Nevertheless, the investigation of the 5'UTR’s regulatory role of COX-2 has never gone beyond its promoter, ranging from -900 to TSS.

In the previous chapter, an important aspect was presented that high fat diet actively manipulates COX-2 gene expression by modifying the landscape of its epigenome. Three hypomethylated regions in the high fat feeding models were identified, two of which situate in the gene body, and the other in the 5'UTR. The hypomethylation of DNA fragments in this particular dietary model perfectly correlates with the transcription level, and may or may not account for the gene activation of COX-2. Little research has been done on these particular regions to determine whether or not they exhibit any regulatory functions or transcriptional activities. In addition to the single CpG sites that are spread over the entire gene body and 5'UTR, two CpG islands are primarily distributed in the proximal promoter region. Methylation of CpG sites within the promoter can lead to gene silencing. However, these two CpG islands are barely methylated. Alteration of them is independent of high fat treatment as opposed to the single CpG sites, the methylation of which can be reduced by high fat diet. Even though the MeDIP-seq provides an overview of the DNA methylation pattern of COX-2, it is quite difficult to pinpoint the CpG site(s) that were truly modified. In addition, we cannot rule out the possibility that the hypomethylated CpG sites varied in the three high fat groups.

The traditionally defined rat COX-2 promoter does not cover this particular region ranging from -1.2kb to -1.1kb. This short DNA fragment contains multiple TF binding elements, several of which have not been studied in the context of COX-2 gene regulation. Moreover, in contrast to the default condition, which is highly methylated, the DNA methylation level in this region was nearly evened out by using high fat diet. In other words, the fold of reduction of methylation intensity in the 5'UTR region exceeds
those in the coding region. On the other hand, methylation of CpG regions downstream of the transcription starting site doesn’t block elongation, which means it may not critically be involved in transcription initiation [286]. Thus, this study is particularly interested in exploring this hypomethylated region in the vicinity of the COX-2 core promoter, because it may also represent an undefined regulatory region (enhancers, silencers, boundary elements/insulators) working in concert with the promoter. In order to answer the questions proposed above regarding methylation of which CpG sites were altered by high fat diet and the degree to which this region contributes to COX-2 gene transcription, bisulfite sequencing and a series of luciferase reporter assays were conducted.

**Materials and Methods**

**Bisulfite Sequencing**
To confirmation DNA methylation at 5’ upstream region (region 1), bisulfite conversion of genomic DNA was performed using EZ Methylation-Gold kit (Zymo Research, Orange, CA) according to the manufacturer’s instructions. Converted DNA was amplified in 5X LongAmp Taq Buffer (XX), 10 μM dNTPs, 5 μM forward and reverse primers (-1533 to -1109), and 1 U of Long Amp Taq polymerase. The cycling conditions used were as follows: 45 cycles of 95°C for 30 seconds, followed by 95°C for 10 seconds, 48°C 30 seconds and 65°C for 30 seconds, then 65°C for 10 minutes. PCR products were elongated with 5X Go Taq Flexi Buffer, 25 mM MgCl, 10 mM dATP, and Go Taq Flexi DNA polymerase at 72°C for 10 minutes. The PCR product was concentrated with a DNA Clean & Concentrator -5 kit (Zymo Research), ligated overnight at 16°C into the TOPO PCR 4.0 TA (Invitrogen) in accordance with the manufacturer’s instructions and transformed into DH5α E.coli (New England Biolabs). Plasmid DNA was isolated with the QiaPrep Spin Miniprep kit (Qiagen XX) and at least 50 independent recombinant clones per sample sequenced at the University of Illinois Core Sequencing Facility (Urbana-Champaign, IL) and the methylation data was compiled using the BISMA (Bisulfite Sequencing DNA Methylation Analysis) software (http://biochem.jacobs-university.de/BDPC/BISMA/). The lower threshold conversion
rate was set to 90%, while all the other analysis parameters were set to the BISMA default values (Mamrut, Harony et al. 2013).

**Cell Culture and Arachidonic Acid Treatment**

The human prostate cancer cell lines (PC3) was obtained from the American Type Culture Collection (Manassas, VA) and was cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 5% (v/v) fetal bovine serum (Mediatech; Herndon, VA) and 100 IU/mL Penicillin, and 100 μg/mL Streptomycin (Mediatech; Herndon, VA). Cells were maintained at 37 °C in 95% humidity and 5% CO2. For Arachidonic acid (AA) treatment, cells were first seeded at a density of 0.5×10^6 cells in a 6-well plate, grown for 16 h, and then starved with 0.3% FBS supplemented with 1.25mg/mL fatty acid free BSA (Sigma) for 48 hours before treatments. AA was neutralized with 1N NaOH and dissolved in the starvation media. Cells were incubated with 10μg/ μL AA for 30min, 1h, 2h and 3h to induce COX-2 expression.

