

THE EFFECTS OF MATERNAL NUTRITION ON OFFSPRING GENE
REGULATION VIA EPIGENETIC MODULATION

BY

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DISSERTATION

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ABSTRACT

Maternal nutrition during pregnancy is an important intrauterine factor that results in persistent alteration of the offspring epigenome and associates with health outcome later in life. Both DNA methylation and histone modifications have been reported in a range of rat tissues including liver, kidney, skeletal muscle and adrenal gland following a restricted diet during gestation. These epigenetic events have also been shown to affect tumor suppressor loci in mammary gland cancer cells. However, it is not known how these tumor-suppressive genes are regulated by epigenetic changes in the mammary glands in vivo under maternal low-protein (LP) diet. One of my published projects examined the effect of maternal low-protein diet on the regulation of the p16 cell-cycle gene expression in the mammary gland of offspring rats. Timed-pregnant Sprague-Dawley rats were fed during gestation one of two isocaloric diets, control (18% casein) or low protein (LP, 9% casein). The expression of p16 mRNA in the mammary gland of the LP offspring was decreased by 75% vs. control. We also detected decreased p16 protein content in the mammary glands of pups gestated under the LP diet. Chromatin immunoprecipitation (ChIP) assay demonstrated that the altered p16 mRNA level and transcription rate in LP offspring resulted from histone modification changes, including the reduced acetylation of histone H4 and the dimethylation of histone H3 at lysine 4 residues within the p16 promoter region. I also found maternal protein restriction down-regulate p21 gene expression in the mammary gland of rat offspring. p21 is a key factor responsible for tumor suppressor p53-dependent cell cycle arrest and has been accepted as a prognostic marker of breast cancer. DNA methylation analysis using bisulfite sequencing did not detect a differing pattern at the p21 promoter between the offspring of control and LP groups. Reduced acetylation of histone H3 and dimethylation of H3K4 within the p21 promoter region was observed. These results supported the hypothesis that maternal protein restriction during pregnancy programs p16 and p21 expression through histone modification alterations in offspring mammary gland.

Maternal nutrition restriction can also lead to low birth weight, which is associated with increased risk of type II diabetes. In our recent study, we investigated the impact of a

maternal low protein diet on the expression of the transcription factor CCAAT/enhancer-binding protein (C/EBP β) in offspring skeletal muscle. C/EBP β belongs to a family of transcription factors that regulates the expression of genes involved in energy homeostasis and muscle development. We observed sex-dependent differences in C/EBP β expression in offspring skeletal muscle subjected to a maternal protein-restricted diet. In female offspring skeletal muscle, both C/EBP β mRNA and protein levels were increased by maternal protein restriction. However, C/EBP β expression was not altered in other tissues or male offspring. Analysis of transcriptional and epigenetic regulation showed acetylated histone 3 and acetylated histone 4 at significantly increased levels at the C/EBP β promoter region in female LP pup's muscle. Correspondingly, downstream phosphoenolpyruvate carboxykinase (PEPCK) gene transcription was also up-regulated in female LP pups through the increased binding of C/EBP β at its promoter. On the other hand, GLUT4 is an insulin-regulated glucose transporter involved in insulin sensitivity and carbohydrate metabolism in muscle cells. We observed sex-dependent GLUT4 mRNA expression and increased GLUT4 protein content in female pup skeletal muscle with maternal low protein. The protein level of myocyte enhancer factor 2A (MEF2A), which has been known as an activator of GLUT4 transcription via the ability to carry out specific binding to the GLUT4 MEF2 binding sequence, increased in female LP pups. Modifications of chromatin structure, including acetylated histone 3, acetylated histone 4 and di-methylated histone 3 at lysine 4, were detected at a significantly increased level at the GLUT4 promoter region in female pup muscle following a maternal low protein diet. Glycogen content was also detected as up-regulated, accompanied by increased glycogen synthase in LP female offspring muscle. The induction of C/EBP β and GLUT4 expression in female offspring skeletal muscle by maternal protein restriction during pregnancy may indicate sex-dependent signaling response in energy metabolism to a low maternal protein diet.

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Chapter 1. Introduction

The importance of maternal nutrition during gestation with regard to pregnancy outcome has long been acknowledged. The tendency of mothers to briefly overhaul their lifestyle and diet is a classic one, with many women changing their habits, and abandoning many of their vices, such as alcohol and caffeine drinking or cigarette smoking. This importance has been further emphasized in the recent new understanding of fetal programming on adult outcomes demonstrated by numerous laboratory and epidemiological studies. We are only beginning to understand how maternal nutrition and intrauterine environment may impact not only immediate pregnancy outcomes, but also life and health course of the offspring. We need a better understanding of diet and dietary supplements during pregnancy and of whether diets are particularly low in some nutrients. Also, we need to understand how common epigenetic variations influence nutrient requirements during these periods. Associations between maternal nutrition and infant growth and development suggest that improving the diets of women of child-bearing age might be an important component of public health strategies aimed at improving the health, nutrition and well-being of women themselves, as well as reducing the burden of chronic disease in their offspring.

The concept that epigenetic alterations occur during gestation and may have the ability to program adult diseases has led to a multitude of studies focusing on maternal behavior and health during pregnancy. Epigenetics is the study of heritable alterations in gene expression patterns not caused by changes in genomic DNA sequence. Genetic imprinting and X chromosome inactivation are two well-studied examples of epigenetic mechanisms related to gene expression regulation. Genomic imprinting controls gene expression depending on the parent of origin, and X chromosome inactivation controls gene expression by silencing one of the two copies of the X chromosome within each cell in females. Maintenance of different gene expression patterns among the diverse cell types also relies on epigenetic modifications using the molecular mechanisms such as DNA methylation, micro RNA expression and covalent modifications of the histone proteins that package DNA into chromatin structure in the nucleus.

Epigenetical alterations of chromatin structure via covalent modifications of histones allow for heritable gene regulation without altering the DNA sequence. Genomic DNA exists as a highly folded chromatin polymer wrapped around an octamer core of histones H2, H3 and H4 with a protruding charged 15-38 amino acid N terminus histone tail. These histone tails influence DNA-histone interactions and nucleosome assembly into higher order chromatin structure not readily accessible to gene activation. Covalent modifications of the H3 and H4 histone tails, such as acetylation, phosphorylation, methylation, ubiquitination, and poly-ADP ribosylation, alter the interaction between histones and DNA, which consequently affects nucleosome locations as well as higher order chromatin folding. In doing so, these post-translational modifications likely enable the regulation of contact with the underlying DNA.

Chapter 2. Literature Review

2.1 Maternal Nutrition and Offspring Disease Risk Overview

Many adult diseases have fetal origins including obesity, diabetes and cancers. Links between maternal nutrition, intrauterine environment of the fetus and susceptibility to these adult diseases have attracted numerous attentions. An unfavorable prenatal environment can trigger epigenetic changes that increase the risk of developing those diseases and reduce the chances of postnatal survival. Major epigenetic programming events take place during the fetal development. This chapter describes investigations that targeting the relationship between maternal nutrition and offspring's obesity, diabetes, and cancer risks.

2.1.1 Maternal Nutrition and Obesity

Obesity is among those adult diseases that have roots in the fetal programming and is studied extensively because of its prevalence in the modern world {Stocker *et al.* 2005; Fernandez-Twinn *et al.* 2006}. Maternal nutrition status is one of the most important causes of programming that influence the risk of obesity of later life. Both maternal undernutrition and overnutrition are all linked to abnormalities of fetal growth and their postnatal obesity risk. The intrauterine environment for fetal growth can be altered by maternal calorie/protein restriction, high fat feeding, iron intake, mothers' smoking status and other maternal factors. Fetal response and adaptation to the altered intrauterine environment during the critical period of development may leads to long-term changes and prevalence of many chronic diseases in their postnatal life including obesity (Table 2.1).

Maternal undernutrition during the pregnancy has been demonstrated to result in intrauterine growth restriction (IUGR) and low birth weight. Malnutrition of pregnant women is still an important public health problem in the world, especially in the developing countries. In order to adapt to the maternal undernutrition environment and

increase the chance of postnatal survival, the fetus responds with a number of strategies, such as changing its metabolic rate, altering the production of hormones, storing nutrients as fat, and redistribution of fetal blood flow to protect brain at the expenses of other tissues such as muscle. These factors lead to a slower fetal growth and low birth weight.

The term intrauterine growth restriction (IUGR) is often used and assigned to newborns with a birth weight and/or length below the 10th percentile for their gestational age and whose abdominal circumference is below the 2.5th percentile with pathologic restriction of fetal growth {Wollmann *et al.* 1998}. During recent years significant progress has been made in the understanding of IUGR-associated pathophysiology. Data have shown that IUGR is associated with a late life increased prevalence of metabolic syndrome, like obesity. Large cohorts in epidemiological research programs have also studied IUGR phenomena. For example, an Indian cohort study showed that babies who are thin and lack muscle at birth gain weight rapidly in childhood, leading to a disproportionately high fat mass in later life {Yajnik *et al.* 2003}. It is also shown that timing of maternal nutrient restriction has a major influence on the outcome in terms of predisposing the offspring to adult obesity. In a Dutch Hunger Winter Study, maternal undernutrition in the first and second trimesters of pregnancy is associated with higher obesity rates in young men {Ravelli *et al.* 1976} and higher BMI and waist circumference in 50-y-old women {Ravelli *et al.* 1999}.

A 'thrifty phenol-type' hypothesis was proposed by Hales and Barker in order to link the fetal intra-uterine environment to the susceptibility to chronic diseases in later life {Hales *et al.* 1992}. It is believed that adaptations associated with fetal malnutrition become detrimental to the health of the offspring if they experience a period of adequate or plentiful nutrition leading to postnatal obesity.

Maternal calorie restriction to 50% of ad-lib in the last week of pregnancy retards beta cell development. Continued restriction of the mother during lactation period results in a permanent reduction of beta-cell mass and impaired glucose tolerance in the offspring. Calorie restriction in pregnancy period was also found to induce hyperphagic behavior and obesity of offspring {Vickers *et al.* 2000}. IGF-I treatment of 6-month-old

undernourished offspring was found to alleviate hyperinsulinemia and obesity. Leptin treatment of neonatal rats normalized caloric intake, body weight, fat mass and insulin concentrations in later life.

The maternal low-protein model is one of the most-extensively studied models. Ozanne and coworkers tested ‘thrifty phenotype hypothesis’ and investigate the effects of fetal programming and post-natal catch-up growth on obesity and longevity. The offspring of low protein-fed dams were switch to mothers receiving control diets for lactation. The body weights of those rats caught up and exceeded that weight of control group by 7 days of age and this pattern persisted in adulthood {Ozanne *et al.* 2004}.

Impacts of Western-style diet, which contain a high percentage of saturated fats on offspring risk, have attracted attentions of researchers in developed countries. Animal studies have shown the adverse effects caused by high fat feeding including fetal insulin resistance, gender-specific hypertension and increased adiposity. A markedly obesity in male and female offspring of Virgin Wistar rats can be induced by maternal high fat intake during the pregnancy. These phenomena are independent of postnatal nutrition {Howie *et al.* 2009}.

Data from human studies comparing breast-fed and bottle-fed infant also suggest that the lactation period is a critical time window for determination of obesity risk in humans. Breast-fed babies are at reduced risk of obesity compared to those who were formula fed as bottle-fed infants have higher total and protein caloric intake {Locke *et al.* 2002}. Higher circulating leptin levels in breast fed infants may contribute to the subsequent reduced obesity risk. Breast milk is also rich in long chain polyunsaturated fatty acids, which are thought to be protective against the development of obesity.

Leptin plays a major role in the regulation of metabolism and neuroendocrine functions. It increases energy expenditure, and modulates appetite by inhibition of hypothalamic arcuate nucleus neurons through the leptin receptor {Ashworth *et al.* 2000}. Obese individuals have sustained elevated adipocyte-derived leptin levels, which may cause selective leptin resistance at the hypothalamic level. In rodents, leptin levels are very low

at birth and then display a surge at the end of the second postnatal week. Injecting leptin into the offspring of undernourished mothers prevents hyperphagia, and excessive body weight and fat mass gain of neonatal rat offspring {Vickers *et al.* 2005}. In contrast, treating male offspring of normally nourished mothers caused an induced weight gain and increased total body adiposity {Vickers *et al.* 2008}. Altering leptin levels during early postnatal key periods of hypothalamic development may induce long-lasting susceptibility to a postnatal obesity depending on the pre-natal maternal nutrition status. In humans, fetus in gestational age and known to be at risk of obese in later life had lower levels of cord leptin {Ong *et al.* 2006}.

About 50% of pregnant women are iron deficient. The main causes of iron deficiency are poor absorption of iron due to insufficient vitamin C levels and inadequate daily intake of iron or high menstrual blood loss. Maternal anemia has been shown to associate with low birth weight. The rodent maternal iron restriction model resulted in low birth weight offspring and programs hypertension and obesity through adult life {Lewis *et al.* 2001}.

In humans, glucocorticoids are administered during pregnancy for the treatment of neonatal respiratory morbidity and maternal asthma. But overexposure of glucocorticoids results in the fetus with reduced birth weight and programme responses that lead to later adult disease. Glucocorticoid is regulated by its receptor in the cell. 11 β -HSD-2, an isoform of 11- β -hydroxy-steroid dehydrogenase, inactivates corticosterone by converting it to corticosterone. Dietary protein restriction attenuates 11 β -HSD-2 in the placenta and may provide a mechanism relating the maternal nutrition to fetal programming {Lindsay *et al.* 1996}. In an epidemiology study, it was found that overexposure to maternal glucocorticoids in human are associated with reduced birth weight and an increase in central adiposity {Gillman *et al.* 2006}.

In a British cohort study, Power C and Jefferies BJMH studied insult effect of maternal smoking during pregnancy on fetal growth and the influence of obesity risk through childhood to age 33. It was found the infants of mothers who smoked in pregnancy have lower birth weight than infants of non-smokers and had an increased risk of obesity from adolescence to age 33 {Power *et al.* 2002}.

2.1.2 Maternal Nutrition and Type II Diabetes

Diabetes influences more than 180 million people worldwide and this number is most likely to double by 2030 (<http://www.who.int/mediacentre/factsheets/fs312/en>). Type II diabetes, a major prevalent form of diabetes, is suggested to have its origin during fetal development. Maternal nutrition during all the stages of gestation and lactation plays an important role in the control of type II diabetes risk. Although type 1 diabetes has been suggested to also have its origins in the fetal period as well, most epidemiological studies have confirmed the association between low birth weight and adult impairment of glucose metabolism and increased predisposition to type II diabetes in adult life. It was also suggest that some physical parameters of baby, such as abdominal circumference, body length, head circumference, low birth weight up to 1 year of age, and catch-up growth are associated with the development of type II diabetes risk in later life. As we discussed in the relation of maternal nutrition with obesity, it is because maternal undernutrition causes the fetus to adapt through endocrine and metabolic changes that such adaptations result in insulin resistance and a predisposition of offspring to developing type II diabetes.

Barker is the first one who associates the birth size to the later development of metabolic syndrome in adult life {Barker *et al.* 1993}. Over 20,000 newborns between 1911 and 1930 in a county were studied and he demonstrated that men who were smallest at birth (<2.5 kg) were nearly seven times more likely to have impaired glucose tolerance or type II diabetes than those were heaviest at birth (>4.3 kg). Hoffman and coworkers showed that short pre-pubertal IUGR children have a specific impairment in insulin sensitivity compared to their normal birth weight peers {Hofman *et al.* 2006}. A recent study conducted in Minnesota found that excess mortality for the adult onset type II diabetes was concentrated in individuals who have abnormal birth weight. A recent meta-analysis conducted by Harder and coworkers involved a total of 132,280 persons and concluded that low birth weight is associated with the increased risk for the onset of type II diabetes in the later life {Harder *et al.* 2007}. Another study conducted in Preston, UK, performed glucose tolerance tests on 226 men and women and found that an increased ratio of

placental weight to birth weight exhibited impaired glucose tolerance or type II diabetes in adult life {Godfrey *et al.* 2001}.

Further evidences of the contribution of low birth weight to type II diabetes come from some animal studies. Jimenez-chillaron and coworkers performed a study to determine whether insulin resistance or insulin secretion dysfunction is associated with low birth weight and type II diabetes {Jimenez-Chillaron *et al.* 2005}. In offspring of undernourished mothers, comparisons of offspring from mice fed with a control diet and offspring from mice fed with an undernourished diet (restricted to 50% that of controls) were studied to explore whether undernutrition contributes to a defect in β -cell function resulting in abnormal glucose-stimulated insulin release and eventually in type II diabetes. Insulin content of β -cells was reduced by 25% in mice of the undernourished mothers, which was suggested that cause is that reduced nutrient delivery compromised insulin secretion. Others have also shown that nutrient restriction during pregnancy results in impaired glucose tolerance in low birth weight mice. A restriction in the maternal food intake by 50% during the last week of pregnancy led to the generation of IUGR pups with impaired β -cell development and a reduction in plasma glucose and insulin concentrations. Moreover, at approximately 8 months of age, the offspring demonstrated 40% decrease in pancreatic insulin content and an increase in non-fasting plasma glucose concentrations, which eventually led to fasting hypoinsulinemia, hyperglycemia and insulin-to-glucose ratio, all of which contribute to the development of type 2 diabetes {Martin-Gronert *et al.* 2005}.

It is recommended that the mother increase her protein intake during pregnancy to provide the additional nitrogen source that is demanded by both the mother and the fetus because an increase in protein metabolism due to the rapid growth demands of the fetus. In both early and late pregnancy periods, the amount of energy obtained from protein is positively associated with birth weight.

Numerous evidences come from the extensively studied low protein animal model. Some mechanism insights of early growth restriction caused by insufficient protein were

obtained from such a model. The model was established by Snoeck and co-workers where dams were fed a diet containing 8% protein through out pregnancy and lactation and compare offspring to those of a control dam fed an isocaloric 20% protein diet {Snoeck *et al.* 1990}. The under half the protein intake of the control diet reflects the relevance to cultures, economies and social-economic groups for those protein sources are expensive. The effects on pregnancy outcome are therefore expected to have no effect on litter size. The model has firstly shown that protein restriction dams deliver litters with lower birth weight. And there were adverse effect on pancreas development, β cell proliferation. Insulin content was reduced and its secretion was impaired. Giving offspring control diet shown a time-dependent matter. After 6 weeks to 3 months, litters demonstrate improved glucose tolerance. But after 15 months, they had an impaired glucose tolerance and by 17 months, frank diabetes with insulin resistance was observed in male low protein offspring. Insulin-stimulated glucose uptake was reduced in both muscle and adipose tissue of 15-month-old males. The observation was accompanied by a reduction in both GLUT 4 and PKC zeta in muscle {Ozanne *et al.* 2003}. Female offspring only demonstrated hyperglycemia and impaired glucose tolerance. Fernandez-Twinn *et al.* employed a maternal protein restriction rat model throughout gestation and lactation. Offspring were again born smaller than controls and developed diabetes, hyperinsulinemia, and tissue insulin resistance in adulthood {Fernandez-Twinn *et al.* 2003}.

In the mechanistic investigations, in pups delivered from low protein fed Dams, expression of pancreatic duodenal homeobox-1 (pdx-1) and insulin-like growth factor II (IGF-II) in the islet cells were reduced {Arantes *et al.* 2002; Petrik *et al.* 1998}. Pdx-1 is responsible for the regulation of a number of genes that are accountable for the proper development and maturation of the pancreas. IGF II functions in the prevention of apoptosis from development. When these two genes expression were reduced, the development of pancreas and the production of insulin are compromised, thus contributing to an increased predisposition to adult onset type II diabetes. In other animal studies, an increase in hepatic gluconeogenic phosphoenolpyruvate carboxykinase (PEPCK) activity {Desai *et al.* 1997} and reduced glycolytic glucokinase levels {Desai *et*

al. 1997; Rees *et al.* 2000} have been reported in response to a maternal low protein diet. Of a note is the decrease in glycolytic glucokinase activity seen in subjects diagnosed with type II diabetes {Vaxillaire *et al.* 2006}. Over expression of PEPCK has also been shown to cause the development of type 2 diabetes in mice {Franckhauser *et al.* 2006}. Ozanne and coworkers reported that the offspring of low protein diet treated mothers had a reduction in epididymal adipocytes, and an increase in basal and insulin-stimulated glucose uptake. However, both the muscle and adipocyte insulin receptor expression in the restricted protein offspring were similar to that of the control group and that molecular alteration resulting in insulin resistance must occur downstream of the insulin receptor. In this regard, a reduction in the expression of the p110 β subunit of phosphatidylinositol (PI3-kinase) and a reduction of the activity of insulin-stimulated protein kinase B were found in the adipocytes of IUGR animals at 3 and 15 months of age {Ozanne *et al.* 2005}.

Accumulating evidence has shown that epigenetic regulation of transcription is a mechanism for inducing changes in phenotype of fetal programming and the increased risk of diabetes in adulthood. The reversible changes that occur as a result of heritable modifications without involvement of primary DNA sequence alterations are defined as epigenetic changes. Histone modification and DNA methylation are two main molecular events known to initiate and sustain such epigenetic modifications.

Along this line, we investigated the impact of maternal low protein diet on the expression of glucose transporter 4 (GLUT4) in offspring skeletal muscle (S. Zheng & Y.-X. Pan, manuscript submitted). A maternal LP diet during pregnancy and/or lactation was reported in affecting postnatal growth, appetite, triglyceride and cholesterol concentrations, as well as insulin resistance in male but not female offspring {Zambrano *et al.* 2006}. However, the molecular mechanism underlying the sex-differentiated carbohydrate metabolism and insulin sensitivity is rarely explained. The major insulin responsive protein involved in glucose absorption is glucose transporter 4 (GLUT4). We observed sex-dependent GLUT4 mRNA expression and increased GLUT4 protein content in female pup skeletal muscle by maternal low protein. Analysis of transcriptional

and epigenetic regulation underlying increased skeletal muscle GLUT4 expression in offspring rats also revealed the regulatory mechanisms. Increases in regulatory genes associated with carbohydrate metabolism, C/EBP β and Nur77, were observed in female pups whose mothers were fed a low protein diet. Modifications of chromatin structure, including acetylated histone H3, acetylated histone H4 and di-methylated histone H3 at lysine 4, were detected at a significantly increased level at the GLUT4 promoter region in female pup muscle following maternal low protein diet. Such modifications include increased levels of acetylated histone H3, acetylated histone 4 and di-methylated histone H3 at lysine 4 in female offspring rats. However, the restricted diet did not activate the amino acid response pathway or alter GLUT4 expression through DNA methylation. These results demonstrated that maternal protein restriction during pregnancy induces GLUT4 expression in female offspring skeletal muscle but not in males, which may indicate sex-dependent adaptation of insulin sensitivity and of glucose metabolism to low maternal protein diet (Figure 2.1).

In another report, Burdge investigated whether the altered methylation of PPAR α and glucocorticoid receptor (GR) promoters are passed to the F2 generation in response to a maternal low protein diet {Burdge *et al.* 2007}. It was observed that hepatic PPAR α and GR promoter methylation was significantly lower in the protein restriction group in the F1 and F2 generations. There were also trends towards a higher expression of PPAR α , GR, acyl-CoA oxidase, and PEPCK in the F1 and F2 males, although this was significant only for PEPCK. These data showed that the altered methylation of gene promoters in the F1 generation by maternal protein restriction during pregnancy is transmitted to the F2 generation. This may represent a mechanism for the transmission of induced phenotypes between generations. The potential to affect subsequent generations by maternal under nutrition during pregnancy has also been reflected by other studies. The Dutch famine study showed that the pregnant women who experienced undernourishment during the famine period consisted of a 5-month of food deprivation in the winter of 1944-1945 gave birth to offspring what were of average or normal birth weight. Those offspring went on to give birth to offspring of low birth weight, which illustrated again that maternal under nutrition can affect more than one generation {Stein *et al.* 2000}.

Maternal under nutrition can result in the overexposure of the fetus to glucocorticoids. An animal model showed the adverse effects of overexposure to glucocorticoids continue to further generations. Offspring overexposed to dexamethansone (glucocorticoid) during late pregnancy have low birth weight and developed glucose intolerance and hypertension in adulthood {Seckl *et al.* 2004}. When these offspring have birth to offspring of there own, their offspring also had low birth weights and impaired glucose intolerance, thus confirming that adverse events during pregnancy affect not only the first generation but also future generations. A recent study reveals that low protein diet during gestation had adverse effects on glucose, insulin and leptin metabolism, resulting in insulin resistance in adult F2 offspring {Lindsay *et al.* 1996}. Future prenatal and gestational nutritional recommendations may need to be formulated on the basis of region, culture and risk assessment criteria.

Both clinical and experimental studies have shown that maternal obesity has been linked to the alteration in fetal development and increased risk of diabetes. Particularly, human studies have linked maternal diabetes with a higher incidence of type II diabetes in the offspring in adulthood {Hadden *et al.* 2008}. The concept of prediabetes has come recognized again with the worldwide epidemic of type II diabetes. Maternal hyperglycaemia has been related to fetal macrosomia in diabetic pregnancy. The recent large studies about the outcome of pregnancy in type II diabetic mothers in England, Wales and Northern Ireland in 2002-2003 shows that there is still an increase in the numbers of both big babies and small babies born to these diabetic women in comparison with the national population data, babies ≥ 4.5 kg with type II diabetes 5.1%, national population with type II diabetes 1.7% (Confidential Enquiry into Maternal and Child Health Pregnancy in Women with Type 1 and Type II Diabetes in 2002-2003; England, Wales and Northern Ireland)

In a small study of 99 babies with verified birth weight > 5 kg in 45 prediabetic women, 5 years prior to the diagnosis of diabetes, 36% of the mothers producing a big baby were obese, while none of the babies born to non-obese mothers weighed as much as 5 kg. A similar difference persisted at 1.5 years before the onset of diabetes, but was reversed

after the onset of diabetes, where non-obese mothers had a 28% prevalence of babies above 5 kg (Pedersen J. *The pregnant Diabetic and Her Newborn: Problems and Management*; 2nd edition).

Hypotheses exist to explain the relationship between intrauterine nutrition and risk of diabetes relevant to the big baby and the small baby. The Pederson hypothesis, simply stated, is that maternal hyperglycaemia causes fetal hyperglycaemia by direct transplacental passage. After the fetal islet cells become functional, this leads to excess fetal insulin secretion, which facilitates excess fetal growth, particularly the fat component. The alternative but not exclusive concept, known as the Barker hypothesis, is that maternal malnutrition associated with placental insufficiency will lead to impaired fetal growth and small babies, which are associated with neonatal insulin resistance and long-term epidemiological evidence of type II diabetes and hypertension. It is possible to consider these two ends of the nutritional background to type II diabetes in a single concept of transgenerational diabetes. The studies of David McCance in the Pima Indians did demonstrate in that population, where type II diabetes is very prevalent, that there was a 'U-shaped' curve where type II diabetes at aged 30-34 years was more common among those who had been less than 2.5 kg or more than 4.5 kg at birth {McCance *et al.* 1994}.

2.1.3 Maternal Nutrition and Cancers

Cancers are age-related diseases and caused by the interaction of genetic susceptibility and environmental factors {Uauy *et al.* 2005}. In recent years, cancer prevention has attracted numerous attentions. Avoiding exposures to some environmental factors, such as radiation, carcinogenic compounds, infectious agents, some dietary factors and contaminants in food, water and air, has been used to prevent cancers from occurring. However, the knowledge of cancer risk management is still very limited especially in the early life intervention. More recently, approaches to identify early metabolic syndrome and its underlying genetic susceptibility have shown promising prevention of cancers before any symptoms or laboratory indicators. For example, obesity has been recognized

as a contributory risk factor for some types of cancers {Key *et al.* 2004}. Relationships between maternal nutrition and obesity risk have been addressed in previous discussions. There are research evidences indicating that maternal nutrition should be considered as a factor to control postnatal cancer risk. The first human evidence came from a study of transient exposures to chemical substances during embryonic and fetal life on triggering cancer in offspring in 1970 {Herbst *et al.* 1999}. Herbst found seven women were treated with diethylstilbestrol during the first trimester of pregnancy and they have a clear-cell vaginal adenocarcinoma. The diethylstilbestrol was a drug that was believed to reduce the incidence of premature births and neonatal deaths. However, Herbst study demonstrated that the diethylstilbestrol treatment in the pregnancy period have the potential adverse consequences of exposure of fetus to such a toxic substance and may increase fetal susceptibility to cancer during such rapid growth and differentiation period. Along this line, maternal intake of the synthetic estrogen during the pregnancy period increase multi-generational uterine cancer risk in female offspring and such effect is demonstrated in animals as well {Giusti *et al.* 1995}. Thus, recently natural estrogens have been classified as known human carcinogens.

2.2 Maternal Nutrition and Breast Cancer Overview

Maternal nutrition and maternal exposure to environmental agents may change metabolic process during offspring's mammary gland growth and alter epigenome thereby predisposing offspring's mammary gland to the breast cancer risk later in life {Hilakivi-Clarke *et al.* 2006}. Maternal low protein diet, one of most common diets encountered by mothers, has been associated with the risk of a number of adult diseases {McArdle HJ *et al.* 2006}. Despite the large distribution of breast cancer in elder women, the disturbing that may increase the risk of carcinogenesis could occur in younger years when the mammary gland is most susceptible. However limited understanding of the maternal nutrition effect and molecular mechanisms underlying mammary gland cancer retards our further determination of the optimal maternal nutrition in cancer prevention. Epigenetic modifications including cytosine methylation of DNA and post-translational modification of chromosomal structural histones alter gene functions which often

resulting in phenotype changes without altering the DNA sequence. For instance, DNA hypermethylation leads to decreased expression and DNA hypomethylation leads to increased expression of target genes. Cytosine DNA methylation patterns are largely erased in the early embryos and then re-established in a tissue-specific manner {Kafri T *et al.* 1992}. Such epigenetic modifications affect cell proliferation during prenatal and postnatal development and may associate with the regulation of the risk for breast cancers.

Indeed, the association between the breast cancer susceptibility and the fetal environment has attracted relatively extensive attentions recently. Maternal exposure to dietary factors during pregnancy or lactation alters the risk of offspring in developing breast cancer. Some exposures induce epigenetic changes and cause gene expression alteration in the offspring's mammary gland development. However, it still lacks evidences to link the epigenetic changes in the fetal development to the increased vulnerability for malignant transformation in postnatal life. Thus, identification of the target genes in the epigenetic programming, unfolding related mechanism at molecular level and studying its consequences during the life span may lead to the useful approaches to prevent breast cancer for women.

First of all, recent epidemiological studies have indicated that women with either high birth weight (> 4 kg) or very low birth weight (< 2.5 kg) are at increased risk of breast cancer {Troisi R *et al.* 2003}. The effect is more apparent in women who developed pre-menopausal rather than post-menopausal breast cancer. It's been well accepted that birth weight is influenced by maternal nutrition (this part will be discussed later). In a British cohort of 2547 girls followed from birth in 1946 to 1999, girls who were heavy at birth reached menarche earlier than others with similar growth in infancy. The findings suggested that women who grow faster in childhood and reach an adult height above the average for their menarche category are at a particularly increased risk of breast cancer {De Stavola BL *et al.* 2004}. This effect is associated with greater exposure to estrogens, which are likely to be promoters of breast cancer. Elevated estrogenic environment is related to high birth weight in most studies except Asian women, who have a low risk of

breast cancer but have significantly higher estrogen concentrations during pregnancy than Caucasian women {Shibata A *et al.* 2002}. This phenomenon might indicate that the increased risk of breast cancer in daughters caused by high concentration of estrogen during pregnancy could be potentially overridden by other factors during the pregnancy or postnatal life. On the other hand, a very low birth weight may also increase the risk of developing breast cancer {Ahlgren M *et al.* 2003}.

Secondly, in an animal model, exposing the mother to E2, the synthetic estrogen, during pregnancy increases carcinogen-induced mammary tumorigenesis in the offspring. Elevated maternal and /or cord-blood levels of estrogens, leptin, adiponectin and IGF-1 might all increase the risk of a daughter developing breast cancer, but an increase in one of these does not necessarily mean an increase in later breast cancer risk. The finding suggested many alterations in utero could contribute to the later breast cancer risk modification.

Estrogens function through the binding to estrogen receptors, ER α and ER β . These estrogen receptors are nuclear transcription factors that regulate genes expressions. Genes encoding ERs are targets of epigenetic modifications and can be silenced by hypermethylation {Ottaviano YL *et al.* 1994}. In normal mammary epithelial cells, ER α is not located in the cells that proliferate, but close to them; whereas in breast tumors, proliferating cells frequently express ER α {Potten CS *et al.* 1998}. It was suggested that ER β might negatively control cell proliferation and may have a protective role in the normal breast {Cheng G *et al.* 2004}. In utero exposure of increased estrogen could increase or reduce the concentration of ER α in offspring {Newbold RR *et al.* 2004}, and correspondingly trigger the ER-mediated pathways, including cell proliferation and expression of estrogen-regulated genes. It has been shown that, in utero, exposure to elevated E2 increases ER α levels in the offspring mammary gland and up-regulates estrogen-regulated genes such as cyclin D1, pSrc and pAkt and down regulate a suppressor gene Caveolin-1. Caveolin-1 is known that its expression inhibits phosphorylation deactivation of the oncogenes Src and ras and pro-survival factors such as Akt {Acconcia F *et al.* 2005}. Therefore, it was suggested that elevated exposure to

estrogen in utero would alter postnatal susceptibility to malignant transformation in breast tissue by altering the expression of ER α and ER α -mediated downstream genes expression. On the other hand, low concentrations of ER α in the mammary gland are also associated with increased risk of breast cancer. For example, rats with high birth weight have reduced mammary ER α and develop mammary tumors earlier than control rats {De Assis S 2006}.

Maternal diet and nutrition are important factors because these factors during pregnancy may modify pregnancy hormone levels, especially estrogen levels in utero. Therefore, maternal diet may affect the later risk of breast cancer for offspring. High fat diet increases E2 concentration during pregnancy. High fat diet, which is high in n-6 polyunsaturated fatty acids, increases the susceptibility of female offspring to develop carcinogen induced mammary tumors as adults¹². Genistein, a phytochemical in soybeans, induces epigenetic changes and influence estrogenic activity by binding to estrogen receptors. Exposure in utero increases the number of terminal end buds (TEBs) in offspring mammary gland, which are targets for malignant transformation {Hilakivi-Clake L *et al.* 2001}. But maternal soy intake did not increase offspring risk of developing mammary tumors, although the soy diet contained high levels of genistein {Trock BJ *et al.* 2000}. The soy diet also increased pregnancy estrogen levels. These results indicate that soy must contain some additional components, which reverse the effects of genistein on offspring breast cancer risk when administered in utero.

2.3 Maternal Protein Restriction and Mammary Gland Development

The maternal low-protein model is one of the most-extensively studied models for nutrition down-regulation studies. The model was established by Snoeck and co-workers where dams were fed a diet containing 8% protein through out pregnancy and lactation and compare offspring to those of a control dam fed an isocaloric 20% protein diet {Snoeck A *et al.* 1990}. The under half the protein intake of the control diet reflects the relevance to cultures, economies and social-economic groups for those protein sources are expensive. The model has firstly shown that protein restriction dams deliver litters

with lower birth weight. Ozanne and coworkers tested ‘thrifty phenotype hypothesis’ and investigate the effects on fetal programming and post-natal catch-up growth. The offspring of low protein-fed dams was switched to a standard diet that mothers used for lactation. The body weights of those rats caught up and exceeded that weight of control group by 7 days of age and this pattern persisted in adulthood {Ozanne SE *et al.* 2003}. Fernandez-Twinn *et al.* employed a maternal protein-restricted rat model throughout gestation and lactation. Offspring were again born smaller than controls {Fernandez-Twinn DS *et al.* 2003}.

Some mechanism insights of early growth restriction caused by insufficient protein could be potentially revealed by maternal low protein model. It’s been established that amino acid methionine and folate are methyl donors and play the central role in the epigenetic regulation in the process of cytosine methylation. They are essential for establishing genome-wide DNA methylation patterns. During pregnancy, to supply the increased protein synthesis associated with rapid fetal growth, methionine requirements are high and suboptimal status of protein nutrition could result in development defects {Essien FB *et al.* 1993}. Therefore, prenatal development is a sensitive stage when maternal diet may affect DNA methylation and furnish permanent fixation of aberrant methylation patterns that is demonstrated in an agouti mouse model {Waterland RA *et al.* 2003}. In postnatal development, DNA methylation patterns and histone modifications must also be maintained through multiple mitotic divisions to keep the homeostasis of most tissues through constant self-renewal. This process requires maintenance of appropriate quantities of folate or other cofactors such as methionine, vitamin B-12, choline and betaine that may affect normal patterns of DNA methylation and histone modifications {Ingrosso D *et al.* 2003}. Therefore, postnatal development is also a sensitive stage that maternal diets may affect chromatin modification and result in disease susceptibility.

On the other hand, maternal low protein during the pre-implantation period in rodents induced a transient reduction in maternal serum essential amino acid concentrations, which is associated with reduction in embryo cell numbers and led to various abnormal postnatal phenotype changes even through offspring were fed a normal diet. Maternal

serum homocystein levels were elevated in response to low protein diet and therefore interfere with methyl group donation required for DNA methylation. The branch-chain amino acids, leucine, isoleucine, and valine, are significantly depleted in both serum and uterine fluid in response to low-protein diet {Porter R *et al.* 2003}. Depletion of branch-chain amino acids could affect a serine-threonine kinase signaling pathway, the mammalian target of rapamycin (mTOR) {Hentges KE *et al.* 2001}. Histone modification could also be affected by maternal diet. We also observed that maternal low protein diet could furnish the histone modification in the offspring rats for regulating specific gene expression in a tissue specific manner {Zheng S *et al.* 2010}. Therefore, fetal and natal nutritional environment likely directly or indirectly alters epigenetic modifications such as DNA methylation and histone modifications, and thereby affects gene transcription, gene expression and ultimately the development of disease. However, nowadays whether maternal low protein affects the incidence of breast cancer in offspring is undocumented.

Breast cancer is a progressive disease that is the most diagnosed and second most fatal cancer in women (American Cancer Society, Cancer Facts & Figures; Atlanta, 2007). The age-related decline in susceptibility to mammary gland cancer has been observed with carcinogens and ionizing radiation in both human and animals. Patients receiving therapeutic ionizing radiation have shown that treatment increases breast cancer risk when exposure occurs during childhood and young adulthood rather than after the age of forty. The phenomena suggested that breast cancer arises as a consequence of disruption in the normal pathways during the mammary gland development and differentiation.

Mammary gland is in an undifferentiated state in the prenatal gestation period. Maternal nutrient supplementation or exposure to environmental agents may change relevant metabolic processes in mammary gland and alter epigenome thereby predisposing mammary gland to the risk of developing cancers later in life. The development of mammary glands in rodents and humans is largely similar. During the fetal period, the mammary fat pad is formed and contains limited stroma, which is important for morphogenesis of the mammary epithelium {Haslam SZ *et al.* 2003}. In rodents, the epithelium is quiescent until 3 weeks after birth. The club shaped terminal end buds

(TEBs) appears. TEBs are the most actively growing terminal ductal structures, and are believed to contain stem cells that could potentially give rise to breast cancer. TEBs are the only structures in the rat mammary gland that transform to microtumors and eventually form adenocarcinomas on exposure to a carcinogen. The corresponding structures in the human breast, called TDLUs, have also been suggested to be the locations that initiate breast cancer {Russo J *et al.* 2000}. TEBs lead the growth of the epithelial ducts and regress to terminal ducts or differentiate to alveolar buds and then to lobules. Although it's been shown, both in mice and rats, that in utero and neonatal exposure to E2 increases the number of TEBs and delays their differentiation to alveolar buds, it is not clear whether the changes in TEBs are linked to the risk of breast cancer.

All mammary epithelial cells originate from mammary stem cells. The destination of stem cells can be modified epigenetically. It has been proposed that elevated hormone and/or growth factor concentrations in utero increase later breast cancer risk by increasing the total number of replicating, immature stem cells which may be at risk for malignant transformation. Baik have measured the concentrations of hematopoietic stem-cell markers CD34⁺, CD38⁻ and granulocyte-macrophage colony-forming unit in cord blood and found them to be linked to the cord-blood concentrations of IGF-1, IGF-binding protein 3, estriol and testosterone {Baik I *et al.* 2005}. One explanation is that elevated concentrations of hormones and growth factors in utero might increase the later risk of cancer by increasing the number of stem cells during the fetal period. How these findings relate to the risk of developing breast cancer is still to be determined. The finding that high birth weight is associated with increased activation of mitogen-activated protein kinase (MAPK) in adult mammary glands provides indirect evidence to support the involvement of stem cells in the effects of fetal environment on later risk of breast cancer. Activation of MAPK might affect interaction between the polycomb protein complex and chromatin and silence specific sets of genes through chromatin modifications {Voncken JW *et al.* 2005}.

2.4 Tables & Figures

Table 2.1 Maternal Nutrition and Offspring's Risk of Obesity

Subjects	Maternal Nutrition	Observations	Ref.
Human	Malnutrition	Low birth weight but gaining weight rapidly in childhood	Yajnik CS et al.
	Malnutrition in early stages of gestation	Increased obesity rate in young men and 50-year old women	Ravelli GP et al. Ravelli AC et al.
	Overexposure to glucocorticoids	Reduction in body size but an increase in central distribution of fat	Gillman MW et al.
	Smoking during pregnancy	Low birth weight but elevated risk of obesity at age 33	Power C et al.
Animal	Calorie Restriction	Induce hyperphagic behavior and obesity of offspring	Vickers MH et al.
	Protein Restriction	Low birth weight but catch-up growth on obesity.	Ozanne SE et al.
	High Fat	A markedly obesity independent of postnatal nutrition	Howie GJ et al.
	Malnutrition	Leptin treatment of offspring reverses obesity risk resulting from relative fetal undernutrition	Vickers MH et al.
	Iron restriction	Low birth weight	Lewis RM et al.