**Plasmid Construction and Methylation**

Wild type COX-2 upstream fragments -1234/-1199, -1204/-1160, -1164/-1131, -1234/+30, -1234/-1029, -1234/-917 and -1234/-814 were amplified by a Thermal Cycler (Applied Biosystems) using LongAmp Taq DNA polymerase (New England Biolabs). The primers/fragments can be found in Table 3. 1. The reaction was performed at 94°C for 30 sec, followed by 40 cycles of 94°C for 30 s, 67°C for 60 sec and 65°C for 50 sec to reach maximum amplification, and ended with 10 min of 65°C for final extension. The restriction enzymes used below are all acquired from New England Biolabs. The fragment of -1234 to +30 was cut with Nhel, gel-purified, and cloned into the Nhel site of a luciferase reporter vector PGL3-basic (PGL3-b) (Promega Corporation). This generated forward and reverse- orientated products. The forward product was cut with Sacl, deriving a partially truncated promoter region (-774/+30) and upstream region (-1234/-774), and Smal generating a fragment of -1234/-1131. The upstream fragment (-1234/-774) was cloned into the Sacl site of a PGL3-promoter (PGL3-p) vector and generated forward and reverse- orientated products. This forward product was further cut with Smal and generated two upstream regions (-1234/-1131 and -1131/-774) in
PGL3-p vector. The reverse product of PGL3-b (+30/-1234) was cut with Smal and derived a partially truncated region (-1131/-1234), which was cloned into PGL3-p vector. The fragments of -1234/-1029, -1234/-917 and -1234/-814 were cut with SacI, gel-purified, and cloned into the SacI site of PGL3-p, from where a forward-orientated product was generated for each fragment. Selected plasmids were methylated using CpG Methyltransferase (M.SssI) (New England Biolabs).

Transfection and Luciferase Reporter Assay
PC3 cells were plated in 24-well plate for 24hr prior to transfection. Each plasmid (0.5 ug) were transfected into each well using Superfect transfection reagent (Qiagen; Valencia, CA) following manufacturer's instructions. Meanwhile, an empty reporter-gene plasmid was transfected following the same procedure, and used as a control. Cells were maintained in transfection for duration of 3 hr. Cells were then lysed with Passive Lysis Buffer (PLB) and subjected to one round of freeze-thaw. Luciferase activity was measured using Single-Luciferase Assay (Promega) with a luminometer (Fermomaster FB12, Zylux Corporation; Huntsville, AL). For each experimental condition, six replicates were performed.

Prediction of Transcription Factor Binding Sites and Site-directed Mutagenesis of CTCF, RAF and HOXD8 Binding Element
Transcription Element Search System (TESS) was used to predict transcription factor binding sites in DNA sequences. It identifies binding sites using site or consensus strings and positional weight matrices from the TRANSFAC, IMD, and CBIL-GibbsMat database. Mutant -1234/-1131 construct were prepared using QuickChange Lighting Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Primers contained mutations in putative CTCF, RAF and HOXD8 binding site (Table 3.1). PCR products were digested with DpnI restriction enzyme (Agilent Technologies) to remove the template DNA. These DNA constructs were sequenced to confirm the mutation site.

Statistical Analysis
Percent methylation patterns of all CpG sites were extracted and for each site the probability of the observed pattern was calculated assuming a model of stochastic binomial distribution for the calculation of the p-value. A parallel comparison using one-way ANOVA was performed in between different transfections.

**Results**

**Bisulfite Sequencing Showed a Site-specific CpG Hypomethylation**

After MeDIP analysis, the region upstream of the hypomethylated promoter was located. The specific upstream region from -1533 to -1109 was fully analyzed through bisulfite sequencing, and the results for the methylation pattern for all 9 CpG sites are presented on Figure 5.1. The graphical display of the average methylation at each CpG site reveals a differential distribution of methylated cytosines between the control group (C/C) and the high fat groups (HF/HF, HF/C, C/HF); The analysis of the average methylation percentage (Figure 5.2)(Table 5.1) for each clone indicates greater site-specific CpG methylation for the healthy control (C/C), compared to the high fat control group (HF/HF), specifically on the 8th CpG site (-1169). Hence, the 8th CpG site is persistently hypomethylated by high fat diet in this region.

**Identification of An Enhancer Element Located at the 5’ UTR**

To investigate the transcription function of the HF- induced hypomethylated fragment at the 5’ region upstream of COX-2, several constructs were prepared from the rat gene that contain this fragment (-1234/-1131, the hypomethylated region from MeDIP result) as well as downstream genomic sequences through +30 and -774. These sequences were cloned into the PGL3-B luciferase reporter vector and their ability to drive regulated transcription was measured (Figure 5.3). The basic promoter of COX-2 (-774/+30) has greater transcription activity, but not as great as the full fragment does (-1234/+30). Therefore, the fragment of -1234/-774 was cloned into Pgl3-p luciferase reporter for further investigation. This construct containing the portion of the 3’ upstream (-1234/-774) region exhibited a relatively small but reproducible increase in transcription. Deletion in the 3’ direction yielded a construct (-1131/-774) that lost transcription activity
and another construct (-1234/-1131) that had significantly increased transcription activity. The fragment containing the -1234/-1131 region, regardless of the orientation, produced the highest transcription activity. When this DNA sequence was placed in front of the Pgl3-b vector, it resulted in an even higher transcription induction than the traditional promoter of COX-2 (Figure 5.3). Therefore, the DNA fragment of -1234/-1131 exhibits transcriptional enhancer activity, and it appears that this fragment can serve as an alternative promoter to COX-2.