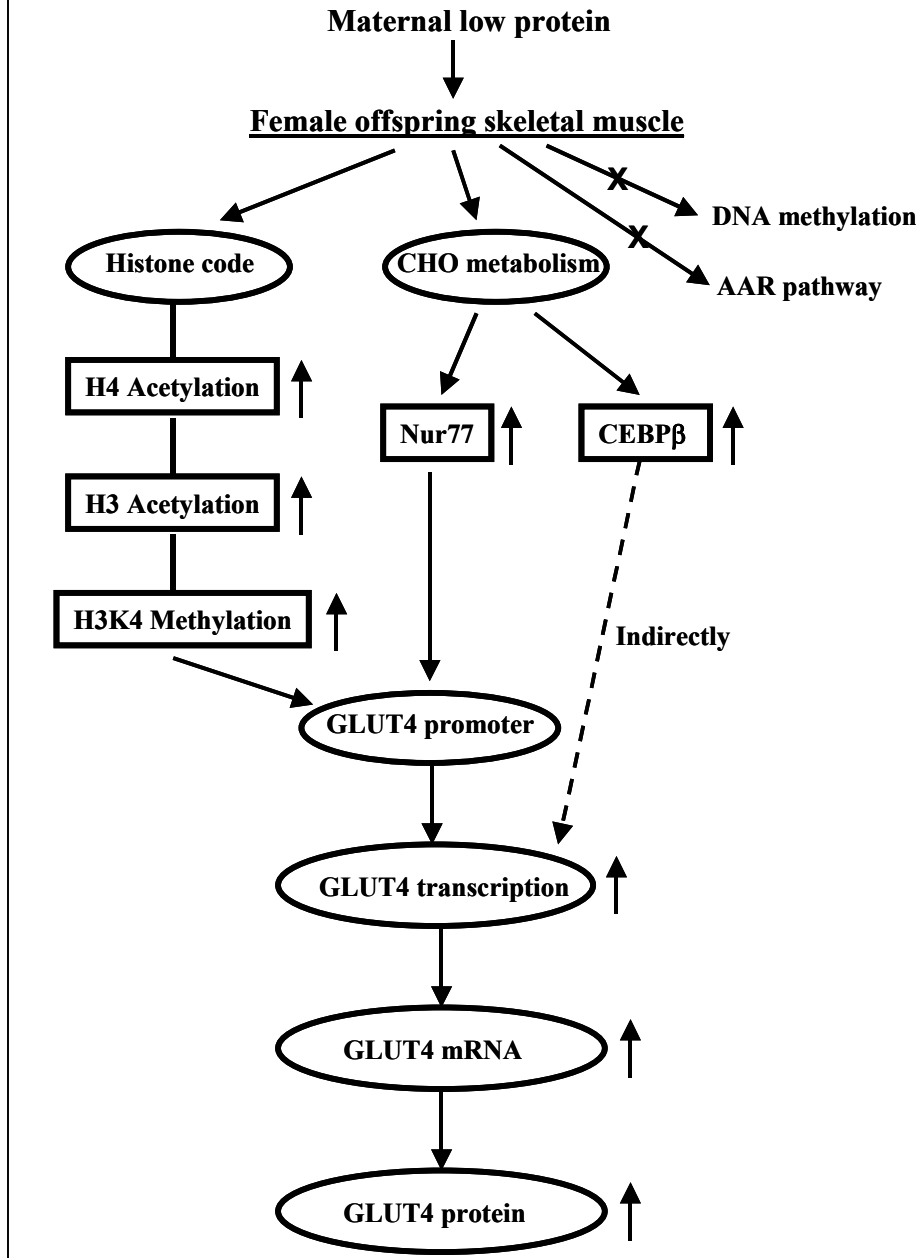
Table 2.2 Maternal Nutrition and Offspring's Risk of Type II Diabetes

Subjects	Maternal Nutrition	Observations or Conclusions	Ref.
Human	Low birth weight	Smallest at birth (<2.5 kg) were more likely to have impaired glucose tolerance or type II diabetes	Baker DJ et al.
		Low birth weight is associated with the increased risk for the onset of type 2 diabetes	Harder T et al.
		An increased ratio of placental weight to birth weight exhibited impaired glucose tolerance or type II diabetes in adult life	Godfrey KM et al.
	IUGR	IUGR children have a specific impairment in insulin sensitivity	Hofman PL et al.
	Obesity	Big babies had the increased risk of developing type II diabetes	Hadden DR et al.
Animal	Calorie Restriction	A defect in β -cell function, reduced insulin content in β -cell, hypoinsulinemia, hyperglycemia and on set of type II diabetes in later life	Jimenez-chillaron JC et al.
	Protein Restriction	Adverse effect on pancreas development, β cell proliferation	Snoeck A et al.
		A reduction in both GLUT 4 and PKC zeta in muscle in males.	Ozanne SE et al.
		Down regulation of pdx-1 and IGF-II	Arantes VC et al. Petrik J et al.
		Upregulation of PEPCK and downregulation of glycolytic glucokinase	Desai M et al. Rees WD et al.
		Downregulation of p110 β subunit of PI3-kinase	Ozanne SE et al.
		Lower methylation level in the promoter regions of PPAR and GR promoter and higher expression of these genes in F1 and F2 generations.	Burdge GC et al.

Table 2.3 Maternal Nutrition and Offspring's Risk of Cancer

Subjects	Maternal Nutrition	Observations	Ref.
Human	Diethylstilbestrol exposure in pregnancy	Vaginal adenocarcinoma in mothers and increased fetal susceptibility to develop uterine cancers.	Herbst AL et al. Giusti RM et al.
	Elevated estrogenic environment	High birth weight and increased risk of breast cancer.	De Stavola BL et al.
	Maternal intake of fruits, vegetables and fish and seafood	Reduced risk of developing acute lymphoblastic leukemia in offspring	Petridou E et al.
Animal	Exposure to synthetic estrogen	Increased carcinogen-induced mammary tumorigenesis in the offspring	Ottaviano YL et al.
	In utero over exposure of estrogen	Increased estrogen receptor α level in the offspring's mammary gland and over expression of estrogen-regulated genes such as cyclin D1, pSrc and pAkt and down regulate a tumor suppressor gene Caveolin-1	Hilakivi-Clarke L et al.
		Rats with high birth weight have reduced mammary ER α and develop mammary tumors earlier than control rats	De Assis S et al.
	High-fat diet	Increased E2 level in utero environment and increased susceptibility of female offspring to develop carcinogen induced mammary tumors as adults	Hilakivi-Clarke L et al.
	Genistein exposure	Increased number of TEBs which are targets for malignant transformation	Hilakivi-Clarke L et al.
	Soy intake	Did not increase offspring's risk of developing mammary tumors	Trock BJ et al.

Figure 2.1



2.5 References

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Chapter 3. Maternal Protein Restriction during Pregnancy Induces CCAAT/Enhancer-Binding Protein (C/EBP β) Expression through the Regulation of Histone Modification at Its Promoter Region in Female Offspring Rat Muscle

3.1 Abstract

Maternal nutrition during pregnancy is an important intrauterine environmental factor that can cause persistent alterations of the offspring genome and is associated with potential disease risk later in life. In the present study, we investigated the impact of a maternal low protein diet on the expression of a transcription factor gene, CCAAT/enhancer-binding protein (C/EBP β) in offspring skeletal muscle. C/EBP β belongs to a family of transcription factors that regulate expression of genes involved in energy homeostasis, including amino acid metabolism. We investigated C/EBP β transcriptional regulation from an epigenetic aspect in this study. We first time observed the sex-dependent difference of C/EBP β expression in offspring skeletal muscle treated by a maternal protein-restricted diet. In female offspring skeletal muscle, both C/EBP β mRNA and protein levels were increased by maternal protein restriction. However, C/EBP β expression was not altered in male offspring. Analysis of transcriptional and epigenetic regulation as the underlying causes of increased skeletal muscle C/EBP β mRNA in female offspring rats with maternal low protein (LP) revealed changes in chromatin structure. Specifically, acetylated histone 3 and acetylated histone 4 were detected at significantly increased levels at the C/EBP β promoter region in female LP pup's muscle. Phosphoenolpyruvate carboxykinase (PEPCK) gene transcription was also up-regulated in female LP pups through the increased binding of C/EBP β at its promoter. The induction of C/EBP β expression in female offspring skeletal muscle by maternal protein restriction during pregnancy may indicate C/EBP β -involvement in signaling response in amino acid metabolism to a low maternal protein diet.

3.2 Introduction

Experimental studies of rodents using a variety of nutritional manipulations in pregnancy

demonstrate that deviations in maternal nutrition during pre- and postnatal periods plays an important role in the etiology of disease in offspring {Langley-Evans *et al.* 2006; Gambling *et al.* 2003; Khan *et al.* 2003}. Dietary protein intake during fetal development has a long-term impact on health outcomes as well as lifespan {Zimmerman *et al.* 2003}. Impaired muscle development with a decreased number of fibre cells was observed in offspring during the gestation stage under maternal nutrition restriction {Zhu *et al.* 2006}. Epidemiology and experimental studies have also indicated that a low protein maternal diet during fetal and neonatal development in both humans and laboratory animals increases offspring susceptibility to the later development of altered carbohydrate metabolism {Dahri *et al.* 1991}. In a recent study, a maternal low protein diet during pregnancy and/or lactation affected the postnatal growth of offspring, their appetitive behaviors, triglyceride and cholesterol concentrations in the blood, and the insulin resistance in male but not female offspring {Zambrano *et al.* 2006}. Although physiological consequences of maternal protein intake on offspring are well studied, the molecular mechanism at the gene transcription level is relatively unknown.

CCAAT/enhancer-binding proteins (C/EBPs) are a family of transcription activating factors that regulate expression of genes involved in a wide range of physiological processes, ranging from the acute-phase response to the control of macronutrient homeostasis {Lekstrom-Himes *et al.* 1998}. C/EBP β is a member of the C/EBP family that also includes C/EBP α , g, d, e and CHOP (C/EBP homology protein) {Ramji *et al.* 2002}. C/EBP β controls gene transcription and metabolic processes in a variety of tissues. For instance, C/EBP β controls the expression of insulin-responsive genes that regulate glucose transport and metabolism in adipocytes {Jain *et al.* 1999}. It is also critical to adipogenesis {Tang *et al.* 2005} and neuron generation {Paquin *et al.* 2005}. Deletion of the C/EBP β gene impaired carbohydrate metabolism and resulted in hypoglycemia {Croniger *et al.* 2001}. Other factors also control the expression of C/EBP β and associated activities in skeletal muscle. For example, glucocorticoids increased C/EBP β expression and its DNA binding activity in muscle cells {Yang *et al.* 2005}. Friedman *et al.* reported that the deletion of C/EBP β increased insulin sensitivity in mice skeletal muscle {Wang *et al.* 2000}. As a transcriptional regulator, C/EBP β binds to the CRE motif in the muscle PEPCK promoter, which has been reported to be critically involved

in the anaplerosis and cataplerosis in amino acid metabolism in muscle {Owen *et al.* 2002}. PEPCK can provide phosphoenolpyruvate (PEP), a source of pyruvate, for re-feeding the citric acid cycle to provide energy for muscle cells. The role of C/EBP β as a transcription factor in muscle provides potential insight for further investigations of amino acid metabolism alteration by maternal nutrition restriction.

Recently, the transcriptional control of C/EBP β has attracted some attention, albeit limited. Dietary protein restriction has been shown to result in higher mRNA content of C/EBP β in rat liver {Marten *et al.* 1996}. Up-regulation of C/EBP β was also observed under histine deprivation in human hepatoma cells {Siu *et al.* 2001}. Subsequently, Chen *et al.* demonstrated that C/EBP β binds to the asparagine synthetase amino acid response element *in vitro* following amino acid deprivation {Chen *et al.* 2004}. Still, the role of maternal protein intake on C/EBP β in offspring skeletal muscle is not yet known. Moreover, to the best of our knowledge, there are no studies that address the epigenetic effects of maternal nutrition including histone code modifications in C/EBP β promoter region through generations. In the present study, we investigate offspring C/EBP β gene transcription response in rat skeletal muscle upon maternal protein restriction. We found that low protein intake during the gestation period resulted in an increase in C/EBP β expression at both the mRNA and protein level in female offspring skeletal muscle. We also confirmed the modification of histones in the C/EBP β promoter region, which could potentially contribute to the up-regulated C/EBP β as well as the changes of downstream genes.

3.3 Material and Methods

Animals and treatment

The animal protocol for this study was approved by the International Animal Care and Use Committee (IACUC) at the University of Illinois. Timed-pregnant Sprague Dawley rats (Charles River Laboratories) were obtained on day 2 of gestation and weight matched into one of two isocaloric diets, control (180 g/kg casein) or low protein diet (LP, 90 g/kg casein), which were modified according to the AIN-76 diet formula throughout gestation (Table 3.1). Each group contained 4 dams. Both groups had free access to rat

chow and drinking water. Food intake was daily monitored by recording food left over. Animals were individually housed in standard polycarbonate cages with corncob bedding and maintained in the humidity and temperature controlled colony room on a 12 hr light-dark cycle. Twenty-four hours after newborn, 6 pups (3 female and 3 male) were selected and used in the litters to minimize variation in pups' nutrition status during suckling. Pups body weight was measured once a week after birth. Both groups of mothers were switched to standard diet through lactation {Lillicrop *et al.* 2005}. At day 24, in both experimental groups, the pups were weaned from the mother to a standard rat chow. To get the enough size of tissues for sample analysis, pups were sacrificed when they were 38 days old. The gastrocnemius muscle of right hind leg, visceral adipose tissue (mesenteric) and liver were then collected, snap-frozen in liquid nitrogen, and stored at -70°C.

RNA isolation and cDNA synthesis

Frozen tissue samples were ground in a mortar and pestle with liquid nitrogen and total RNA was isolated with TRI reagent (Sigma, St. Louis, MO). Following isopropanol (Fisher Scientific, Fair Lawn, NJ) precipitation RNA was resuspended and quantified by spectrophotometry (BIO-RAD Smart Spec Plus, Hercules, CA) at A260/A280. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used for reverse transcription of 2 µg of total RNA. All samples that were to be compared were reversed transcribed from the same reaction mixture at the same time with a no RNA template tube as negative control. The whole procedure was performed in a DNA 2720 Thermal Cycler (Applied Biosystems). The samples at the 20 µl reaction scale were heated at 37°C for 2 hr for reverse transcription procedure following 85°C for 5 seconds to inactive reverse transcriptase and terminate the reaction. The final 20 µl of cDNA was diluted up to 400 µl by nuclease free water and stored at -20°C.

Real time quantitative RT-PCR (qPCR)

To measure the relative amount of mRNA, cDNA samples were run in a 96-well plate on 7300 Real-Time PCR Systems (Applied Biosystem, Foster City, CA)), detected by iTag SYBR Green Supermix with ROX (Bio Red, Hercules, CA), which is a dye binding to

dsDNA generated during amplification. The reactions were activated at 95°C for 10 min accompanied by 35 cycles of 95°C for 15 s and 60°C for 1 min. The abundance of mRNA was quantified according to fluorescence signal by using a cDNA standard with specific concentration gradient versus Ct values. The cDNA standards were made from the same RNA samples of frozen tissues. The PCR primers amplified these standard samples in a linear fashion, and the Ct values of experimental samples were within the Ct values of the standard samples. Ribosomal protein L7a mRNA level was also measured at the same time as the internal control. After PCR, dissociation curve was generated by stepwise increase of the temperature from 55°C to 95°C to ensure that a unique product was amplified. Primers for the qPCR were designed using Vector NTI software (InforMax Inc., Frederick, MD) to amplify the transcriptional regions of the C/EBP β and PEPCK gene (Table 3.2).

Western blot

Frozen muscle sample (25 mg) was ground in liquid nitrogen and put into 500 μ l of protein sample buffer (0.125 M Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 1% SDS, 20% glycerol, 0.4% bromphenol blue, protease inhibitor). Protein was sonicated (model 100 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) on ice with 25 pulses at power setting 2. Lowry assay was used to determine protein content and samples containing 20 μ g of protein were resolved by SDS-PAGE. After electrotransfer to a polyvinylidene fluoride membrane (0.2 μ m) using a wet transfer protocol, 5% milk in TBS/T (30 mM Tris base pH 7.6, 200 mM NaCl and 0.1% Tween 20) was performed to block the membrane for 1 hr at room temperature and then the membrane was incubated with rabbit polyclonal antibody against C/EBP β (Table 3.3) in 5% BSA at a 1:1000 dilution at 4°C overnight. The membrane was washed five times for 5 min with TBS/T on a shaker the next day and then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in 1% milk at a 1:10,000 dilution for 1 hr at room temperature. The membrane was then washed again for 5 min by five times. The bound secondary antibody was detected using a SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL). The blots were then exposed and bands were quantified by a BioRad Chemi Doc machine. The background

signals on the western blots were subtracted from the signal of protein bands when quantification was done by the machine. An anti-actin antibody was used as an internal control to show the equality of protein levels loaded. For each protein sample, the intensity of the C/EBP β bands was normalized against that of the actin band.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed according to a modified protocol {Chen *et al.* 2004}. Briefly, 200 mg frozen skeletal muscle samples were ground in liquid nitrogen, resuspended in PBS and cross-linked in 1% formaldehyde for 10 min at room temperature. The tissue pellet was re-suspended in nuclei swelling buffer (5 mM Pipes [NaOH] pH 8.0, 85 mM KCl, 0.5 % NP40) containing protease inhibitor. The separated nuclei were lysed in SDS lysis buffer (50 M Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS) containing protease inhibitors. The resultant chromatin was sonicated (Fisher Scientific model 100 Sonic Dismembrator, Pittsburgh, PA) on ice with 8 bursts for 40 s at power setting 5 with 2 min cooling interval between each burst. The average length of sonicated chromatin was determined by resolving on a 1.6% agarose gel and found to be around 500 bp. The sample was then centrifuged at 13,000 rpm for 10 min at 4°C to remove cell debris from the crude chromatin lysate. One milliliter-sheared chromatin was diluted in total 10 ml ChIP dilution buffer. Ten percent of the diluted lysate was subsequently incubated overnight on a hematology mixer (model 346, Fisher Scientific) with 2 μ g of primary antibodies at 4°C. Pre-blocked salmon sperm DNA/protein A agarose beads (60 μ l, 50% slurry; Upstate Biotechnology, Lake Placid, NY) were then added to the chromatin for 2 hr, followed by centrifugation at 2000 rpm for 1 min at 4°C. Supernatant of normal rabbit IgG was saved as the input control for PCR after clean up. The pellets containing immunoprecipitated complexes were washed sequentially with 1 ml of low salt solution (0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM pH 8.0 Tris-HCl, 150 mM NaCl), high salt solution (0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM pH 8.0 Tris-HCl, 500 mM NaCl), and LiCl solution (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM pH 8.0 Tris-HCl) and twice with TE (pH 8.0). Antibody/protein/DNA complexes were eluted from Protein A agarose beads by adding twice 250 μ l of the elution buffer (50 mM NaHCO₃ and 1% SDS) followed by shaking at 37°C at 300 rpm

for 15 min and flash spin down at room temperature. The combined supernatants were incubated at 65°C for 4-5hr after addition of 20 μ l 5 M NaCl and 1 μ g of RNase A to reverse the formaldehyde cross-linking and release the DNA fragments. Samples were then treated with Proteinase K at 37°C for 1 hr to remove protein and DNA was purified with QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA). Immunoprecipitated DNA (5%) was quantified then by real time qPCR as described above. The standards and the DNA samples were simultaneously amplified using the same master mixture in a 25 μ l reaction. Primers for the qPCR were designed using Vector NTI software to amplify the C/EBP β and PEPCK proximal promoter sequences (Table 3.2).

Transcriptional activity

To measure the transcriptional activity from the C/EBP β gene, the method of Sandoval et al {Sandoval *et al.* 2004} was used, which relies on ChIP analysis to examine RNA polymerase II (Pol II) binding at the coding region distal to the promoter. For this purpose, primers were designed within the protein coding region of the C/EBP β gene (Table 3.2). The qPCR analysis was then performed using a 7300 realtime PCR system (ABI) and the product was detected with SYBR Green. Serial dilutions of input chromatin were used to generate a standard curve and the results were expressed as the ratio to input DNA.

Statistical analysis

Results are expressed as mean \pm SEM. Comparison of mRNA and protein expression or histone modification between control and treated groups were performed by two-tailed t-test. Significance testing was set at $p < 0.05$ level.

3.4 Results

Mothers food intake and offspring body weight

Pregnant dams were obtained on day 2 of gestation. Figure 3.1A shows mothers' daily food intake from day 3 of gestation to day 21, at which they gave birth. There were no differences in the daily food intake between control and low protein groups ($p > 0.05$), indicating the quantity of food intake per se would not affect experiment results. At birth,

the control pups (6.32 ± 0.19 g) weighed similar to the LP pups (5.92 ± 0.09 g). As the pups grew to 15 days, average body weight was significantly lower in the LP pups than in the control ($p < 0.02$). Figure 3.1B provides the average offspring body weight at birth and at day 7, 15, 22 and 35 of postnatal life. The average weight of the LP offspring was significantly reduced by 12% at day 15, 16% at day 22 and 13% at day 35 ($p < 0.05$) as compared to the control pups. Litter size and litter sex distribution did not differ between the control and LP mothers (not shown).

Maternal low protein induced C/EBP β mRNA level in female offspring muscle

Though it is a potent transcription factor involved in muscle amino acid and carbohydrate metabolisms, C/EBP β has not been previously shown to be affected by maternal nutrition restriction in this specific tissue. In this study, C/EBP β mRNA levels in offspring rats were quantified by qPCR. Figure 3.2A shows the C/EBP β mRNA expression levels in skeletal muscle, visceral adipose tissue and liver of offspring of both sex from control mothers versus low protein (LP) mothers. A 2.14-fold increase ($*p = 0.003$) of C/EBP β mRNA content in skeletal muscle was observed in female offspring of maternal low protein mothers compared to control mothers, while no change was observed in males. Samples from 5 animals were tested in each of the two conditions. The values represent the mean \pm S.E.M. L7a expressions were similar between the samples of control and LP in all of those three tissues (not shown). Results indicate a sex-dependent C/EBP β change by maternal protein restriction, which might suggest a difference in energy metabolism between female and male offspring muscle during early life under this maternal nutrition state. Since C/EBP β gene expression did not change in other tissues (adipose tissue and liver) and in the males, we concentrated further study on the regulatory mechanisms for C/EBP β gene in female offspring skeletal muscle only.