Effects of \textit{in vitro} CpG Methylation on Enhancer Activity

\textit{In vitro} methylation at CG dinucleotides in a transfecting plasmid can inhibit gene expression in mammalian cells. In order to test if the methylated enhancer lost its transcriptional activity, PGL3-P and PGL3-P containing the COX-2 enhancer were methylated (Figure 5.5). The results showed that methylation of both plasmids have deprived their original transcriptional activities. PGL3-P containing the COX-2 enhancer exhibited 3 times greater ability to drive regulated transcription than PGL3-P. After methylation, the transcriptional activity driven by the COX-2 enhancer was only increased by less than 1 time. Therefore, \textit{in vitro} methylation of the plasmid resulted in a significant reduction of the transcriptional activity driven by COX-2 enhancer. This result also suggested that the transcriptional activity of this COX-2 enhancer was not fully dependent on methylation status. Other factors, such as the binding of TFs that are not affected by DNA methylation, may sustain the remaining activity of the enhancer.

Transcriptional Activity of Different Fragment of COX-2 Enhancer

In order to locate the possible critical binding elements within this enhancer, three shorter DNA fragments (Fragment 1, -1234/-1199; Fragment 2, -1204/-1160; Fragment 3, -1163/-1131) (Figure 5.6) were cloned into PGL3-P in both forward and reverse position and were checked for their transcription activity (Figure 5.7). All of them produced significantly higher luciferase signal than PGL3-P. In comparison to the control plasmid, fragment 1 exhibited 3 times higher activity while fragment 2 and 3 exhibited 7 to 8 times higher activities in forward position. Even though the activity was lower when these fragments were placed in reverse orientation, they were still able to
generate 3 to 4 times higher activity than the control plasmid did. These results suggested that each fragment was sufficient to produce transcriptional enhancer activity, and that fragment 2 and 3 might comprise stronger TFs binding elements to drive the transcription initiation.

Site-directed Mutagenesis Revealed Crucial Transcription Factors

In order to characterize the critical and novel binding factors to this enhancer, binding sites for predicted transcription factors were mutated and tested them with a luciferase reporter assay. The predicted TFs have been mapped out in Figure 5.6 based on the TESS analysis. The binding of these TFs tend to be clustered. Therefore, mutation of the DNA sequences that bind to one TF may also affect the binding of the ones in the nearby vicinity. Several TFs have been shown to be associated with COX-2 transcriptional activity in a variety of cell types, including NF-1 [287], Ets-1 [288], c-Myb [289], YY1 [290], PU.1 [291], HSP1 [292] and AP-2 [293]. Mutation of the binding elements for these TFs is prone to alter transcriptional activity. In contrast, there have not been any studies showing that COX-2 gene transcription is subject to the regulation of POU1F1α, NF1/L, TEF, HOXD8, HSTF, GAL4, GCN4, RAF, CTCF and MAZ. Given the fact that POU1F1α, NF1-L, TEF, HSTF and GAL4 share consensus binding sequences with TFs that were known to regulate COX-2 transcription, we decide to begin the tests with RC2/HOXD8, RAF and CTCF. CTCF is of greater interest in the test due to its insulator properties [294, 295] and its close relevance to DNA methylation status [296]. The individual site-directed mutations were performed for each of them, generating three sets of enhancers that carried one mutation site at a time (Figure 5.8 and 5.9). The Luciferase assay showed that mutated CTCF and RAF sites lessen the transcriptional activity of the original enhancer by 25%, whereas mutated HOXD8 sites entirely deplete transcriptional activity (Figure 5.9). These results, taken together, suggested that RC2/HOXD8, RAF and CTCF are potential contributors to enhancer activity.

Enhancer Activity Cannot be Further Induced by Arachidonic Acid
In accordance to the previous publication, AA was used to induce COX-2 expression in PC3 cells. Samples were harvested at different time points. Compared to BSA control cells, the greatest induction of COX-2 mRNA was acquired at a time of 3 hours (Figure 5.10). However, using the same treatment condition, luciferase activity of the enhancer element in both orientations cannot be further induced by AA treatment (Figure 5.11).

**Discussion**

To our knowledge, these results are the first to identify a novel enhancer element at 5'UTR that is independent of the promoter sequence of the COX-2 gene. In high fat treatments, this enhancer element was persistently hypomethylated, which may contribute to the gene activation of COX-2 in the liver. Bisulfite analysis confirmed the MeDIP-seq map and pinpointed the possible hypomethylated CpG sites on this enhancer fragment.

The MeDIP-seq results in Chapter 4 led to the identification of a novel enhancer at the 5' UTR of COX-2. Rat model-based COX-2 gene analysis has not been as extensive as human and mouse analysis. This study preceded the previous research of rat COX-2 gene regulation. It was able to target a specific region based on the fully revealed DNA methylation landscape of COX-2. In both humans and mice, a luciferase assay established the predominant regulatory role of the COX-2 core promoter. Promoter activities in these two species are quite significant. Mutations of these given TF binding elements can result in a dramatically reduced transcriptional activity toward LPS challenge [285, 297]. However, there appears to be a different regulation scenario for rat hepatic COX-2, if one consider that the promoter location of this gene is conservative across species. These results showed that the promoter activity of COX-2 in rats was not as great as what had been seen in both humans and mice. Instead, when the tested fragment was extended and harbored the hypomethylated region at the 5' upstream region of the promoter, this yielded stronger activity. This is an essential piece of evidence that demonstrated that COX-2 activation in rat livers may require the DNA sequence at the region which is 5' upstream of the promoter. A further set of experiments proved this point of view by revealing the enhancer activity of this particular
DNA fragment. Thus, it is speculate that consuming a high fat diet induces COX-2 at least partially by motivating the enhancer activity, and the hypomethylation that occurred on this enhancer could be an underlying mechanism which bridges the high fat signal and enhancer stimulation. This enhancer also presents activity which resembles that of the promoter. Additional investigations will be required to further characterize its properties.

This study also provided the first evidence in rats that high fat diet undermethylated single CpG sites within this transcriptional enhancer. DNA methylation on both CpG islands and single/multiple CpG sites can be closely associated with gene expression and disease development. Many genes contain more than one CpG island on their genomes, and these are specifically associated with the start of the gene when they are within promoter regions. Likewise, methylation of CpG sites within the promoters of genes can lead to gene silencing, whereas reduced methylation intensity of CpG sites has been associated with gene activation. This "on and off" phenomenon has been found frequently in cancer [298, 299] and has also been observed in numerous other types of diseases, such as heart disease [300], chronic kidney disease [301] and NAFLD [302]. Activation of the human COX-2 gene was previously found to be inversely correlated with the DNA methylation of a single CpG site within the promoter of patients with chronic periodontitis [303]. In contrast to this study, rat COX-2 was induced when methylation of a single CpG site within the enhancer was decreased, which means it is with fewer relevancies to promoter methylation. This observation supports the increasing amount of emerging evidence regarding mice [304] and human studies [305, 306] that DNA methylation state could be closely associated with gene expression alteration. Furthermore, these cell type- [307] and lineage-specific [308] DNA methylations of a given enhancer were expected to produce gradients of expression in a wide range of tissues. Thus, DNA hypomethylation of this enhancer of hepatic COX-2 might be a very particular and unique consequence of a high fat diet.

Nevertheless, it was wondered how DNA hypomethylation of this enhancer might be associated with COX-2 induction. Distal methylation sites that were associated with
expression were in a particular class of enhancers [306]. These enhancers were bound to transcription factors depending on the DNA methylation state [306]. In other words, altered DNA methylation at a given CpG site in these enhancers may affect the downstream gene transcription by changing the interaction between DNA structure and transcription factors. TESS analysis revealed a number of TFs that can potentially bind to this enhancer sequence. Among them, CTCF generally binds to the hypomethylated region [296], which partially protects surrounding genetic sequences from methylation [309]. It can form boundaries by binding to the regions that are between enhancers and promoters, and thereby block enhancer function [294, 295]. CTCF shares consensus sequences with many other TFs, such as Sp1 and AP-2. Therefore, even though mutation of CTCF binding sequence reduced transcriptional enhancer activity, it is not assured that CTCF truly interacted with the enhancer. Additional sets of experiments and analysis may allow revealing this point. The DNA sequence containing the 8th CpG that is hypomethylated in high fat groups constitutes a binding spot for RAF. Mutation of this binding site undermined the transcriptional activity of COX-2 enhancer, although it is not clear as to whether DNA methylation of this CpG site affected RAF binding. There hasn’t been a solid conclusion concerning the regulatory function of HOXD8 during COX-2 activation. Our research is the first to reveal that removal of HOXD8 in COX-2 enhancer not only reduced the enhancer activity, but also diminished SV40 promoter activity carried by PGL3-P. However, there hasn’t been a reasonable explanation provided for this phenomenon. Although these DNA sequences appear to contain elements that are essential to enhancer activity, further characterizations are indispensable for fully defining this methylation-sensitive enhancer of COX-2.

In addition to characterizing the transcriptional function of hypomethylation region, the current study is interested in finding a possible physiological cause that can explain why and how it happened in the first place. In chapter 3, increased arachidonic acid content of the livers of high fat treated pups was showed. Given that it was the sole substrate of COX-2 enzymatic reaction, we want to determine whether arachidonic acid can promote enhancer activity in order to induce COX-2 gene expression. However, the supply of arachidonic acid did not further elevate enhancer activity in the PGL3-P construct.
There are multiple explanations why this might happen. First, arachidonic acid does not affect this enhancer. Second, the induction condition for human PC3 cells cannot be applied to rat COX-2. Third, arachidonic acid might induce enhancer activity by reducing the methylation intensity, which cannot be fulfilled in this plasmid because the inserted DNA sequence is demethylated. Given these concerns, an alternative experimental design, such as using a rat cell line, might offer better insights that can reveal the physiological causes.

**Conclusion**

In summary, the current study showed that a single CpG site at 5'UTR was persistently hypomethylated by high fat diets. This study also identified the enhancer property of this hypomethylated region by comparing its transcriptional activity to other DNA sequences. It is speculated that altered DNA methylation is associated with enabled enhancer activity, which triggers COX-2 transcription. Additional characterization and exploration of physiological motivators of this enhancer will be conducted in future research.