Expression of genes related to amino acid response (AAR) pathway

The limitation of amino acids to mammalian cells modulates gene expression through the AAR pathway either at the transcriptional level through activating transcription factor 3 (ATF3), or at the translational level through ATF4, which is enhanced in stress conditions that lead to the increase of eukaryotic initiation factor 2a (eIF2a) phosphorylation

{Kilberg *et al.* 2005}. To identify whether or not the increased C/EBPb expression in female pup muscle by maternal low protein is regulated through this AAR programming, we tested the mRNA expression of ATF3, ATF4 as well. C/EBPb acts as a cofactor for ATF3 and ATF4 in the AAR pathway. The mRNA expression levels of ATF3 or ATF4 did not differ between the two female pup groups (Figure 3.2B; $p>0.05$), suggesting that the alteration of C/EBPb mRNA in female offspring muscle through maternal protein restriction may not be mediated by the AAR pathway. Samples from 5 animals were tested in each of the two conditions. The values represent the mean \pm S.E.M. The results implicate C/EBPb as a mediator in regulating amino acid or carbohydrate metabolism in skeletal muscle independent of the AAR pathway.

Maternal low protein induced the transcription rate of the C/EBP β gene

To confirm the transcriptional activity from the C/EBP β gene, ChIP DNA samples were used to monitor RNA Polymerase II (Pol II) binding to a region distal to the promoter, as demonstrated by Sandoval *et al.* {Sandoval *et al.* 2004}. The measurement of Pol II binding within the coding region of a gene reflects the transcriptional activity. For the C/EBP β gene, this analysis showed that the transcription rate increased 2-fold in female LP pup skeletal muscle relative to control pups (* $p=0.02$; Figure 3.2C). Therefore, increased transcription appeared to account for most of the induction in C/EBP β mRNA following subjection to maternal low protein. Samples from 5 animals were tested in each of the two conditions. The values represent the mean \pm S.E.M..

C/EBP β protein level was increased in female offspring by maternal low protein

To examine C/EBP β protein content in female pup skeletal muscle, we performed Western blot analysis using an anti-C/EBP β antibody. According to studies *in vitro*, C/EBP β mRNA can produce at least three isoforms, liver-enriched transcriptional activator protein, LAP A, LAP B and LIP (liver-enriched transcriptional inhibitory protein), detected as multiple bands with molecular mass from 36 to 45 kDa {Chen *et al.* 2004; Ramji *et al.* 2002}. This may be the result of post-translational modification {Ramji *et al.* 2002; Kim *et al.* 2002}. As shown by our results presented in Figure 3.3, two LAP isoforms of C/EBP β protein were both induced by maternal low protein in

female offspring skeletal muscle. The ratios of C/EBP β protein levels in female pup control to LP group were determined to be 1:1.58 for LAP A (*p=0.01) and 1:1.74 for LAP B (*p=0.005). Significantly higher C/EBP β protein content in female LP pups' skeletal muscle was consistent with the trend observed in its mRNA expression. The LIP isoform was not expressed in rat skeletal muscle. An anti-actin antibody was used as an internal control to show the equality of protein levels loaded. Samples from 5 animals were tested in each of the two conditions. The values represent the mean \pm S.E.M..

Chromatin structure modification at C/EBP β promoter in female offspring muscle

Changes in chromatin structure affected by chemical modifications of histone proteins such as methylation and acetylation regulate gene transcription. RNA polymerase II (also called Pol II) is an enzyme found in eukaryotic cells {Roeder *et al.* 1969}. It catalyzes the transcription of DNA to synthesize precursors of mRNA and most snRNA and microRNA. A wide range of transcription factors are required for it to bind to its promoters and begin transcription {Conaway *et al.* 1997}. To investigate Pol II binding status at the transcription factor C/EBP β promoter, we performed a ChIP assay, which refers to a procedure used to determine whether a given protein binds to or is localized to a specific DNA sequence in vivo. Normal rabbit IgG antibody was used as the negative control, which indicates non-specific binding. Antibodies with equal or less binding than IgG (\leq 0.05 ratio to input) were considered negative for binding. The supernatant isolated from the IgG solution was used as the input control to determine the total protein background. The binding of Pol II and modified histones were expressed as ratios to the input. Figure 3.4A demonstrates the Pol II binding at C/EBP β promoter region in female pup skeletal muscle with maternal low protein in comparison to control. Recruitment of Pol II at the promoter of the C/EBP β was increased in female pups due to maternal low protein (2.53-fold; *p=0.01). Data was in agreement with the increased C/EBP β mRNA expression.

To determine whether the altered transcriptional level of C/EBP β gene was regulated by changes of chromatin structure modification that resulted in the Pol II binding change at the C/EBP β promoter, methylated histone or acetylated histone antibodies were

incorporated into the ChIP assay. Histone acetylation is associated with increased gene transcription, and therefore with transcriptionally active chromatin domains. Although histone methylation is generally associated with transcriptional repression, methylation of different lysine or arginine residues of histone also results in transcriptional activation. For example, the acquisition of active chromatin markers, such as H3K4Me₂, leads to opened chromatin structure, which correlates with transcriptional activation of a target gene, while H3K9Me₃ induces transcription repression. To test the effect of maternal low protein on chromatin remodeling at the C/EBP β promoter, ChIP assay was performed with antibodies specific for histone modifications associated with enhanced gene transcription: anti-acetyl-H3, anti-acetyl-H4 and anti-H3K4Me₂. To examine if the increased C/EBP β mRNA expression results from diminished methylation of histone at residues, which lead to transcriptional repression, we also included the antibody of anti-H3K9Me₃. Figure 3.4B shows the histone code modifications at C/EBP β promoter region in female pup skeletal muscle of maternal low protein versus control. Increases in both acetyl-H3 and acetyl-H4 that associated with the C/EBP β gene were observed in female pups muscle with maternal low protein versus control, by 1.82- and 2.01-fold (*p<0.05), respectively. Di-methylation of H3K4 did not differ between the two groups and no difference was detected in H3K9me₃. Data suggests that the altered C/EBP β gene transcription might be regulated by the changes of histone modification at its promoter region, histone acetylation at lysine 4 and lysine 9 residues, which results in the change of Pol II binding at the promoter. There was no any change of histone modifications within the C/EBP β promoter in male offspring skeletal muscle or in female offspring liver samples (Figure 3.4C and 3.4D). Samples from 5 animals were tested in each of the two conditions. The values represent the mean \pm S.E.M..

C/EBP β binding at the PEPCK promoter and PEPCK expression were increased by maternal low protein

To further investigate the physiological role of C/EBP β in nutritional regulation in muscle, ChIP DNA samples that were pulled down by anti-C/EBP β antibody were used to determine the amount of C/EBP β binding at the PEPCK promoter. Quantification of PEPCK DNA samples was obtained through qPCR assay using the PEPCK primer set

(Table 3.2) to amplify its promoter region, which was bound with the C/EBP β transcriptional factor. Figure 3.5A shows the increased C/EBP β binding at the PEPCK promoter in female offspring skeletal muscle (*p<0.05), suggesting the potentially enhanced role of PEPCK on pyruvate synthesis via the generation of PEP to re-feed the citric acid cycle in muscle. Since C/EBP β was shown to bind at the PEPCK promoter, we used qPCR to measure mRNA expression of PEPCK in order to confirm the effect of C/EBP β binding. Figure 3.5B demonstrates the up-regulation of PEPCK mRNA in female offspring muscle by maternal low protein treatment (*p<0.05). PEPCK primers used for checking its coding region are shown in Table 3.2. Samples from 5 animals were tested in each of the two conditions. The values represent the mean \pm S.E.M..

3.5 Discussion

The results presented in this study document for the first time that C/EBP β is up-regulated in both mRNA and protein levels in the skeletal muscle of female rat offspring in response to a low protein diet during the maternal gestation period. Consistent with this observation, the ChIP results showed chromatin modifications and increased recruitment of RNA polymerase II at the C/EBP β promoter region in female offspring muscle. The transcription of PEPCK was up-regulated via increased binding of C/EBP β within its promoter, which contributes to amino acid metabolism in muscle and represents an important physiological impact.

Maternal diet and nutrition may modify pregnancy hormone environment {Hilakivi-Clarke *et al.* 2006}. Therefore, maternal diet may affect the different adaptation of female or male offspring to the altered utero hormone environment, especially estrogen levels. It is known that C/EBP β is a key mediator of estrogen-response cell proliferation and differentiation in many tissues {Mantena *et al.* 2006}. The findings in our study revealed the sex-specific difference of C/EBP β gene expression, which reflects one of the effects of maternal protein restriction in offspring skeletal muscle. This sex-specific difference may stem from sex-dependent responses to early developmental perturbations including altered utero estrogen levels. In addition, maternal protein restriction may alter

circulating estrogen level in offspring rats and results in higher blood pressure {Musha *et al.* 2006}. On the other hand, it was reported that insulin regulates some stress-responsive genes through induction of C/EBP β {Du *et al.* 2009}. But how insulin regulates C/EBP β is still not understood. Chamson-Reig A and colleagues had reported a sex-dependent difference in offspring pancreatic β -islet cell insulin synthesis/secretion and circulating insulin concentrations due to the reduced diet protein during gestation and lactation {Chamson-Reig *et al.* 2006}. In one of our previous studies, we observed increased glucose transporter 4 expression in female offspring skeletal muscle only by maternal low protein (not published), which may be a result of insulin-responsive gene expressions through regulating C/EBP β {Liu *et al.* 2006}. Better understanding the role of C/EBP β as transcription factor in muscle nutrient metabolism could also provides potential insight to explain the sex-specific difference in responding to the varied hormone levels in utero. We also considered that maternal behaviors may be affected by gestation diet. It was reported that well-nourished dams spend more time with LP pups in passive nursing and pup grooming than with control pups {Galler *et al.* 1991}. However, in our animals, we didn't observe any obvious variation in maternal behaviors between LP and control groups.

Maternal nutrition may also affect fetal skeletal muscle development, which involves myogenesis, adipogenesis, and fibrogenesis. C/EBP β has been known as a transcription factor regulating adipogenesis {Du *et al.* 2010}. The sex-specific C/EBP β expression in our study may associate with the different phenotypes of adipogenesis in offspring skeletal muscle. C/EBP β promotes the differentiation of fibroblastic cells into adipocytes. C/EBP β is induced during the initial phase of adipogenesis {Du *et al.* 2010}, but males and females have similar muscle growth until puberty {Tipton *et al.* 2001}. Maternal under-nutrition has been reported to manipulate the expression of myostatin, a negative regulator of muscle development, has recently been shown to effect fat deposition {Peiris *et al.* 2010}. High concentration of intramuscular fat content is highly related with type 2 diabetes {Greco *et al.* 2002}. In our study, the induced C/EBP β expression that occurred in female puberty rats by a maternal low protein diet, may provide more information about the effect of maternal nutrition on fat deposition during postnatal muscle

development, potentially implying the risk of type II diabetes.

C/EBP β acts as a transcription factor for many genes, such as those involved in nutrient metabolism. PEPCK, for example, is a very important enzyme participating in not only gluconeogenesis in liver but also significant local metabolisms in muscle, kidney, adipose and small intestine {Owen *et al.* 2002}. Following an overnight fast or caloric restriction, there is a net release of amino acid from the skeletal muscle. The released amino acids, however, do not reflect the composition of muscle protein, suggesting local metabolism and interconversion of amino acids in the skeletal muscle {Owen *et al.* 2002}. During exercise, although TCA intermediates increase from the entry of amino acids, the source of pyruvate needs to be replenished to maintain feeding into the TCA cycle. In muscle, PEPCK catalyzes the conversion of oxaloacetate to PEP, which is one of the sources of pyruvate, for re-feeding of the TCA cycle.

C/EBP β mRNA can produce at least three isoforms in rat liver, 38 kDa (LAP*), 35 kDa (LAP) and 20 kDa (LIP). LAP* and LAP contain both an activation domain and the bZIP domain, where LIP only contains the bZIP domain {Descombes *et al.* 1991}. Therefore, LIP forms a non-functional heterodimer with the other C/EBP members and inhibits C/EBP function. It was reported that the LIP content was increased and LAP content remained unchanged in the rat liver in response to reduced dietary protein {Marten *et al.* 1996}. In our case, however, the LIP was not detectable in rat skeletal muscle. LAP A and LAP B were up-regulated, which was consistent with the elevated mRNA expression of C/EBP β in LP female offspring muscle.

We observed up-regulation of C/EBP β at both mRNA and protein levels in offspring skeletal muscle in response to maternal protein restriction. An increased C/EBP β mRNA level was found in rat hepatoma cells following incubation in amino acid limiting medium {Marten *et al.* 1994}. Also, Dr. Kilberg *et al.* has demonstrated that amino acid deprivation of mammalian cells leads to increased transcription of C/EBP β , synthesis of total C/EBP β protein and binding to the AARE within the ASNS promoter {Chen *et al.* 2004}. The increase in C/EBP β mRNA expression following amino acid response (AAR)

pathway activation is primarily due to increased transcription controlled by a 93 bp sequence containing a regulatory element that activates transcription in response to amino-acid-deprivation and to elevated activating transcription factor 4, ATF4. But in vivo, we did not observe altered ATF4 gene transcription under maternal low protein condition in offspring muscle (not shown).

In conclusion, the data presented here provides evidence that a maternal low protein diet during gestation alters C/EBP β expression in female offspring muscle at both the transcriptional and translational levels, as show through by QPCR and Western blotting. ChIP results reveal that the altered C/EBP β gene expression was culminated from the mediation of histone code modifications at its promoter region. The alteration of PEPCK transcription via the enhanced binding of C/EBP β at its promoter could potentially impact amino acid metabolism as well as energy homeostasis in skeletal muscle.

3.6 Tables & Figures

Table 3.1 Composition of the two isocaloric diets

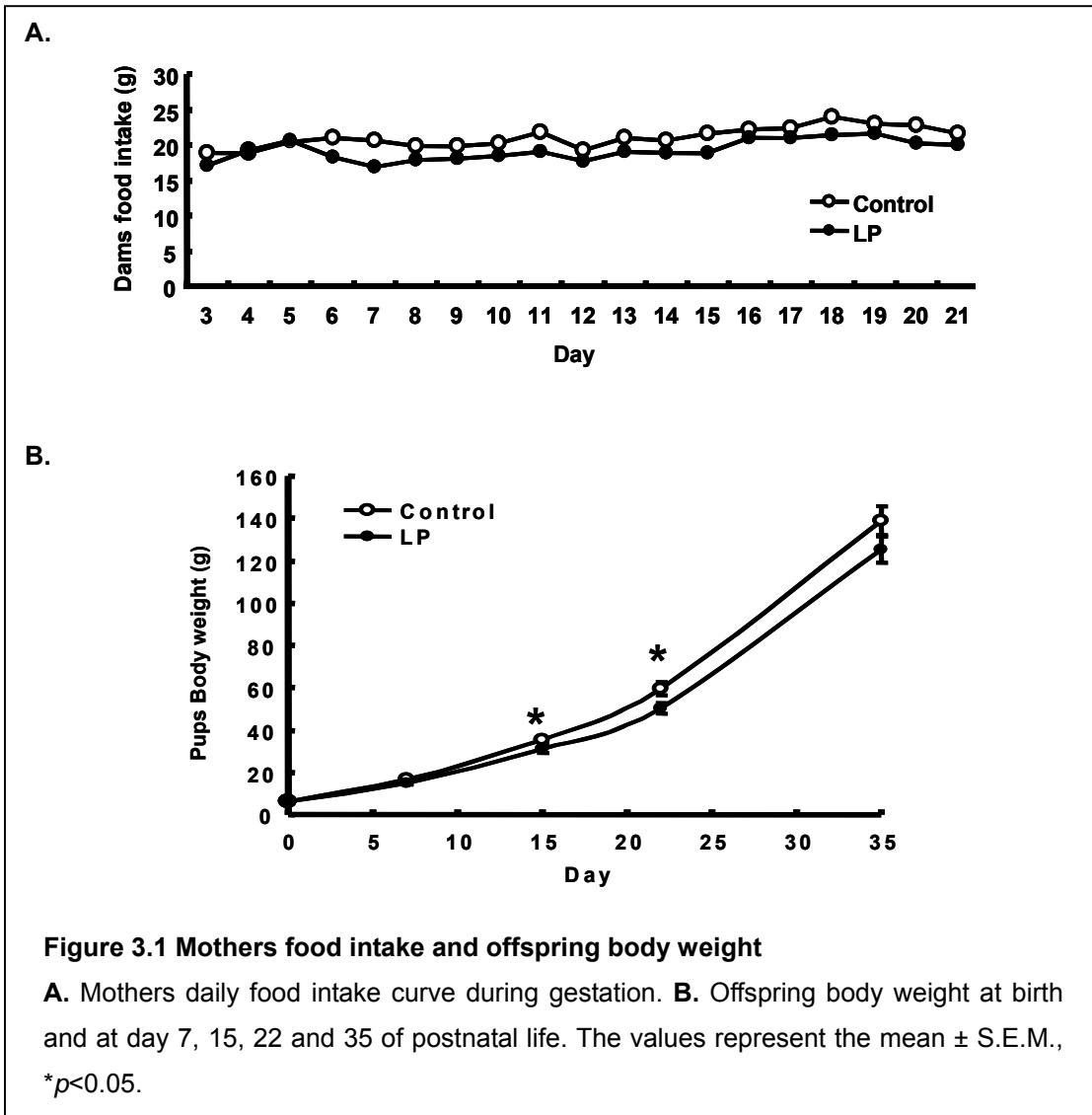
	C, g/kg	LP, g/kg
Casein	180	90
Folic acid	0.001	0.001
Cornstarch	405	465
Sucrose	213	243
Choline	2	2
Cellulose	50	50
Corn oil	100	100
D,L-Methionine	5	5
Vitamin mix	10	10
Mineral mix	35	35
Total energy, kcal/kg	3868	3868

Table 3.2 Primer sequence used in qPCR and ChIP assay

Gene (Ensembl ID)	Forward primer (5' → 3') Reversed primer (5' → 3')	Purpose
C/EBPβ (ENSRNOG00000025114)	(+822) AGAACGAGCGGCTGCAGAAGA	mRNA expression
	(+889) GAACAAGTTCCGCAGCGTGC	
	(-834) ATGTTCCACCCGCAACCACC	Histone modification at C/EBPβ promoter
	(-766) GACCCCAAGAAAGACCCGCAC	
PEPCK (ENSRNOG00000028616)	(+822) AGAACGAGCGGCTGCAGAAGA	Transcription rate
	(+889) GAACAAGTTCCGCAGCGTGC	
	(+1177) CGAACGCCATTAAGACCATCCAG	mRNA expression
	(+1253) CCAGTAAACACCCCATCACTTGTC	
	(-148) TGTTAGGTCAGTTCCAAACCGTGC	C/EBPβ binding at PEPCK promoter
	(-66) TGGACCTGGAGGCTCGCC	

Table 3.3 Antibodies used in Western blot and ChIP assay

Antibody	Introduction & company	Purpose
C/EBPβ	sc-150, Santa Cruz Biotechnology, Santa Cruz, CA	Protein analysis
H3Ac	Anti-acetylated histone 3 at lysine 9, 14 residues; 06-599, Upstate, Temecula, CA	Histone modifications
H4Ac	Anti-acetylated histone 4 at lysine 5, 8, 12 and 16 residues; 06-866, Upstate, Temecula, CA	analysis
H3K4Me2	Anti-di-methylated histone 3 at lysine 4 residues; 07-030, Upstate, Temecula, CA	
H3K9Me3	Anti-tri-methylated histone 3 at lysine 9 residues; cs200604, Upstate, Temecula, CA	
IgG	sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA	
Pol II	sc-899, Santa Cruz Biotechnology, Santa Cruz, CA	Pol II analysis



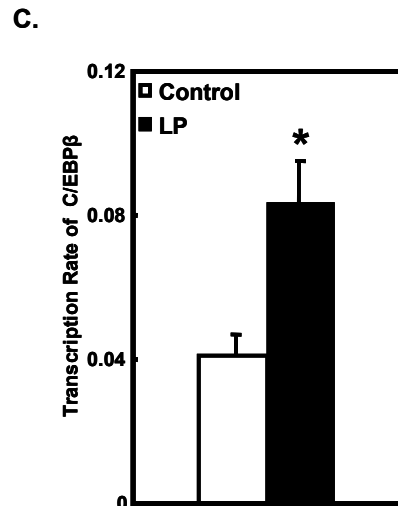
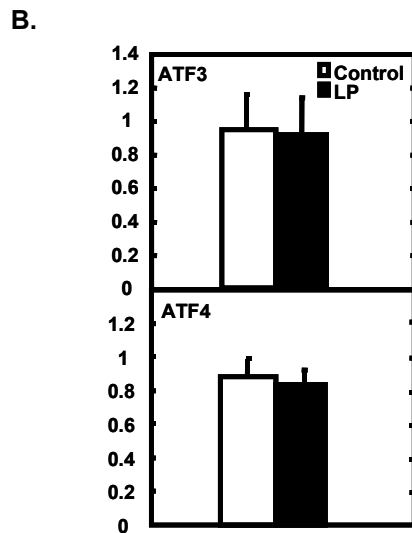
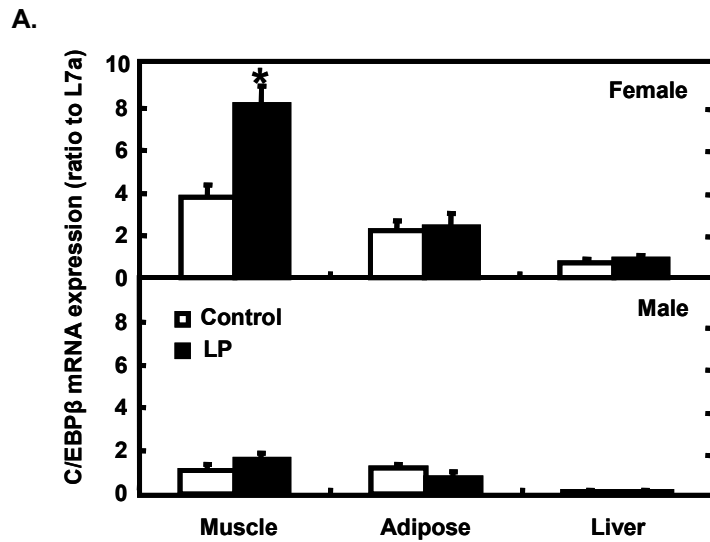


Figure 3.2 C/EBPβ and ATFs expression analysis

A. C/EBPβ mRNA expression levels in offspring skeletal muscle, visceral adipose tissue and liver (n=5, samples from 5 animals were tested in each of the two conditions). The bar values represent the mean ± S.E.M.; *indicates comparison of C/EBPβ mRNA in pups of control and LP groups, $p < 0.05$. **B.** Expression of transcription factors in AAR pathway (n=5, samples from 5 animals were tested in each of the two conditions). All the values represent the mean ± S.E.M. after normalization by L7a. **C.** Real transcription rate of the C/EBPβ gene (n=5, samples from 5 animals were tested in each of the two conditions). The values represent the mean ± S.E.M., * $p < 0.05$.