Undermethylated genomic regions, specifically promoters or enhancers, have been associated with the elevated expression of downstream genes. However, previous work may have overlooked the roles that enhancer DNA methylation, and single CpG site demethylation, has played in regulating gene expression. These regions may be actively involved in other scenarios that provoke COX-2 transcription, so correcting misconstructed epigenomes on these enhancers/regions may efficiently reduce transcription and subsequent physiological responses. COX-2 inhibitors have long been used in the anti-inflammatory drug category, but produce a significant increase in heart attacks and strokes [310]. Studies using epigenetic tools may offer a different perspective in inhibiting overproduction of COX-2 in such diseases by correcting transcription levels using epigenetic therapeutic strategies.
Figure 3.1 Growth curve (A) and weekly caloric intake (B) of control (C/C) offspring and offspring fed a high fat diet during gestation and lactation (HF/C), postweaning (C/HF) or throughout life (HF/HF). Values are mean ± SEM, n=10 animals. Some error bars are too small to be seen.
Table 3.1 Body weight (BW) and caloric intake analyzed by Repeated ANOVA$^{1,2}$

<table>
<thead>
<tr>
<th>BW</th>
<th>HF/C</th>
<th>C/HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>0.5</td>
<td>0.05</td>
</tr>
<tr>
<td>HF/HF</td>
<td>0.009*</td>
<td>0.11</td>
</tr>
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</table>

<table>
<thead>
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<th>Kcal</th>
<th>HF/C</th>
<th>C/HF</th>
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</thead>
<tbody>
<tr>
<td>C/C</td>
<td>0.79</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>HF/HF</td>
<td>&lt;0.0001*</td>
<td>0.64</td>
</tr>
</tbody>
</table>

$^1$Comparison are made between each two treatment groups

$^2$*P<0.05
Figure 3.2. Representative images of H&E staining (upper panel) and ORO staining (lower panel) in livers of C/C, HF/C, C/HF and HF/HF offspring. Objective, 20X, Scale bar, 200µm.
Figure 3.3. Scattered plot of hepatocellular vacuolation counting (upper panel) and ORO evaluation (lower panel). Each symbol represents one individual sample. Horizontal lines represent mean value. n=8 individual animals. Individual treatment with different letters differ (P<0.05)
Figure 3.4. Hepatic (top) and plasma (bottom) TAG levels were measured in four treatment groups. Data are presented as means ± SEM, n= 6. Letters are assigned by LSD. Individual bars with different letter differ (p<0.05).
Figure 3.5 Expression of COX-2 mRNA, pre-mRNA and Δ6Desaturase mRNA were measured by qPCR and normalized to internal control gene β-actin. Data are presented as means ± SEM, n= 8. Letters are assigned by LSD. Individual bar with different letter differ (p<0.05).
### Table 3.2. Gas Chromatograph analysis of fatty acid composition in liver\(^1,2\)

<table>
<thead>
<tr>
<th>Fatty Acid Composition</th>
<th>CC (%)</th>
<th>HFC (%)</th>
<th>CHF (%)</th>
<th>HFHF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Lauric acid)12:0</td>
<td>44.6±0.03</td>
<td>46.8±0.03</td>
<td>49.8±0.03</td>
<td>57.0±0.03</td>
</tr>
<tr>
<td>(palmitic acid)16:0</td>
<td>14.5±0.006</td>
<td>15.3±0.01</td>
<td>9.6±0.01</td>
<td>5.9±0.01</td>
</tr>
<tr>
<td>(palmitoleic acid, n-7)16:1</td>
<td>6.7±0.004(a)</td>
<td>3.2±0.005(a,b)</td>
<td>0.3±0.001(b)</td>
<td>0.2±0.001(b)</td>
</tr>
<tr>
<td>(stearic acid)18:0</td>
<td>4.7±0.004</td>
<td>4.7±0.003</td>
<td>3.9±0.2</td>
<td>3.5±0.01*</td>
</tr>
<tr>
<td>(oleic acid, n-9)18:1</td>
<td>16.7±0.02</td>
<td>13.4±0.02</td>
<td>15.8±0.008</td>
<td>15.1±0.01</td>
</tr>
<tr>
<td>(linoleic acid, n-6)18:2</td>
<td>8.7±0.004(b)</td>
<td>10.0±0.004(a,b)</td>
<td>12.2±0.007(a)</td>
<td>11.1±0.01(b)</td>
</tr>
<tr>
<td>(Arachidonic acid, n-6)20:4</td>
<td>4.3±0.005(b)</td>
<td>6.4±0.003(a)</td>
<td>8.3±0.005(a)</td>
<td>7.3±0.1(a)</td>
</tr>
</tbody>
</table>

\(^1\)Values are means ± SEM, n=6.

\(^2\)Letters were assigned to indicate significant different between treatment groups.
Table 3.3. Two-Way Factorial Interaction\(^1\)