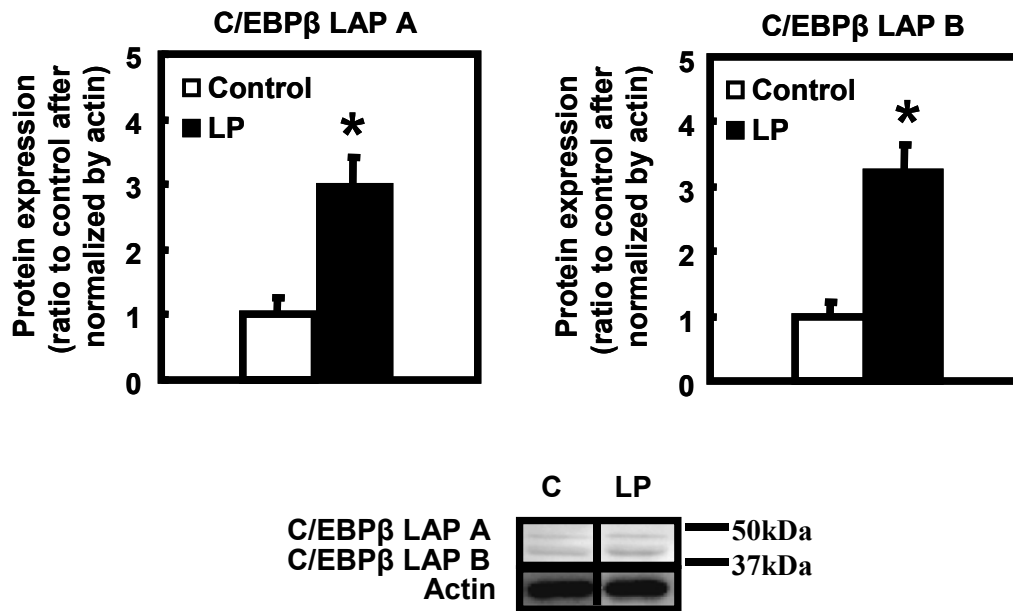
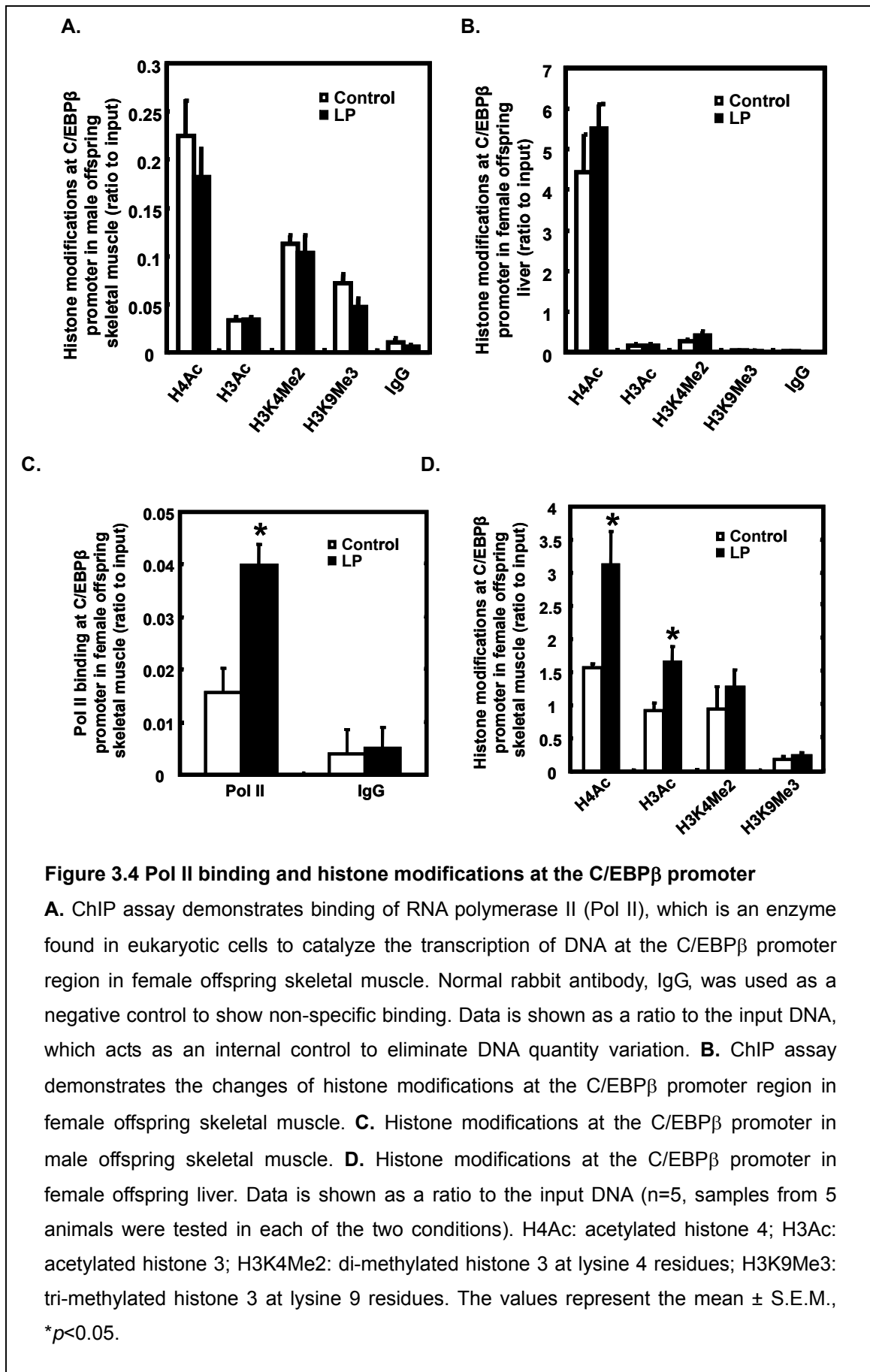
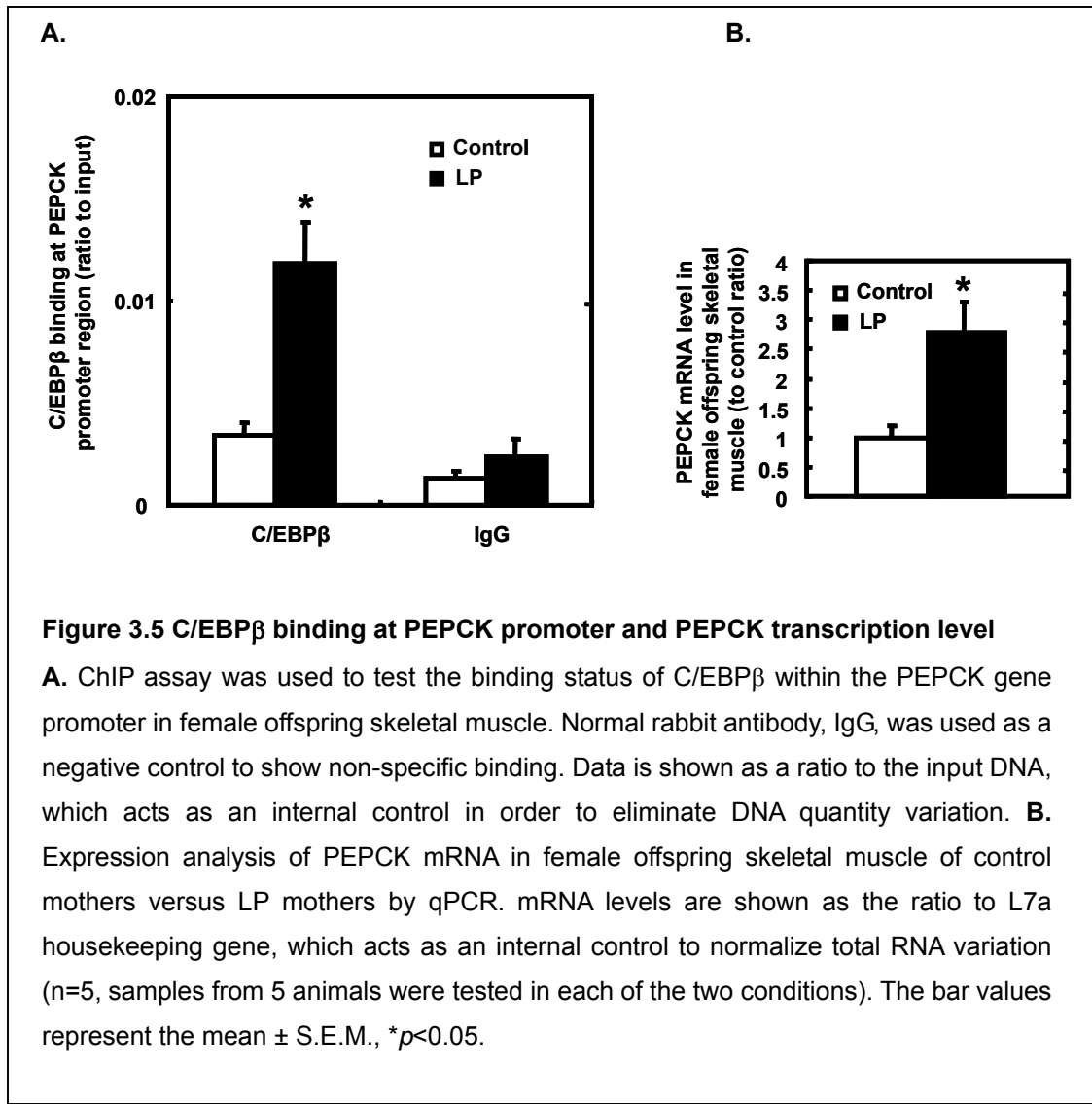


Figure 3.3 C/EBPβ protein content in female pup skeletal muscle by Western blot

Expression of C/EBPβ protein, LAP A and LAP B, in female offspring skeletal muscle from LP mothers versus control (n=5, samples from 5 animals were tested in each of the two conditions). The bands represent the average level of C/EBPβ protein in control and LP groups. LAP A and LAP B are isoforms of C/EBPβ protein, detected as multiple bands with molecular mass from 36 to 45 kDa. For each protein sample the intensity of the C/EBPβ bands were normalized against that of the actin band. LP protein levels were then expressed as a ratio to control. The values represent the mean ± S.E.M., * $p < 0.05$.





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Chapter 4. Protein Restriction during Gestation Alters Histone Modifications at the Glucose Transporter 4 (GLUT4) Promoter Region and Induces GLUT4 Expression in Skeletal Muscle of Female Rat Offspring

4.1 Abstract

Maternal nutrition during pregnancy is an intrauterine factor that results in alteration of the offspring genome and associates with disease risk in the offspring. Maternal nutrition restriction can lead to low birth weight, which is associated with increased risk of type II diabetes and cancer in offspring adult life. We investigated the impact of a maternal low protein (LP) diet on the expression of glucose transporter 4 (GLUT4) in offspring skeletal muscle. GLUT4 is an insulin-regulated glucose transporter involved in insulin sensitivity and carbohydrate metabolism in muscle cells. We observed sex-dependent GLUT4 mRNA expression and increased GLUT4 protein content in female pup skeletal muscle with maternal low protein. Analysis of transcriptional and epigenetic regulation of increased skeletal muscle GLUT4 expression in offspring rats revealed the regulatory mechanisms involved. The protein level of myocyte enhancer factor 2A (MEF2A), which has been known as an activator of GLUT4 transcription via the ability to carry out specific binding to the GLUT4-MEF2 binding sequence, increased in female pups whose mothers were fed a low protein diet. Modifications of chromatin structure, including acetylated histone H3, acetylated histone H4 and di-methylated histone H3 at lysine 4, were detected at a significantly increased level at the GLUT4 promoter region in female pup muscle following a maternal low protein diet. Glycogen content was also detected as up-regulated, accompanied by increased glycogen synthase in LP female offspring muscle. However, the restricted diet did not activate the amino acid response pathway. These results document that maternal protein restriction during pregnancy induces GLUT4 expression in female offspring skeletal muscle but not in males, which may indicate sex-dependent adaptation of glucose metabolism to a low maternal protein diet.

4.2 Introduction

Maternal nutrition deviation in pre- and postnatal periods is an important determinant of disease risk in adult life {Langley-Evans *et al.* 2006}. Nutrition restriction during fetal development can result in low birth weight, which has long-term consequences in postnatal life, including predisposition of offspring to obesity and type II diabetes {Ozanne *et al.* 2005}. About 13% of U.S. births involve teen mothers, who are less likely to gain adequate weight during pregnancy, leading to low birth weight (CDC National Vital Statistics Report; Volume 57, No. 7). Through the use of animal models, nutritional deficiency research has demonstrated the development of insulin resistance in the skeletal muscle of offspring that suffered from intra-uterine growth restriction (IUGR) established by dietary calorie restriction {Raychaudhuri *et al.* 2008}. Epidemiology and experimental studies have indicated that a maternal LP diet during fetal and neonatal development in both humans and experimental animals impacts an offspring's susceptibility to later development of altered carbohydrate metabolism {Dahri *et al.* 1991}.

In a recent study, a maternal LP diet during pregnancy and/or lactation affects postnatal growth, appetite, triglyceride and cholesterol concentrations, as well as insulin resistance in male but not female offspring {Zambrano *et al.* 2006}. However, the molecular mechanism underlying the sex-differentiated carbohydrate metabolism and insulin sensitivity is rarely explained. The predominant adaptive mechanism could be aberrant glucose uptake into insulin-sensitive tissue; either skeletal muscle or adipocyte tissue {Thamotharan *et al.* 2005}. The major insulin responsive protein involved in glucose absorption is glucose transporter 4 (GLUT4). Insulin controls glucose homeostasis by regulating the translocation of GLUT4 from the intracellular pool to the plasma membrane {Shepherd *et al.* 1999}. The disruption of GLUT4 results in glucose intolerance and insulin resistance. Macronutrient content and composition of the diet has proven to influence the expression of the glucose transporter genes and insulin sensitivity. A recent study examined mechanisms involved in the reduction of skeletal muscle GLUT4 mRNA in IUGR female rat offspring from dams that received 50% daily food intake during pregnancy and lactation {Raychaudhuri *et al.* 2008}. In contrast, Kahn *et al.* found that calorie restriction in fasting adult rats down-regulates GLUT4 expression in adipose cells but up-regulates it in skeletal muscle {Charron *et al.* 1990}. Tissue-specific

regulation of glucose transporter expression was proposed to be an adaptive response to altered nutrient availability {Kahn *et al.* 1994}, although the molecular mechanism is less understood. Moreover, associations of maternal nutrition restriction with gene expression in the offspring of carbohydrate metabolism-related genes create more complications.

There is growing evidence that maternal nutritional status can alter the epigenetic state of the offspring. The insulin resistance phenomena in IUGR female offspring has been shown to be transgenerationally transmitted {Thamotharan *et al.* 2007}. Histone modifications at the GLUT4 promoter have been observed, which result in the transcriptional repression in female offspring when adult pregnant rats were exposed to a low calorie diet {Raychaudhuri *et al.* 2008}. Therefore, the present study investigated the consequence of maternal protein restriction during teenage pregnancy on GLUT4 transcriptional regulation and epigenetic modifications in offspring skeletal muscle, particularly histone modifications and DNA methylation. Raychaudhuri *et al.* showed that in female IUGR pups, skeletal muscle GLUT4 transcription was repressed, which is accompanied by de-acetylation and di-methylation of specific amino-acid residues in the N-tail of histone H3. Such histone modifications contribute to co-repressor complex formation and destruction of co-activator complex, decreasing GLUT4 transcription at both birth and through later life, which increases the offspring's risk for pre-diabetes {Raychaudhuri *et al.* 2008}. In contrast, the present data demonstrates that maternal low protein intake (but not calorie restriction) up-regulates GLUT4 gene expression through chromatin modification programming specifically in females and alters glycogen content in offspring skeletal muscle.

4.3 Material and Methods

Animals and treatments

The animal protocol for this study was approved by the International Animal Care and Use Committee (IACUC) at the University of Illinois. Timed-pregnant Sprague Dawley rats (Charles River Laboratories) were obtained on day 2 of gestation and weight matched into one of two isocaloric diets, control (180 g/kg casein) or low protein diet (LP,

90 g/kg casein), which were modified according to the AIN-76 diet formula throughout gestation (Table 4.1). Each group contained 4 dams. Both groups had free access to rat chow and drinking water. Food intake was daily monitored by recording food left over. Animals were individually housed in standard polycarbonate cages with corncob bedding and maintained in the humidity and temperature controlled colony room on a 12 hr light-dark cycle. Twenty-four hours after newborn, 6 pups (3 female and 3 male) were selected and used in per litter to minimize variation in pups' nutrition status during suckling. Pups body weight was measured once a week after birth. Both groups of mothers were switched to standard diet through lactation {Lillicrop *et al.* 2005}. At day 24, in both experimental groups, the pups were weaned from the mother to a standard rat chow. To get enough sizes of tissues for sample analysis, pups were sacrificed when they were 38 days old. The gastrocnemius muscle of right hind leg, visceral adipose tissue (mesenteric) and liver were then collected, snap-frozen in liquid nitrogen, and stored at -70°C.

RNA isolation and cDNA synthesis

Frozen skeletal muscle samples were ground in a mortar and pestle with liquid nitrogen prior to total RNA isolation using TRI reagent (Sigma, St. Louis, MO). Following isopropanol (Fisher Scientific, Fair Lawn, NJ) precipitation, RNA was re-suspended and quantified by spectrophotometry (BIO-RAD Smart Spec Plus, Hercules, CA) at A260/A280. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used for reverse transcription of 2 µg of total RNA. All samples that were to be compared were reverse transcribed from the same master reaction mixture at the same time with a 'no RNA template' tube as negative control. The whole procedure was performed in a DNA 2720 Thermal Cycler (Applied Biosystem). The samples at the 20 µl reaction scale were heated at 37°C for 2 hr for reverse transcription procedure following 85°C for 5 seconds to inactive reverse transcriptase and terminate the reaction. The final 20 µl of cDNA was diluted up to 400 µl by nuclease free water and stored at -20°C.

Real time quantitative RT-PCR (qPCR)

To measure the relative amount of mRNA, qPCR was performed with a 96-well plate in a 7300 Real-Time PCR System (Applied Biosystem). The reactions were activated at 95°C for 10 min followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. The mRNA level of ribosomal protein L7a was measured at the same time as the internal control. After PCR, a dissociation curve was generated by stepwise increase of the temperature from 55°C to 95°C to ensure that a unique product was amplified in the reaction. Primer efficiency was determined by a standard curve from a dilution series and used for calculations of Δ CTs and $\Delta\Delta$ CTs. Primers used for PCR are shown in Table 4.2.

Protein isolation and Western blotting

Twenty-five milligrams of frozen muscle sample was ground in liquid nitrogen and put into 500 μ l protein sample buffer (0.125 M Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 1% SDS, 20% glycerol, 0.4% bromophenol blue, protease inhibitor). Protein was sonicated (Fisher Scientific model 100 Sonic Dismembrator, Pittsburgh, PA) on ice with 25 pulses at power setting 2. Lowry assay was used to determine protein content. Briefly, 1 ml ice-cold TCA was used to precipitate protein from 10 μ l of sample buffer, and the supernatant was completely pouring out followed by 30 min speed dry. 100 μ l of 0.2% SDS / 0.2 M NaOH was used to resolve protein pellet, 650 μ l of Lowry Copper Reagent and 60 μ l of 1 M Folin-Ciocalteu Reagent were then added for absorbance test. Samples containing 20 μ g of protein were resolved by SDS-PAGE. After electrotransfer to a polyvinylidene fluoride membrane (0.2 μ m, Bio-Rad) using a wet transfer protocol, 5% milk in TBS/T (30 mM Tris base pH 7.6, 200 mM NaCl and 0.1% Tween 20) was used to block the membrane for 1 hr at room temperature. The membrane was then incubated at 4°C overnight with a 1:1000 dilution in 5% BSA of rabbit polyclonal antibody against GLUT4 or glycogen synthase; or 1:500 dilution in 5% BSA for antibody against MEF2 (Table 4.3). The next day, the membrane was washed five times for 5 min with TBS/T on a shaker and then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in 1% milk at a 1:10,000 dilution for 1 hr at room temperature. The membrane was then washed five times for 5 min each wash. The bound secondary antibody was detected using a SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL) and Western blot

images were captured and analyzed by a Chemi Doc system (Bio-Rad). An anti-actin antibody was used as an internal control to show the equality of protein levels loaded.

Glycogen content assay

Glycogen content in female offspring muscle was tested by using a Glycogen Assay Kit (BioVision #K646-100, Mountain View, CA). Frozen muscle tissue (8 mg) was ground in liquid nitrogen and homogenized with 200 μ l dH₂O on ice. The homogenates were boiled for 5 min to inactivate the enzymes. The boiled samples were spun at 13,000 rpm for 5 min to remove insoluble material. Hydrolysis Enzyme Mix was added into supernatant and the standard that comes with the kit, and then the samples were incubated for 30 minutes at room temperature. Development Reaction Mix (50 μ l) was added to each well containing Glycogen Standard or samples. The mixtures were incubated at room temperature for another 30 minutes, protected from light. The samples and glycogen standard were measured with the colorimetric OD value at 570 nm. The sample's readings were applied to the standard curve to get the amount of glycogen.

ChIP-qPCR

ChIP (Chromatin immunoprecipitation) analysis was performed according to a modified protocol {Chen *et al.* 2004}. Briefly, 200 mg of frozen skeletal muscle samples were ground in liquid nitrogen and resuspended in PBS and cross-linked in 1% formaldehyde for 10 min at room temperature. The tissue pellet was re-suspended in nuclei swelling buffer (5 mM Pipes [NaOH] pH 8.0, 85 mM KCl, 0.5 % NP40) containing protease inhibitor. The separated nuclei were lysed in SDS lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS) containing protease inhibitors. The resultant chromatin was sonicated (Fisher Scientific model 100 Sonic Dismembrator, Pittsburgh, PA) on ice with 6 bursts for 40 s at power setting 5, with 2 min cooling interval between each burst. The average length of sonicated chromatin was determined by resolving on a 1.6% agarose gel and found to be around 500 bp. The sample was then centrifuged at 13,000 rpm for 10 min at 4°C to remove cell debris from the crude chromatin lysate. One milliliter of sheared chromatin was diluted in ChIP dilution buffer to total 10 ml. Ten percent of the diluted lysate was subsequently incubated overnight on a hematology mixer (346, Fisher

Scientific) with 2 µg of primary antibodies at 4°C (see Table 4.3 for antibody information). Pre-blocked salmon sperm DNA/protein A agarose beads (60 µl, 50% slurry; Upstate Biotechnology, Lake Placid, NY) were then added to the chromatin for 2 hr, followed by centrifugation at 2000 rpm for 1 min at 4°C. Supernatant of normal rabbit IgG was saved as the input control to reflect the total chromatin content for each sample. Chromatin pull down with antibodies for each sample was normalized as a ratio to its total chromatin content after real-time qPCR analysis. The pellets containing immunoprecipitated complexes were washed sequentially with 1 ml of low salt solution (0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM pH 8.0 Tris-HCl, 150 mM NaCl), high salt solution (0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM pH 8.0 Tris-HCl, 500 mM NaCl), and LiCl solution (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM pH 8.0 Tris-HCl) and TE (pH 8.0), twice. Antibody/protein/DNA complexes were eluted from Protein A agarose beads by twice adding 250 µl of the elution buffer (50 mM NaHCO₃ and 1% SDS) followed by shaking at 37°C at 300 rpm for 15 min and a flash spin down at room temperature. The combined supernatants were incubated at 65°C for 4-5 hr after addition of 20 µl of 5 M NaCl and 1 µg of RNase A to reverse the formaldehyde cross-linking and release the DNA fragments. Samples were then treated with proteinase K at 37°C for 1 hr to remove protein and purified with a Wizard SV Gel and PCR Clean up System (Promega).

Quantitative real-time PCR was then performed to study modified histones and transcription factor binding to DNA. Enrichment of ChIP-ed DNA, which was pulled down by specific antibodies, then reflected the binding status of protein of interest within the GLUT4 promoter. 5% of immunoprecipitated DNA was used for each real-time PCR reaction. The standards and the ChIP-ed DNA samples were simultaneously amplified using the same reaction master mixture at 25 µl scale. Real-time qPCR data was then optimized for the amount of input material (total protein) and expressed as the ratio to input. Primers used to amplify genomic sequences at the promoter region of GLUT4 are shown in Table 2. Normal rabbit IgG antibody was used as the negative control, which indicates non-specific binding. Other antibodies were determined to be 'no binding' if their level was equal to or less than IgG (ratio to input). All antibodies were

from Upstate, except C/EBP β (Santa Cruz Biotechnology, Santa Cruz, CA).

Transcriptional activity

To measure the transcriptional activity from the GLUT4 gene, the method of Sandoval et al. {Sandoval *et al.* 2004} was used, which relies on ChIP analysis to examine RNA polymerase II (Pol II) binding at the coding region distal to the promoter. For this purpose, primers were designed within the protein coding region of the GLUT4 gene (Table 4.2). qPCR analysis was then performed using a 7300 real-time PCR system (ABI) and the product was detected with SYBR Green. Serial dilutions of input chromatin were used to generate a standard curve and the results were expressed as the ratio to input DNA.

Statistical analysis

Results are calculated as mean \pm SEM. Comparisons of mRNA/protein expressions and histone modification between control and treated groups were performed by two-tailed t-test. All of the data in LP groups was expressed as the ratio to control. Significance testing was set at the $p < 0.05$ level.

4.4 Results

Birth and body weight of offspring

At birth, the control pups weighed 6.32 ± 0.19 g while the LP pups weighed slightly less, 5.92 ± 0.09 g. As the pups grew to 15 days, average body weight was significantly lower in the LP pups than in the control ($p=0.02$). Table 4.1 compares the average of body weight at birth and at day 7, 15, 22 and 35 of postnatal life, showing that the average weights of the LP offspring were reduced by 12% at day 15, 16% at day 22 and 13% at day 35 ($p<0.05$) as compared to the control pups. There were no differences in the daily food intake, and the body weight of the dams during pregnancy (not shown). Litter size and litter sex distribution did not differ between the control and LP mothers (not shown).

GLUT4 mRNA expression followed sex-dependent manner in offspring skeletal muscle under maternal low protein intake

GLUT4 mRNA expression has been reported to be decreased in female skeletal muscle by semi-restricted maternal food intake {Raychaudhuri *et al.* 2008}. However, whether regulation of GLUT4 gene expression by maternal protein restriction is sex-dependent in skeletal muscle has not yet been reported. We investigated GLUT4 mRNA levels in 38-day-old female and male offspring skeletal muscle and adipose tissue by qPCR (Figure 4.1A). In female pups, a 1.59-fold increase ($p=0.001$) in GLUT4 mRNA content in skeletal muscle was observed in pups from litters of mothers with a restricted protein diet when compared to those of control mothers. However, in male offspring skeletal muscle, there was no change of GLUT4 mRNA level related to maternal low protein. Also, in female offspring adipose tissue, a 1.45-fold increase ($p=0.03$) of GLUT4 mRNA was detected by maternal protein restriction, but it did not alter in male offspring. Since the amount of visceral adipose in a 38-day-old rat is not enough to do all of the assays involved in this study, we focused on skeletal muscle to examine the molecular mechanisms of GLUT4 regulation by maternal protein restriction.

The affect of maternal low protein on the transcription rate of GLUT4

To confirm the real transcriptional activity for the GLUT4 gene, ChIP DNA samples were used to monitor RNA polymerase II binding within the coding region. This analysis showed that the transcription rate was increased approximately 2.89-fold in female pup muscle as the result of maternal low protein diet (Figure 4.1B). Therefore, the increased transcription appears to account for most of the induction in GLUT4 mRNA following maternal low protein diet.

Maternal low protein alters GLUT4 protein level in female offspring

To detect GLUT4 protein content in pup skeletal muscle, protein samples were subjected to Western blot analysis using an anti-GLUT4 antibody. In all muscle samples, GLUT4 was detected as one major band with a molecular mass of about 45 kDa (Figure 4.2). The ratio of GLUT4 protein level between female control and LP groups was determined to be 1:1.78 (Figure 4.2A; $p=0.008$). A significantly higher GLUT4 protein content in the skeletal muscle of female LP pups was consistent with the trend observed in its mRNA expression. Moreover, GLUT4 protein levels in male pup muscle were unchanged, which

also corresponds to the unchanged male offspring mRNA expression levels (Figure 4.2B).

Glycogen synthase expression in rat offspring muscle

Glycogen synthase is an enzyme that incorporates glucose molecules into the glycogen particle. Recent studies suggested that glucose transport is the rate-limiting step for muscle glycogen synthesis {Price *et al.* 1996}. Therefore, the expression level of glycogen synthase was investigated in the offspring of maternal LP and control diets. Glycogen synthase levels were enhanced in the muscles of female offspring in the LP group, with mRNA and protein levels increase by 1.29- and 7.56-fold, respectively (Figure 4.3A and 4.3B). In male pups, no significant variation was observed. Since glucose-6-phosphate is the pre-substrate used for glycogen synthesis and is converted from glucose by the enzyme hexokinase, the hexokinase expression level was checked as well (not shown). However, there was no difference in hexokinase expression between LP and control groups in female offspring.

Glycogen content increased in female offspring muscle by maternal protein restriction

Skeletal muscle is the main tissue for glycogen storage, and contains 2 to 4 times more glycogen than the liver. The amount of glycogen was tested using a Glycogen Assay Kit. The results show that the glycogen content in female offspring skeletal muscle was increased by 35% (Figure 4.3C; $p=0.018$) in the maternal LP group. Thus, the potential physiological impact was demonstrated as corresponding to the altered GLUT4 expression in the offspring muscle.

No interaction of C/EBP β protein at the GLUT4 gene promoter

According to the data that we previously published, amino acid response (AAR) pathway was not activated in female offspring muscle by this maternal protein-restricted animal model {Zheng *et al.* 2011}. But as a cofactor for ATF3 and ATF4 in the AAR pathway, we did observe significantly increased expression of C/EBP β mRNA in female offspring muscle from the maternal low protein group. To identify whether or not the increased C/EBP β expression could contribute to the altered GLUT4 transcription in female

offspring muscle, because C/EBPb also acts as a mediator in regulating carbohydrate metabolism in skeletal muscle independent of the AAR pathway, ChIP assay was performed using rabbit polyclonal antibody against C/EBPb (see Table 4.3 for antibody information). In Figure 4.4, the ATF3 gene was used as a positive control as it has been known to be regulated through direct binding by C/EBPb. The binding of C/EBPb at the GLUT4 promoter was found to not be present (<IgG of 0.02 ratio to input), suggesting that C/EBPb does not bind to the GLUT4 promoter in our case.

Transcriptional enhancer - MEF2A was increased in female offspring muscle

To further investigate the potential mechanism that contributed to the up-regulated GLUT4 expression, myocyte enhancer factor 2A (MEF2A), which is known as a transcriptional enhancer to carry out specific binding to the GLUT4 MEF2 binding sequence {Moreno *et al.* 2003}, was tested by western blot. Figure 4.5 shows that MEF2 protein content was also increased 1.7-fold in female offspring of protein-restricted mothers. Since the binding of MEF2 is required for normal GLUT4 expression, the increased MEF2 protein could contribute to the up-regulated GLUT4 expression. We also tried to test the exact binding of MEF2 at GLUT4 promoter by using CHIP assay. There was, however, no commercial working antibody for us.

Histone modifications on GLUT4 promoter in rat offspring muscle

ChIP assays were performed to investigate the *in vivo* Pol II binding status at the GLUT4 promoter. Pol II binding at the GLUT4 promoter region in female offspring skeletal muscle with maternal low protein was increased 1.46-fold compared to control (Figure 4.6A; $p=0.04$). Data were consistent with the increased GLUT4 mRNA expression. Since Pol II binding status at a promoter is often regulated by chromatin structure, methylated-histone or acetylated-histone antibodies were used in the ChIP assay at the GLUT4 promoter from maternal low protein and control diet offspring.

ChIP assays were performed with antibodies specific for histone modifications associated with either enhanced gene transcription [acetylated histone H3 (H3Ac), acetylated histone H4 (H4Ac), and di-methylated histone H3 at lysine 4 residues (H3K4Me2)] or

transcriptional repression through diminished methylation [tri-methylated histone H3 at lysine 9 residues (H3K9Me3)]. Increases in both acetylated histone H3 and acetylated histone H4 were observed in the muscle of female pup from dams fed a LP diet versus the control, by 2.11- and 1.81-fold ($p < 0.05$), respectively (Figure 4.6A). Methylation of histone H3 lysine 4 was also increased with maternal low protein (1.66-fold; $p < 0.05$), but no difference was detected at lysine 9 of histone H3. In male offspring, histone code modifications did not differ at the GLUT4 promoter region (Figure 4.6B). Comparison of histone modifications between female and male offspring from control mothers also revealed that the basal acetylations of histone H3 and H4 were lower in males, while the methylation levels of histone H3 were similar between sex.

4.5 Discussion

Skeletal muscle is the most important tissue responsible for whole body glucose uptake in humans {Shulman *et al.* 1990} and rodents {Kraegen *et al.* 1985}. Insulin resistance is associated with decreased glucose uptake in insulin-sensitive tissues, i.e., skeletal muscle {Bell *et al.* 2001}. In the present study, we investigated GLUT4 gene expression and modifications of histones within the promoter in female and male pups with maternal protein restriction. This study demonstrates for the first time that GLUT4 gene expression is significantly higher in the skeletal muscle of female offspring subjected to in utero maternal protein restriction. We also showed that there are sex-dependent modifications to the GLUT4 promoter region and that this epigenetic change significantly increases the expression of GLUT4 mRNA only in female pups with maternal low protein. This new observation suggests that skeletal muscle in female pups might have greater capability to make GLUT4 transporter when more glucose transporters are needed, reflecting an adaptation to protein restriction first, and only, encountered during gestation. It is interesting to think that an estrogen-involving signaling pathway may influence this response in a sex-specific manner. Estrogen has been reported to increase expression of plasma membrane GLUT4 and to boost carbohydrate metabolism {Barros *et al.* 2006}. Considering the critical role environment plays in fetus development, the observed sex difference in gene expression in offspring may also be due to gender-specific perturbation

of placental function, an idea supported by the reported alteration in gene expression patterns, including increased GLUT4 expression, that occurred in the placentas of male fetuses upon early pregnancy stress {Mueller *et al.* 2008}.

In this study, we investigated the potential involvement of a cofactor, C/EBP β protein, in amino acid response pathway on altering GLUT4 expression as a consequence of maternal LP diet. C/EBP proteins are also involved in a wide range of cellular processes, such as cellular proliferation, carbohydrate metabolism, adipocyte differentiation and inflammation {Ramji *et al.* 2002}. In our case, however, we previously found no change in ATF gene expression upon maternal protein restriction in offspring muscle. We did observe a significant increase of C/EBP β in LP female offspring muscle. However, ChIP assays revealed that the C/EBP β protein does not interact with the GLUT4 promoter. The xChIP assay we performed is designed to cross-link all the interacting factors, including those that are not directly bound with the DNA sequence. Therefore, the mechanism regulating the GLUT4 gene may not involve C/EBP β as a transcription factor.

It was established through transgenic investigations that conserved GLUT4 promoter regions play a crucial role in its expression in skeletal muscle. Myocyte enhancer factor 2 (MEF2) is a required transcription factor for normal GLUT4 expression. In skeleton muscle, MEF2A has the ability to specifically bind to the GLUT4 MEF2 binding sequence to activate GLUT4 transcription in conjunction with the binding of GLUT4 enhancer factor to domain 1, located upstream of the MEF2 binding site {Knight *et al.* 2003}. Disruption of the MEF2-binding site turned off tissue-specific GLUT4 expression {Thai *et al.* 1998}. In our rat model, we observed the total MEF2A protein level increased in LP offspring muscle. In another rat study, Holmes B. F. et al. also observed roughly two-fold increase in total MEF2 protein upon stimulation of AMP-activated protein kinase in muscle {Holmes *et al.* 2005}. Correspondingly, nuclear MEF2A protein increased five-fold and MEF2 binding to the GLUT4 consensus sequence increased two-fold. In a transgenic mice model, the protein levels of MEF2, determined by western blot using a MEF2A antibody were decreased in the diabetic state and recovered following insulin treatment {Thai *et al.* 1998}. The binding activities of MEF2 to GLUT4

reflect its protein level changes. Thus, increase of MEF2A total protein level we observed might suggest that MEF2A is an important transcription factor that activates GLUT4 in LP pup's skeleton muscle in response to maternal protein restriction.

The myogenic differentiation factor D (MyoD) was reported to bind MEF2 and thyroid hormone receptor $\alpha 1$ (TR $\alpha 1$), synergistically driving gene expression for myogenesis, which would include skeletal muscle GLUT4 transcription {Santalucia *et al.* 2001}. There is also a MyoD binding site in the GLUT4 promoter region, proximal to the MEF2-binding site. However, we only observed marginally enhanced binding of MyoD protein to the GLUT4 promoter region in LP offspring with results close to significant (not shown).

Sex-specific histone modifications at the GLUT4 promoter, as observed in our study, may prove to be the key changes in nuclear protein-GLUT4 promoter interaction. Such modifications include increased levels of acetylated histone H3, acetylated histone H4 and di-methylated histone H3 at lysine 4 in female offspring rats following a maternal LP diet. Most importantly, the histone status perturbations may be memorized in the fetus and remain in the adult female offspring. The trans-generation transmitted insulin resistant phenotype of the IUGR adult female offspring has suggested similar epigenetic alternations on histones. In contrast to our study, diminished skeletal muscle GLUT4 mRNA in IUGR female rat offspring was related to H3K14 de-acetylation and di-methylation of H3K9. It is worth to note that histone hypermethylation has both stimulatory and inhibitory effects on gene transcriptions, dependent on methylation sites on different lysines in histone H3. H3K4 methylation will generally induce gene transcription whereas H3K9 methylation will silence gene transcription. Nevertheless, both studies showed that histone modification caused by maternal diet could alter offspring GLUT4 expression and potentially disturb carbohydrate metabolism in skeleton muscle. However, the epigenetic consequences could differ substantially based on diet constitutions and diet quantities. On the other hand, the basal acetylations of histone H3 and H4 were low in male offspring of control mothers with comparison to female offspring. This might suggest that, within GLUT4 gene promoter, females have relatively

more opened chromatin structure than males to recruit other transcription factors.

The rate limiting reaction for the formation of muscle glycogen has been generally considered to be the transfer of glucose from UDP-glucose to an amylose chain, a reaction mediated by glycogen synthase {Ren *et al.* 1993}. However, it has been suggested that glucose transport rather than glycogen synthase activity is rate limiting for muscle glycogen synthesis. For example, total GLUT4 protein content in muscle has been investigated in numerous studies to understand the long-term metabolic and functional consequences on intracellular substrate utilization, glycogen synthesis and glycogen content for example. In transgenic mice, over expression of GLUT4 resulted in an increase in muscle glycogen concentration 20% above the level of the non-transgenic mice {Hansen *et al.* 1995}. Glycogen synthesis rate was 50% faster in the muscle of transgenic mice that over expressing GLUT4 than control mice. In addition, up-regulation of GLUT4 in fast-twitch muscle resulted in a 2.5-fold increase in glycogen storage in muscle {Tsao *et al.* 1996}. In our data, total expression of glycogen synthase and glycogen content were both up-regulated in female offspring by a low protein maternal diet. This up-regulation in glycogen storage may be closely associated with the up-regulation of total GLUT4 content. According to our previous study, gestational protein restriction did not cause any difference on either dams' or pups' glucose or insulin level (data unpublished). In this study, the low protein diet was used for gestation period only and all of dams were put onto a standard rat chew throughout the lactation. Thus, we studied total GLUT4 content and glycogen storage, which may reflect a sex-dependent adaptation in offspring to gestational protein restriction on long-term metabolic and functional consequences.

Overall, maternal low protein intake (but not calorie restriction) up-regulates GLUT4 gene expression through chromatin modification programming specifically in female offspring skeletal muscle and induces an increase of glycogen synthase and glycogen content. Expressions of regulatory genes associated with carbohydrate metabolism, C/EBP β and MEF2A, increased in LP female pup skeletal muscle. These results strongly indicate sex-dependent adaptation of glucose metabolism to a low maternal protein diet.