<table>
<thead>
<tr>
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<th>Maternal</th>
<th>Postweaning</th>
<th>Maternal * Postweaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2 mRNA</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<tr>
<td>COX-2 Protein</td>
<td>0.004</td>
<td>0.12</td>
<td>0.01</td>
</tr>
<tr>
<td>Δ6Desaturase</td>
<td>0.0008</td>
<td>0.0014</td>
<td>0.09</td>
</tr>
<tr>
<td>TAG</td>
<td>0.22</td>
<td>&lt;0.0001</td>
<td>0.03</td>
</tr>
<tr>
<td>Vacuolation</td>
<td>0.39</td>
<td>0.0012</td>
<td>0.1</td>
</tr>
<tr>
<td>ORO</td>
<td>0.55</td>
<td>&lt;0.0001</td>
<td>0.1</td>
</tr>
<tr>
<td>(Lauric acid)12:0</td>
<td>0.19</td>
<td>0.07</td>
<td>0.6</td>
</tr>
<tr>
<td>(palmitic acid)16:0</td>
<td>0.26</td>
<td>0.0003</td>
<td>0.17</td>
</tr>
<tr>
<td>(palmitoleic acid, n-7)16:1</td>
<td>0.22</td>
<td>0.01</td>
<td>0.34</td>
</tr>
<tr>
<td>(stearic acid)18:0</td>
<td>0.93</td>
<td>0.25</td>
<td>0.54</td>
</tr>
<tr>
<td>(oleic acid, n-9)18:1</td>
<td>0.4</td>
<td>0.81</td>
<td>0.59</td>
</tr>
<tr>
<td>(linoleic acid, n-6)18:2</td>
<td>0.53</td>
<td>0.13</td>
<td>0.02</td>
</tr>
<tr>
<td>(Arachidonic acid, n-6 )20:4</td>
<td>0.26</td>
<td>0.003</td>
<td>0.04</td>
</tr>
</tbody>
</table>

\(^1\)Interaction of main effects (maternal X postweaning)
Figure 3.6 Expression of COX-2 protein content in the liver of male offspring by Western blot (n=6). The bands presented the average level of OCX-2 protein in control and all high fat groups. Data are present as means ±SEM, n=6. Letters are assigned by LSD. Individual bar with different letter differ (p<0.05). Actin was used as loading control to indicate total protein quantity.
Figure 3.7 Correlation of Δ6Desaturase mRNA (top), COX-2 pre-mRNA (middle) and protein (bottom). Each symbol represents one individual sample. Regression line was calculated after the analysis.
Figure 3.8. Representative images of COX-2 protein content in livers of C/C, HF/C, C/HF and HF/HF offspring. Protein content of COX-2 in liver was analyzed by immunofluorescent staining using an antibody against COX-2 protein and an Alexa Fluor 647-labeled secondary antibody (red, middle panel). Nuclei were counterstained with Hoechst 33342 fluorescent stain (blue, top panel). The two pictures were merged to show the cytoplasmic location and distribution of COX-2. Objective, 63x. Scale bar, 20 μm.
Table 3.4. Primer sequences used in real-time PCR analysis of genes