4.6 Tables & Figures

Table 4.1 Offspring body weight

Age (days)	Body weight (g)		
	C (4)	LP (4)	<i>p</i>
Birth (day 0)	6.32 ± 0.19	5.92 ± 0.09	0.11
7	16.55 ± 0.69	14.89 ± 0.46	0.10
15	35.06 ± 1.14	30.73 ± 0.70	0.02
22	59.37 ± 1.40	49.98 ± 1.63	0.006
35	138.56 ± 2.58	125.21 ± 3.54	0.006

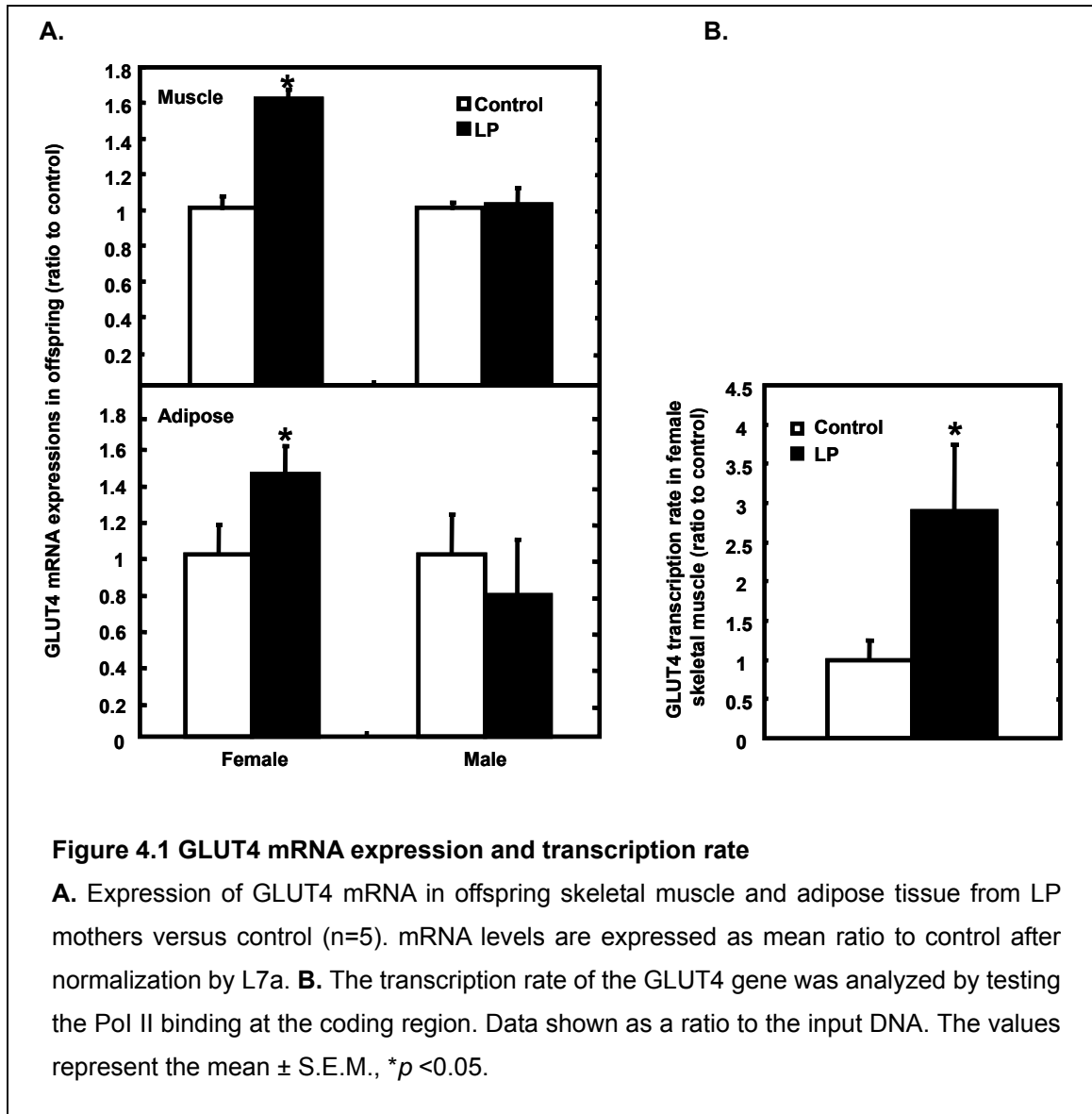
Number of litters shown in parentheses. Body weight was shown as the average value at the specific day. Data are means with ± S.E.M. Significance testing was set at $p < 0.05$ derived from two-tail t-test.

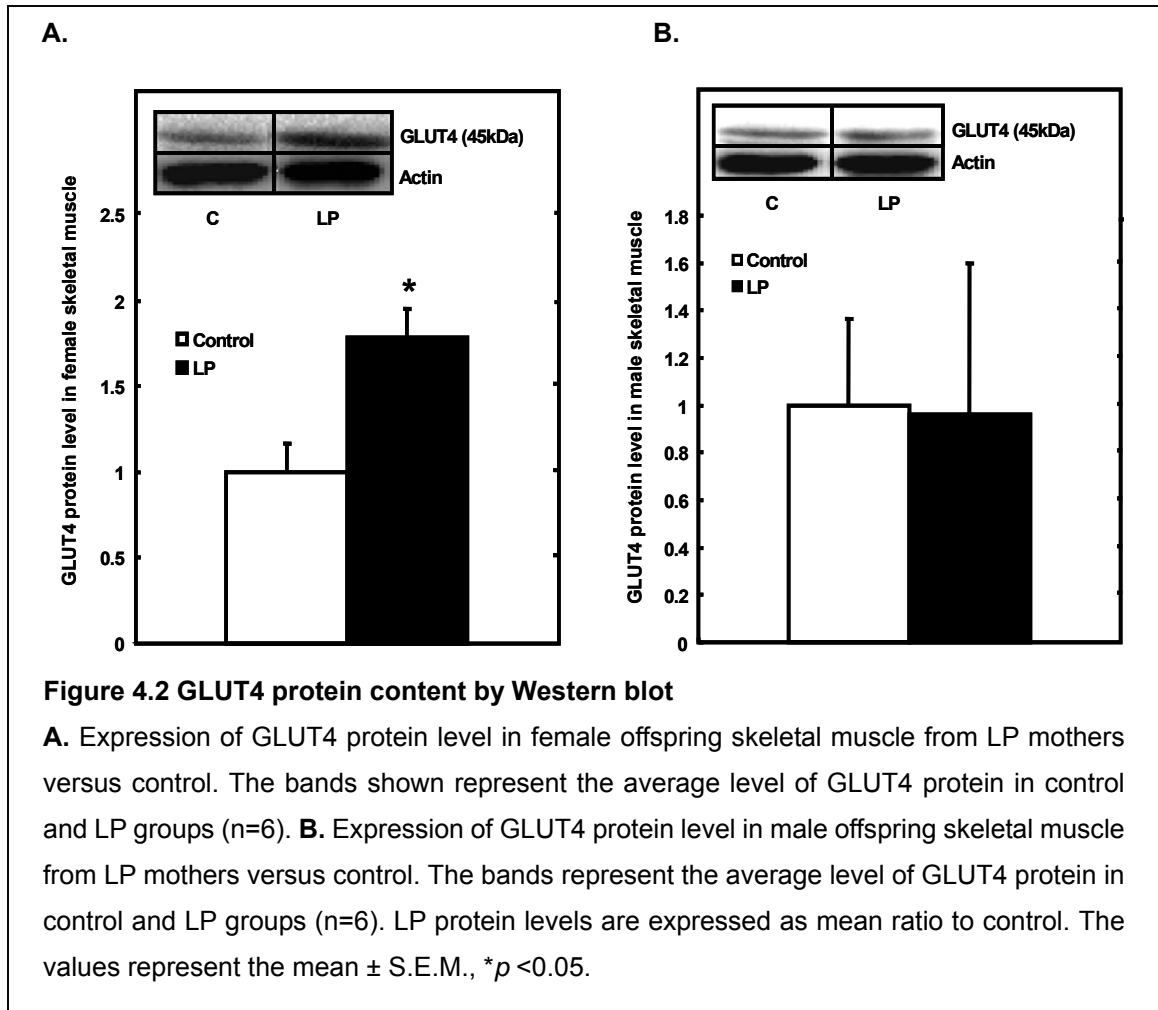
Table 4.2 Primer sequence used in qPCR and ChIP assay

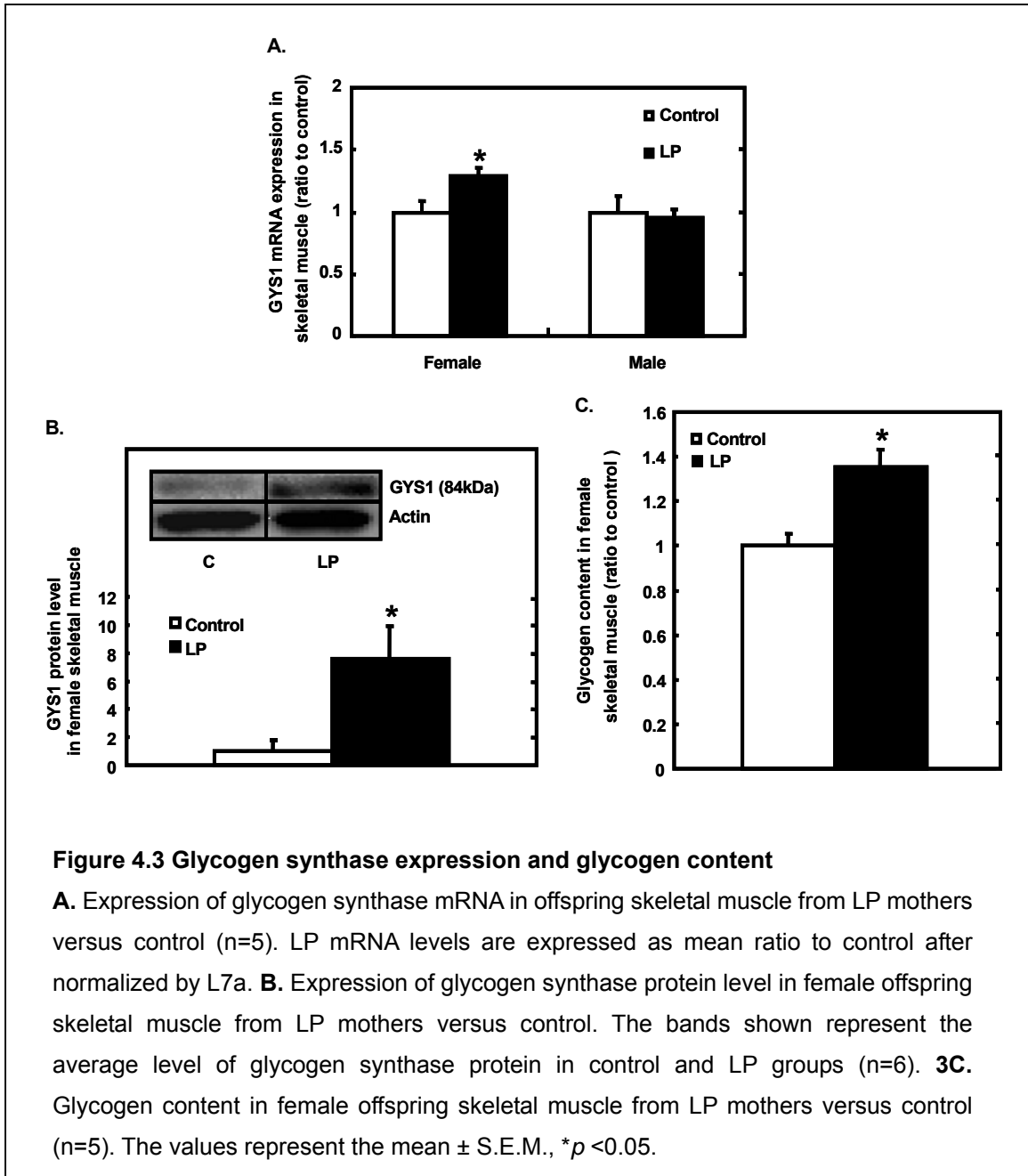
Gene (Ensembl ID)	Forward primer (5' → 3')	Purpose
	Reversed primer (5' → 3')	
GLUT4 (ENSRNOG00000017226)	CCATAGGAGCTGGTGTGGTCAATAC TCGCCAGCTCGCTCTACTAAG	mRNA expression
ATF3 (ENSRNOG00000003745)	GGTGTCTGCACTGTGGGATGTTAAC GCTGAAATACTCTGGACCGCATCTC	
ATF4 (ENSRNOG00000017801)	GTTCCCCAGGGTTTCTGTCTTC CAACCTCACTTCCCAGCTCTAAAC	Gene promoter
C/EBPβ (ENSRNOG00000025114)	AGAACGAGCGGCTGCAGAAGA GAACAAGTTCCGCAGCGTGC	
L7a (ENSRNOG00000005071)	GAGGCCAAAAAGGTGGTCAATCC CCTGCCCAATGCCGAAGTTCT	Gene promoter
GYS1 (ENSRNOG00000020812)	TTGGGACACCTGCAACATC ACAGGACGGCATCATTGG	
ATF3 (ENSRNOG00000003745)	GGCCAGTTCTCCCTGGAAGCTAT CCCGCTTAACCTGGTTACCAAT	Gene promoter
GLUT4 (ENSRNOG00000017226)	CTTCAGCTCTCCGCATCTTTCC CACTGCCACAACCCAAGGAAC	
GLUT4 (ENSRNOG00000017226)	CTTCAGCTCTCCGCATCTTTCC CACTGCCACAACCCAAGGAAC	histone modification
GLUT4 (ENSRNOG00000017226)	GGGTGCCTTGGGAACACTCAAC CAACACCTGGGCAACCAGAATG	transcription rate

Table 4.3 Antibodies used in Western blot and CHIP assay

Antibody	Introduction & company	Purpose
GLUT4	Cat # 2299, Cell Signaling Technology, Beverly, MA	Protein analysis by Western
Glycogen synthase	Cat # 3893, Cell Signaling Technology, Beverly, MA	
MEF2	Cat # sc-313, Santa Cruz Biotechnology, Santa Cruz, CA	
C/EBP β	sc-150, Santa Cruz Biotechnology, Santa Cruz, CA	C/EBP β binding at GLUT4 gene promoter
H3Ac	Anti-acetylated histone 3 at lysine 9, 14 residues; 06-599, Upstate, Temecula, CA	Histone modifications analysis
H4Ac	Anti-acetylated histone 3 at lysine 5, 8, 12 and 16 residues; 06-866, Upstate, Temecula, CA	
H3K4Me2	Anti-di-methylated histone 3 at lysine 4 residues; 07-030, Upstate, Temecula, CA	
H3K9Me3	Anti-tri-methylated histone 3 at lysine 9 residues; cs200604, Upstate, Temecula, CA	
IgG	sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA	
Pol II	sc-899, Santa Cruz Biotechnology, Santa Cruz, CA	Pol II analysis







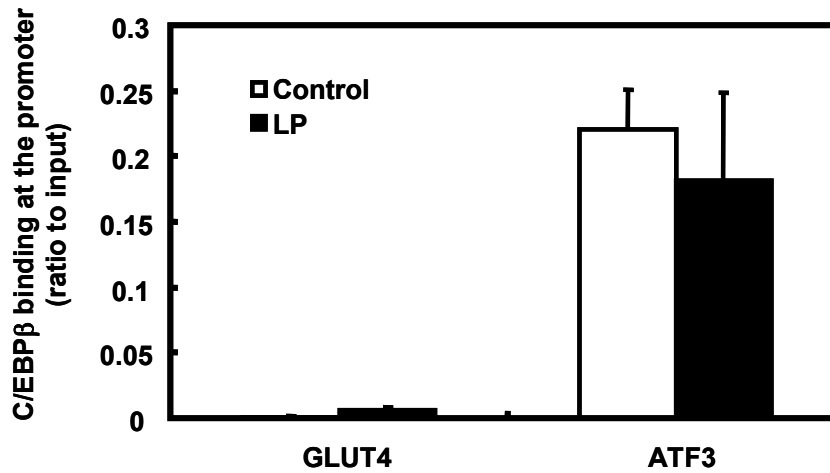


Figure 4.4 Interaction of C/EBP β protein at the GLUT4 gene promoter

Binding of C/EBP β at the GLUT4 promoter in female offspring muscle. Data is shown as a ratio to the input DNA. The ATF3 gene was used as a positive control. All the values represent the mean \pm S.E.M.

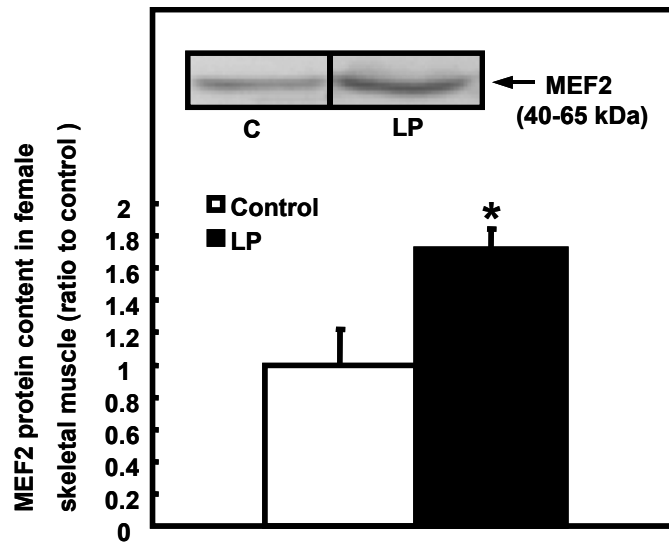


Figure 4.5 MEF2 protein content by Western blot

Expression of MEF2 protein level in female offspring skeletal muscle from LP mothers versus control. The bands shown represent the average level of MEF2 protein in control and LP groups (n=6). LP protein levels are expressed as mean ratio to control. The values represent the mean \pm S.E.M., * $p < 0.05$.

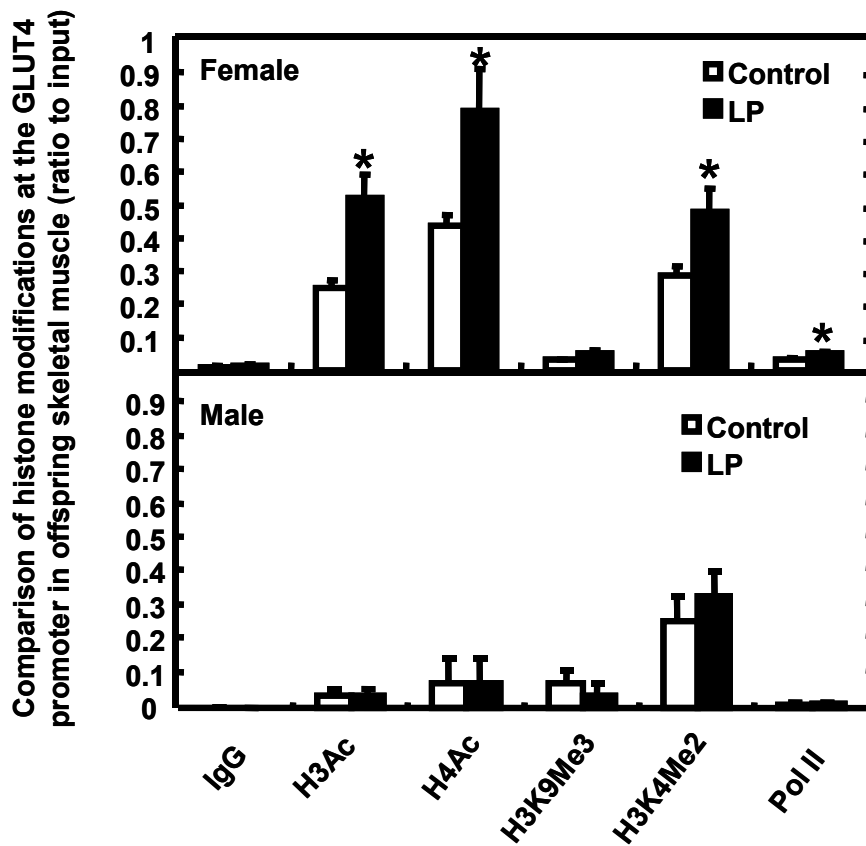


Figure 4.6 Chromatin modifications within GLUT4 gene promoter

ChIP assay demonstrating histone modifications and binding of Pol II at GLUT4 promoter region in offspring skeletal muscle. Data is shown as a ratio to the input DNA. Data is plotted as the increase relative to control. NC was negative control by testing the up-stream 5kb region of GLUT4 genome. H4Ac: acetylated histone H4; H3Ac: acetylated histone H3; H3K4Me2: di-methylated histone H3 at lysine 4; H3K9Me3: tri-methylated histone H3 at lysine 9; Pol II: RNA polymerase II. All the values represent the mean \pm S.E.M., * $p < 0.05$ (n=5).

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Chapter 5. Histone Modifications, not DNA Methylation, Cause Transcriptional Repression of p16 (CDKN2A) in the Mammary Glands of Offspring of Protein-Restricted Rats

5.1 Abstract

Maternal nutrition during pregnancy is an important intrauterine factor that results in persistent alteration of the offspring epigenome and that associates with health outcome in later life. This study examined the effect of maternal low-protein diet on the regulation of p16 (CDKN2A) gene expression in the mammary gland of offspring rats. Timed-pregnant Sprague Dawley rats were fed during gestation one of two isocaloric diets, control (18% casein) or low-protein (LP, 9% casein). The expression of p16 (CDKN2A) mRNA in mammary gland of the LP offspring was decreased 75% versus control. We also detected declined p16 (CDKN2A) protein content in the mammary glands of pups gestated under the low-protein diet. Analysis of transcriptional and epigenetic regulation in offspring rats with maternal low-protein diet revealed the regulatory mechanisms underlying decreased p16 (CDKN2A) expression. Chromatin immunoprecipitation (ChIP) assay demonstrated that the altered p16 (CDKN2A) mRNA level and transcription rate in LP offspring resulted from histone code changes, including the reduced acetylation of histone H4 and the di-methylation of histone H3 at lysine 4 residues within the p16 (CDKN2A) promoter region. These results supported the hypothesis that maternal protein restriction during pregnancy programs p16 (CDKN2A) expression through histone code alterations in offspring mammary gland.