<table>
<thead>
<tr>
<th>Gene Name (Ensembl ID #)</th>
<th>Sequences</th>
</tr>
</thead>
</table>
| rActb mRNA (ENSRNOE00000310180) | Forward (+451), 5’- GAG ACC TTC AAC ACC CCA GC-3’  
|                           | Reverse (+526), 5’- CAG TGG TAC GAC CAG AGG CA -3’ |
| rΔ6D mRNA (ENSRNOE00000222342) | Forward (+165), 5’- TGA CAG GCT CAG GAA ATG TC-3’  
|                           | Reverse (+257), 5’- TTG CGA TCA ATC ACC AGC-3’ |
| rCOX-2 mRNA (ENSRNOT00000003567) | Forward (+1048), 5’- ATTACTGCTGAAGCCACC -3’  
|                           | Reverse (+1115), 5’- TTGTCAGACTCCAATTTGAG -3’ |
| rCOX-2 pre-mRNA | Forward (+2972), 5’- CAGGCATCGGACTCTGCTAT -3’  
|                           | Reverse (+3052), 5’- AGCAGCACAATGAGGGGA -3’ |
| rCOX-2 ChIP | Forward (-1664), 5’- CAGTCTGTGCACTCAGTTGAGATGG -3’  
|                           | Reverse (-1598), 5’- TTCCGTGTATGGCACTCCCAA -3 |
| rCOX-2 ChIP | Forward (-1320), 5’- TTGCCCAGACTGCTTCAAC -3’  
|                           | Reverse (-1217), 5’- CTGTCTTGGCAGCTAATGGGA -3’ |
| rCOX-2 ChIP | Forward (-135), 5’- AACTGAGCGGGAGCTCCAGGAG -3’  
|                           | Reverse (+68), 5’- TGGCAGGACACAGAGCTGAGTTC -3 |
| rCOX-2 ChIP | Forward (+1847), 5’- ATTACTGCTGAAGCCACC -3’  
|                           | Reverse (+1115), 5’- TTGCCAGACTCCCTTGAAG -3 |
| rCOX-2 ChIP | Forward (+135), 5’- TCTCCAACCTCTCTACTACAC -3’  
|                           | Reverse (+1934), 5’- TGTACTCACCTTTACACCC -3 |
| rCOX-2 ChIP | Forward (+2972), 5’- CAGGCATCGGACTCTGCTAT -3’  
|                           | Reverse (+3052), 5’- AGCAGCACAATGAGGGGA -3 |
| rCOX-2 ChIP | Forward (+3967), 5’- CTCAAAACAGGAGCATCCTG -3’  
|                           | Reverse (+4031), 5’- ATCAGTATGAGCCTGCTGG -3 |
Figure 4.1 MeDIP-seq results of COX-2. Displayed tracks include DNA methylation (MeDIP-seq) for rat COX-2 in liver tissue from C/C, HF/C, C/HF and HF/HF offspring. The dashed box highlights the hypomethylated region at COX-2 5’ upstream in all HF treated groups. Blue line illustrates COX-2 gene structure, with each square box represents an exon. Two CpG islands are marked at the bottom at their relative location.
Figure 4.2 Landscape of DNA methylation patterns (A) and gene structure (B) of COX-2. A. Y axils represents the MeDIP reads, X axils represents the relative location to TSS. Individual comparison was made between each high fat group (HF/C, top; C/HF, middle, HF/HF, bottom) and control. Hypomethylated region in 5'UTR was highlighted in the dashboxed as it may closely associated with gene transcription of COX-2. B. Ten exons were labeled according to the relative location. Two CpG islands were marked with arrows in gene structure illustration. Each hypomethylated region was tagged with hyphen.
Figure 4.3. MeDIP-seq results of β-actin. Displayed tracks include DNA methylation (MeDIP-seq) for rat β-actin in liver tissue from C/C, HF/C, C/HF and HF/HF offspring. Blue line illustrates β-actin gene structure, with each square box represents an exon. Two CpG islands are marked at the bottom at their relative location.
Figure 4.4 Landscape of DNA methylation patterns (A) and gene structure (B) of β-actin. A. Y axils represents the MeDIP reads, X axils represents the relative location to TSS. Individual comparison was made between each high fat group (HF/C, top; C/HF, middle, HF/HF, bottom) and control. B. Six exons were labeled according to the relative location. One CpG island was marked with arrows in gene structure illustration.
Figure 4.5. Mapping of the histone structure of COX-2 gene in the liver of C/C (n=8) and HF/HF (n=8) groups. Acetylation of histone H4 (top), H3 (middle) and binding of HDAC3 (bottom) at upstream, promoter and coding regions of COX-2 were detected. Data are represented as the ratio to the value obtained with input DNA. The normal rabbit IgG was used as negative control to mark the non-specific association background for each tested region. Values are means ± SEM. *P<0.05
Figure 4.6 Mapping of the histone structure of COX-2 gene in the liver of C/C (n=8) and HF/HF (n=8) groups. Di-methylation of histone H3 Lysine 4 (top), tri-methylation of H3 Lysine 9 (middle) and Lysine 27 (bottom) at upstream, promoter and coding regions of COX-2 were detected. Data are represented as the ratio to the value obtained with input DNA. The normal rabbit IgG was used as negative control to mark the non-specific association background for each tested region. Values are means ± SEM. *P<0.05
<table>
<thead>
<tr>
<th>Antibody Name</th>
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<tbody>
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<td>Santa Cruz</td>
</tr>
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<td>06-599</td>
<td>Millipore</td>
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<tr>
<td>H4Ac</td>
<td>06-866</td>
<td>Millipore</td>
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<tr>
<td>HDAC3</td>
<td>sc-11417</td>
<td>Santa Cruz</td>
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<td>H3K4Me2</td>
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<td>Millipore</td>
</tr>
<tr>
<td>H3K9Me3</td>
<td>07-442</td>
<td>Millipore</td>
</tr>
<tr>
<td>H3K27Me3</td>
<td>07-449</td>
<td>Millipore</td>
</tr>
<tr>
<td>IgG</td>
<td>sc-2027</td>
<td>Santa Cruz</td>
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</table>
Figure 5.1 CpG methylation within -1533 to -1109 upstream region of COX-2 coding region in liver of offspring from high fat fed groups. Distribution of methylation levels of individual CG sites within the analyzed region. *Indicates significant difference compared to the C/C group (P≤0.05).
Figure 5.2 Methylation statuses of the nine CpG sites. Each column corresponds to one CpG site in the studied region, and each row represents average methylation of 50 individual clones.
Table 5.1 Percentage of Methylation at each CpG site

<table>
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<tr>
<th>CpG site#</th>
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<th>2</th>
<th>3</th>
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<th>6</th>
<th>7</th>
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<tbody>
<tr>
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<td>-1441</td>
<td>-1431</td>
<td>-1410</td>
<td>-1217</td>
<td>-1213</td>
<td>-1169</td>
<td>-1134</td>
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<tr>
<td>Methylatio n (%)</td>
<td>C/C</td>
<td>HF/C</td>
<td>C/HF</td>
<td>HF/HF</td>
<td>C/C</td>
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<td>88.9</td>
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</tbody>
</table>

*p<0.05 comparing to C/C
Figure 5.3 Identification of the essential elements in rat COX-2 by luciferase assay. The diagrams on the top show the firefly luciferase reporter constructs driven by various rat COX-2 fragments of -774/ +30, -1234/+30 and -1234/-1131 in Pgl3-basic. The luciferase activities from firefly were assayed and the relative luciferase activity was obtained by normalizing the firefly luciferase activity of constructed plasmids against the control plasmid luciferase activity. The results are expressed as mean ± the SEM of six replicated experiments.
Figure 5.4 Identification of the essential elements in rat COX-2 by luciferase assay. The diagrams on the top show the firefly luciferase reporter constructs driven by various rat COX-2 fragments of -1234/-774, -774/-1234, -1131/-774, -774/-1131, -1234/-1131 and -1131/-1234 in Pgl3-promoter. The luciferase activities from firefly were assayed and the relative luciferase activity was obtained by normalizing the firefly luciferase activity of constructed plasmids against the control plasmid luciferase activity. The results are expressed as mean ± the SEM of six replicated experiments.
Figure 5.5. Luciferase activity of methylated constructs. Values of control are normalized to PGL3-p, values of +Me are normalized to PGL3-p+Me. The results are expressed as mean ± the SEM of six replicated experiments.
Figure 5.6. Transcription Element Search System (TESS) analysis of COX-2 enhancer. Three segments representing the left, middle and right part of this enhancer was constructed. The starting and ending nucleotide position was noted. The potential binding transcription factors are labeled at its predicted site.
Figure 5.7 Characterization of COX-2 enhancer by luciferase assay. The diagrams on the top show the firefly luciferase reporter constructs driven by various rat COX-2 fragments of -1234/-1199 (forward and reverse), -1204/-1160 (forward and reverse), -1163/-1131 (forward and reverse) in Pgl3-promoter. The luciferase activities from firefly were assayed and the relative luciferase activity was obtained by normalizing the firefly luciferase activity of constructed plasmids against the control plasmid luciferase activity. The results are expressed as mean ± the SEM of six replicated experiments.
Figure 5.8. Diagram shows the site-directed mutagenic sites of COX-2 enhancer. Three different mutations were conducted separately. From left to right: ΔHOXD8, TTA to GGG; ΔRAF, CGG to ATT; ΔCTCF, CCCT to TTTC.
Figure 5.9 Characterization of COX-2 enhancer by luciferase assay. The diagrams on the top show the firefly luciferase reporter constructs driven by various rat COX-2 fragments that contains different mutation sites. Relative location of each mutation site is marked. The luciferase activities from firefly were assayed and the relative luciferase activity was obtained by normalizing the firefly luciferase activity of constructed plasmids against the control plasmid luciferase activity. The results are expressed as mean ± the SEM of six replicated experiments.
Figure 5.10 Time course of the Arachidonic acid treatment. PC3 cells were grown in RPMI-1640 medium and starved with 0.3% FBS supplemented with 1.25mg/mL fatty acid free BSA for 48 hours before treatment. Cells were then incubated with 10μg/μL AA for 30min, 1h, 2h and 3h to induce COX-2 expression. Values are means ± SEM. *P<0.05
Figure 5.11 Luciferase activities of enhancer constructs (Forward, F; Reverse, R) in response to Arachidonic acid. Values are normalized to PGL3-p. The results are expressed as mean ± the SEM of six replicated experiments.
### Table 5.2 Primer sequences used in cloning and sequencing

<table>
<thead>
<tr>
<th>COX-2 (ENSRNOT00000003567)</th>
<th>Sequences</th>
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| -1234 to -1199, +SmaI      | top, GGG CCATTCACTGCCAAGAAGGTACGGTTTAATTGAAATGT CCC  
bottom, GGG ACATCAAATAACCCGTATCTTGCTGGCATGAATGG CCC |
| -1204 to -1160, +SmaI      | top, GGG GAATGTTTTAGTTTCTCATTTTTCTTTGTGGTTATTCAACTGGTTTTCTAC CCC  
bottom, GGG GTAAAAACCGAGTAAAAACAGAAAAATGAGGAAAAACTAAAAACATTC CCC |
| -1163 to -1131, +SmaI      | top, GGG CACTATTCCCTCTAGATCCCCTCCCGTGGGC CCC  
bottom, GGG GCCGGGGAGGGGATCTGAAGATGGAATAGTC CCC |
| -1234, +Sacl              | Forward, ATTATA GAGCTC CCATTCTGCAAGAAGGTACGGT |
|                           | Reverse, ATTATA GAGCTC ATCCA CTCT GTTCTG AAACA TTGCT TGA |
| -1029, +Sacl              | Reverse, ATTATA GAGCTC GACTTATTFTTGTCCACGTTTACGTTACAAG |
| -906, +Sacl               | Reverse, ATTATA GAGCTC GGTATTTTCTCCCTGCTGTCTACATAGC |
| -814, +Sacl               | Reverse, ATTATA GAGCTC GGCTTTTCTCTCCCTGGCTGTCTACATAGC |
| -1234, +Nhel              | Forward, ATTATAGCTAGCCATCTAGCAAGAAGGTACGGT |
| +30, +Nhel                | Reverse, ATTATAGCTAGCAGCGCAAGACAGACAGCGCTCGGAAGAGC |
| deltaRAF, cgg to att      | Forward, TTTAGTTTTCTCATTTTTTGTGTTTTTACTATTGTTTTTCATATTCTACCCATCCTAGNTCGATATCCC  
Reverse, GGGGATCTGAGGATGGAATGTAAGAAAAATGAAAAAGAAAAATGAGGAAAACTAAA |
| deltaHOXD8, tta to ggg    | Forward, TCCCATTTCTGCAAGAAATGCTACGTTGGGATTTGAATGTTTTTACCTTTTCTCATTTTTTCCC  
Reverse, GAAATAGGGAAATCTAAACACCTTCTTCCCGTACGTCTTCTGGCATGAAATGGGA |
| deltaCTCF, ccct to ttcc   | Forward, CTATTCCATCTCAGATCTCTCCCGGGGCTCAGATCTGGG  
Reverse, CGCAGATCTCGAGCGCCGGGAAAGGATCTGAGGATGGAATAG |
| -1533, bisulfite seq      | Forward, AGAATTAAATTAGTATGATGATATGAAAGTAAA |
| -1109, bisulfite seq      | Reverse, TTCCCACTATATTTAATATTAACCC |