5.2 Introduction

Recently documented developmental origins of adult human diseases have emphasized the effects of maternal nutrition during fetal development on long-term health outcome in offspring. Epigenetic regulations mediated by macro- or micro-nutrients are increasingly recognized as prenatal mechanisms that program offspring susceptibility to disease development {Chavatte-Palmer *et al.* 2008}. Offspring gene expression alterations, stemming from changes in either DNA methylation or histone modifications, have been

reported in the rat liver, kidney, skeletal muscle and adrenal gland following a restricted diet during gestation {Lillicrop *et al.* 2005; Raychaudhuri *et al.* 2008; Burdge *et al.* 2007}. These epigenetic events have been shown to affect tumor suppressor loci in mammary gland cancer cells, including p16 (CDKN2A), E-cadherin and BRCA1, {Foster *et al.* 1998; Hiraguri *et al.* 1998; Dobrovic *et al.* 1997}. However, it is not known how these tumor suppressive genes are regulated by epigenetic changes in the mammary glands in vivo under maternal low-protein diet.

The prenatal period is critical in the development of the mammary gland. Findings with experimental rodent models revealed that exposures to dietary factors during the in utero and pubertal periods, when the mammary gland is undergoing extensive modeling and re-modeling, alter susceptibility to mammary tumor development {Hilakivi-Clarke *et al.* 2007}. p16 (CDKN2A), a cyclin-dependent kinase inhibitor encoded by CDKN2A (INK4a/ARF), binds to cyclin/cdk4 or cyclin/cdk6 kinase complexes, thereby blocking their kinase activity and inhibiting cell-cycle progression at the G1/S boundary {Voorhoeve *et al.* 2004; Voorhoeve *et al.* 2003; Tsellou *et al.* 2008}. Although recent studies demonstrated that regulation of p16 (CDKN2A) and cyclin D1 is critical for growth arrest during mammary involution {Gadd *et al.* 2001}, the role of p16 (CDKN2A) in mammary gland growth before puberty is poorly understood. p16 (CDKN2A) and p53 are two examples of genes that are frequently inactivated in many tumor types, including mammary carcinomas {Rocco *et al.* 2001}. The inactivation of p16 (CDKN2A) and p53 occurs through a variety of mechanisms, including point mutation, deletion and epigenetic silencing {Zhang *et al.* 2006}. Recently, the Ozanne group {Fernandez-Twinn *et al.* 2007} reported that compensatory mammary growth in the offspring following protein restriction during both pregnancy and lactation increased the number of early-mammary tumors. The increased incidence of mammary tumors was accompanied by elevated expression of receptors to insulin, IGF-1, epidermal growth factor and estrogen. This report became the first study on the effect of maternal low-protein on mammary cancer development and provided an extremely relevant model for further study of such processes and, ultimately, the development of potential interventions.

In the present study, we employed a well-established model {Lillycrop *et al.* 2005; Fernandez-Twinn *et al.* 2007} to measure the effect of maternal protein restriction during pregnancy on the promoter chromatin status and expression of p16 (CDKN2A) in the mammary gland of the offspring after weaning. Our present study took advantage of the clinical features of the rat maternal low-protein model and addressed the mechanistic question by uncovering changes in epigenetic regulation related to p16 (CDKN2A). The results obtained from our analysis support that maternal low-protein programs p16 (CDKN2A) gene expression in offspring mammary glands through histone modifications and transcriptional repression.

5.3 Material and Methods

Animal and diet

Timed-pregnant Sprague Dawley rats (Charles River Laboratories) were obtained at day 2 of gestation and fed one of two isocaloric diets, control (180 g/kg casein) or low-protein (LP, 90 g/kg casein) as described by Lillycrop, et al. {Lillycrop *et al.* 2005} throughout gestation. Both groups had ad lib access to food and drinking water. Animals were individually housed in standard polycarbonate cages and maintained in a humidity- and temperature-controlled room on a 12-hour light-dark cycle. Pups were weighed on a scale within 12 hours of birth and returned to their mother. Twenty-four hours after birth, the litters were limited to 6 pups to minimize variation in pups' nutritional status during suckling. Dams were all fed a standard diet through lactation {Lillycrop *et al.* 2005}. At day 24, in both experimental groups, the pups were weaned from the dams and maintained with the same standard diet. When the pups were 38 days old, female pups were sacrificed, and the fourth abdominal mammary gland was collected, snap-frozen in liquid nitrogen, and stored at -70°C.

RNA isolation and one-step real time quantitative PCR (qPCR)

Frozen mammary gland samples were ground with a mortar and pestle in liquid nitrogen before total RNA was isolated with TRI reagent (Sigma, St. Louis, MO). For quantification, 25ng template RNA per well was mixed with iTag SYBR Green Supermix

with ROX (Bio-Rad, Hercules, CA), which is a dye that can bind to dsDNA generated during amplification, in a 96-well plate on a 7300 Real-Time PCR System (Applied Biosystem, Foster City, CA). The samples were first incubated at 48°C for 30 min for reverse transcription to take place. To determine the exponential phase of amplification, kinetic analysis was conducted for the different primers. Total cDNA was denatured and hot start RNA polymerase was activated at 95°C for 10min, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. To ensure that a unique product was amplified in the reaction, dissociation curves were generated after PCR by stepwise increase of the temperature from 55°C to 95°C. Primers used for PCR are shown in Table 5.2.

Protein isolation and Western blotting

25 mg of frozen mammary gland sample was ground in liquid nitrogen and put into 500 µl of protein sample buffer (0.125 M Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 1% SDS, 20% glycerol, 0.4% bromphenol blue, protease inhibitor). Protein was sonicated (Fisher Scientific model 100 Sonic Dismembrator, Pittsburgh, PA) on ice with 25 pulses at power setting 2. Lowry assay was used to determine protein content and samples containing 20 µg of protein were resolved by SDS-PAGE. After electrotransfer to a polyvinylidene fluoride membrane (0.2 µm, Bio-Rad) using a wet transfer protocol, incubation in 5% milk in TBS/T (30 mM Tris base pH 7.6, 200 mM NaCl and 0.1% Tween 20) was performed for 1 hr at room temperature to block the membrane. The membrane was then incubated with mouse monoclonal antibody against p16 (CDKN2A) (sc-81156, Santa Cruz Biotechnology, Santa Cruz, CA) in 5% BSA at a 1:200 dilution at 4°C overnight. The next day, the membrane was washed five times for 5 min with TBS/T on a shaker and then incubated with peroxidase-conjugated goat anti-mouse secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in 1% milk at a 1:5,000 dilution for 1 hr at room temperature. The membrane was then washed 5 times for 5 min. The bound secondary antibody was detected using a SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL) and western blot images were captured and analyzed by a Chemi Doc system (Bio-Rad).

Methylation sensitive PCR

Methylation analysis was performed using a modified Methylation Sensitive PCR (MSP) protocol, based on digesting DNA with methylation sensitive enzymes and subsequently performing qPCR to detect DNA methylation. Following cleavage with methylation-specific endonucleases, promoter-specific PCR primers amplify intact genomic DNA, while genomic DNA that had been cleaved by one of the two restriction endonucleases remained unamplified. Briefly, DNA was isolated from the mammary gland samples and quantified using a Spectrophotometer (Bio-Rad, SmartSpec Plus). DNA was digested with either *AciI* or *HhaI* restriction enzymes, which are unable to cut at methylated sites (New England BioLabs, Ipswich, MA), using buffer 3 and 4, respectively. Both “cut” and “uncut” samples were incubated in the appropriate buffer for 1 hr at 37°C and then incubated at 65°C to halt enzyme activity. Digestion longer than 1 hr has been employed without any deleterious consequence (data not shown). Following enzyme digestion, samples were analyzed by qPCR using a 7300 Real-Time PCR System (Applied Biosystem). Detection of amplified products was enabled by SYBR Green fluorescent dye. To ensure specificity, a no-template control was included. The PCR amplification was performed in a 96-well optical plate with a 20 µl reaction volume. PCR was performed at the following conditions: 95°C for 10 min, followed by 45 cycles at 95°C for 1 min; 60°C for 10 s. To ensure amplification of desired products, a dissociation curve was generated following the real-time reaction with a temperature range of 55°-95°C. The amount of methylation is shown as the % ratio of cut/uncut DNA normalized to control in each dietary treatment.

Chromatin immunoprecipitation (ChIP)

In order to determine whether a given protein binds to a specific DNA sequence *in vivo*, ChIP analysis was performed according to a modified protocol {Chen *et al.* 2004}. Briefly, 200 mg of frozen mammary gland was ground in liquid nitrogen, re-suspended in PBS and cross-linked in 1% formaldehyde for 10 min at room temperature. The tissue pellet was re-suspended in nuclei swelling buffer (5 mM Pipes [NaOH] pH 8.0, 85 mM KCl, 0.5 % NP40) containing protease inhibitor. The separated nuclei were lysed in SDS lysis buffer (50 M Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS) containing protease inhibitors. The resultant chromatin was sonicated (Fisher Scientific, model 100 Sonic

Dismembrator, Pittsburgh, PA) on ice with 10 bursts for 40 s at power setting 5, with 2 min cooling interval between each burst. The average length of sonicated chromatin was determined to be around 500 bp by resolving on a 1.6% agarose gel. The sample was then centrifuged at 13 000 rpm for 10 min at 4°C to remove cell debris from the crude chromatin lysate. Sheared chromatin (1 ml) was diluted in ChIP dilution buffer (total 10 ml). Ten percent of the diluted lysate was subsequently incubated overnight at 4°C on a hematology mixer (Model 346, Fisher Scientific) with 2 µg of primary antibodies of interest. Pre-blocked salmon sperm DNA/protein A agarose beads (60 µl, 50% slurry; Upstate Biotechnology, Lake Placid, NY) were then added to the chromatin for 2 hr, followed by centrifugation at 2 000 rpm for 1 min at 4°C. Supernatant of normal rabbit IgG was saved as the input control for PCR after clean up. The pellets containing immunoprecipitated complexes were washed sequentially with 1 ml of low salt solution (0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM pH 8.0 Tris-HCl, 150 mM NaCl), high salt solution (0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM pH 8.0 Tris-HCl, 500 mM NaCl), and LiCl solution (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM pH 8.0 Tris-HCl) and twice with TE (pH 8.0). Antibody/protein/DNA complexes were eluted from Protein A agarose beads by adding 250 µl of the elution buffer (50 mM NaHCO₃ and 1% SDS) followed by shaking at 37°C at 300 rpm for 15 min and a flash-spin down at room temperature. The combined supernatants were incubated at 65°C for 4 – 5 hr after addition of 20 µl 5 M NaCl and 1 µg of RNase A, to reverse the formaldehyde cross-linking and release the DNA fragments. Samples were then treated with proteinase K at 37°C for 1h to remove protein. DNA was purified with a Wizard SV Gel and PCR Clean up System (Promega, Madison, WI). 5% of immunoprecipitated DNA was used for each real time PCR reaction. The standards and the samples were simultaneously amplified using the same reaction master mixture at the 25 µl scale. Primers were used to amplify genomic sequences at the promoter region of p16 (CDKN2A) gene (Table 5.2).

Transcriptional activity assay

To measure the transcriptional activity from the p16 (CDKN2A) gene, the method of Sandoval et al. {Sandoval *et al.* 2004} was used, which relies on ChIP analysis to

examine RNA polymerase II (Pol II) binding at a coding region distal to the promoter. For this purpose, primers were designed within the protein coding region of the p16 (CDKN2A) gene (Table 5.2).

Statistical analysis

Results are expressed as mean \pm SEM. Comparison of mRNA expression or histone modification between either control and treated groups were performed by t-test. Significance testing was set at $p < 0.05$ level (two-sided).

5.4 Results

Birth and body weight of offspring

On the 21 day of gestation, the control dams delivered all pups after noon while the LP dams delivered 43% of the pups before noon and 57% pups after noon (Table 5.1). At birth, the control pups weighed 6.32 ± 0.19 g while the LP pups weighed slightly less, 5.92 ± 0.09 g. As the pups grew to 15 days, average body weight was significantly lower in the LP pups than in the control (at a p value of 0.02). Table 1 compares the average of body weight at birth and at day 7, 15, 22 and 35 of postnatal life, showing that the average weights of the LP offspring were reduced by 12% at day 15, 16% at day 22 and 13% at day 35 ($p < 0.05$) as compared to the control pups. There were no differences in the daily food intake, body weight of the dams during pregnancy (data not shown), litter sizes or litter sex distribution (Table 5.1) between control and LP mothers.

Maternal low-protein diet reduced p16 mRNA level in offspring mammary gland

The effect of maternal low-protein diet on offspring gene expression was examined through real time qPCR. The p16 (CDKN2A) expression in mammary gland of the LP pups notably decreased by 75.8% at day 38 relative to the control pups (Figure 5.1B; $p < 0.001$). The p16 (CDKN2A) mRNA level didn't differ between the two groups of dams (Figure 1.5A). Because tumor protein 53 (p53) and p16 (CDKN2A) function following a compensatory mechanism in cell cycle arrest {Serrano *et al.* 1997}, the expression of p53 mRNA was examined. However, there was no significant change of p53 expression

between the LP and control pups (Figure 5.1C).

p16 protein content was decreased by maternal low-protein regime

To examine if the altered p16 (CDKN2A) mRNA produces a change in protein content in offspring mammary gland, we performed Western blot analysis using an anti-p16 (CDKN2A) antibody. p16 (CDKN2A) protein was reduced by maternal low-protein diet in female offspring mammary gland (Figure 5.2) by 71% ($p = 0.004$), normalized with internal control. Significantly lower p16 (CDKN2A) protein content in female LP pups' mammary gland was consistent with the trend observed in its mRNA expression.

Restricted maternal low-protein didn't change p16 gene methylation

DNA methylation has been reported as a mechanism to regulate p16 (CDKN2A) gene expression in mammary cells {Bean *et al.* 2007}. Figure 5.3A represents the positions of the p16 gene promoter and its CpG island on the intact genomic sequence. In this study, the p16 methylation status was assessed by methylation sensitive PCR (MSP), presented in Figure 5.3B and 5.3C. A methylation-specific endonuclease, AciI, was used to digest genomic DNA. The MSP primers we designed covered the whole CpG island as shown in Table 5.2. Figure 5.3B shows that a PCR product was amplified, indicating that the p16 promoter was methylated. However, CpG methylation levels were consistent between offspring of both control and low-protein dams. HhaI endonuclease, which has no cutting site within the p16 promoter, was used as a positive control (Figure 5.3C). The expression levels of the three DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3b, were analyzed utilizing qPCR (Figure 5.3D, 5.3E and 5.3F). The expression levels of the methyltransferases were not altered in LP offspring mammary glands. Therefore, the reduced p16 regulation in offsprings was DNA methylation-independent under the treatment of maternal protein restriction.

Programming chromatin structure at the p16 promoter through maternal diet

Changes in chromatin structure caused by chemical modifications of histone proteins, such as methylation and acetylation, regulate gene transcription by affecting the ability of eukaryotic RNA polymerase II (Pol II) to transcribe DNA in order to synthesize the

precursors of mRNA and most snRNA and microRNA {Roeder *et al.* 1969; Conaway *et al.* 1997}. ChIP assays were performed to investigate Pol II binding status at the p16 (CDKN2A) promoter. Recruitment of Pol II to the p16 (CDKN2A) promoter was decreased by 82.8% in LP pups compared to control (Figure 5.4A; $p=0.04$). Data was well associated with the declined p16 (CDKN2A) mRNA expression. Normal rabbit IgG antibody was used as negative control, indicating non-specific binding. Other antibodies were classified as “no bindin” if resulting value was equal or less than rabbit IgG value (≤ 0.003 ratio to input).

To further determine if the altered transcriptional level of p16 (CDKN2A) was regulated by the changes of chromatin structure at the p16 (CDKN2A) promoter, antibodies to either methylated histone or acetylated histone were employed in the ChIP assay. Histone acetylation is associated with increased gene transcription, and hence, with transcriptionally active chromatin domains. Although histone methylation is generally associated with transcriptional repression, methylation of different lysine or arginine residues of histone also results in transcriptional activation. For example, methylation of lysine residue 4 of histone H3 (H3K4) is an activator, while methylation of lysine 9 of histone H3 (H3K9) is a repressor of gene transcription {Bartova *et al.* 2008}. Figure 4B shows the histone modifications at the p16 (CDKN2A) promoter in the mammary gland of offspring rats. An 84.6% decrease in acetylated H4 ($p = 0.01$) and a 92.5% decrease of methylation at H3K4 ($p = 0.02$) were detected in the LP pups, correlating to the 75.8% transcript repression (Figure 5.1). No difference in histone H3 acetylation or methylation at lysine 9 residues was detected. These data suggest that the altered p16 (CDKN2A) gene transcription might be regulated by the changes in histone modification at its promoter region, specifically histone H4 acetylation as well as histone H3 methylation at lysine 4.

Maternal low-protein reduced the real transcription rate of p16 (CDKN2A)

To confirm the real transcriptional activity from the p16 (CDKN2A) gene, ChIP DNA samples were used to monitor RNA polymerase II translocation to a region distal to the promoter, as demonstrated by Sandoval *et al.* {Sandoval *et al.* 2004}. The measurement

of Pol II binding within the coding region of a gene reflects the transcriptional activity at that gene, which usually has a short half-life. For the p16 (CDKN2A) gene, it has been reported having a short half life {Shapiro *et al.* 1995} and by using this analysis the results showed that the transcription rate decreased by approximately 85% in LP pup mammary gland relative to control pups ($p = 0.02$; Figure 5.4C). Therefore, decreased transcription appears to account for most of the reduction in p16 (CDKN2A) mRNA following maternal low-protein regime.

5.5 Discussion

The nutritional environment at the fetal and neonatal stages has been suggested to be a critical factor in breast cancer susceptibility. Our data showed for the first time that maternal protein restriction during gestation affected not only the growth of the pups but also p16 (CDKN2A) gene expression through histone code modifications. These observations were consistent with a previous report {Fernandez-Twinn *et al.* 2007} showing that a low-protein maternal diet resulted in offspring with not only low birth weight and a slow growth rate during lactation, but also retarded mammary gland development at three weeks of age. A rapid compensatory mammary epithelial growth in offspring pups after weaning with normal diet was observed, and led us to hypothesize that maternal protein restriction resulted in the repression of some cell cycle regulators, which could contribute to cancer risk.

The protein p16 (CDKN2A) is known to negatively control cell cycle and to retain pRb in a hypo-phosphorylated form in order to inhibit cellular growth {Voorhoeve *et al.* 2004}. Down-regulated p16 (CDKN2A) expression has been related to aggressive cell growth, leading to many types of tumors in humans {Enders *et al.* 2003}. In the epithelial cells from female mice, the p16 (CDKN2A) expression level has been found to be differentially expressed in a DNA methylation-independent manner during normal mammary gland development {Tsellou *et al.* 2008}. The regulatory mechanism of p16 (CDKN2A) remains to be elucidated. As a comparison, in this study, the expression of p16 (CDKN2A) in the mammary gland of offspring with maternal protein restriction is

substantially reduced, to 24% of the control group. Potentially, down-regulation of p16 (CDKN2A) resulting from maternal low-protein diet may increase the offspring's breast cancer risk later in life.

Tumor protein 53 (p53, or also known as protein 53) is a transcription factor encoded by the human TP53 gene. p53 can promote cell-cycle arrest or apoptosis in response to a variety of cellular stresses, including DNA damage and hypoxia {Graeber *et al.* 1996}. p16 gene activity inversely modulates p53 status and function in primary human mammary epithelial cells. p53 and p16 (CDKN2A) are mutated at high frequency in many tumor types, implying that their action is central to limiting tumor development. In fact, oncogenic Ras activates p53 and p16 (CDKN2A) to provoke premature cell senescence. Constitutive activation of the MEK/MAPK cascade induces p53 and p16 (CDKN2A) simultaneously {Lin *et al.* 1998}. p53 and p16 (CDKN2A) can also act in a compensatory mechanism that suppresses Ras-induced transformation {Serrano *et al.* 1997}. Disruption of either p53 or p16 (CDKN2A) prevents cell cycle arrest and is sufficient for Ras-induced transformation. In our study, although p16 (CDKN2A) expression is significantly suppressed, there is no detectable alteration in the regulation of p53 between control and maternal low-protein groups. This implies the presence of a nutritional signaling pathway that regulates p16 (CDKN2A) and p53 differently in the maternal protein restriction scenario.

There is growing evidence that maternal nutritional status can alter the epigenetic state of the fetal genome through histone modifications {Burdge *et al.* 2007}. Histone modifications, such as acetylation or methylation of specific amino acid residues in the N-terminal tails, exhibit diversified effects on gene transcriptional regulation {Kouzarides *et al.* 2007}. Histone acetylation or methylation can alter the histone-DNA interactions and the affinity of histone binding to DNA, thereby affecting gene expression {Jaenisch *et al.* 2003}. Deacetylation at specific lysine residues in the histone tails is generally associated with transcriptional activity {Turner *et al.* 2000}. In contrast, methylation of lysine has been linked to either activation or repression, depending on which lysine residue is modified. Methylation of lysine 4 of histone H3 (H3K4) promotes

transcriptional activity {Zegerman *et al.* 2002, while di- and tri-methylation of lysine 9 on histone H3 (H3K9) has been shown to inhibit gene transcription {Nakayama *et al.* 2001}. However, the histone epigenetic response to environmental stimuli is much less studied. Our results indicated that, at the p16 (CDKN2A) promoter region, maternal low-protein diet resulted in deacetylation of histone H4, but not histone H3, and dimethylation of H3K4. A number of proteins have been identified that are recruited to histone modifications and bind via specific domains {Kouzarides *et al.* 2007}. The difference we observed between acetylation of histone H4 and histone H3 might be due to the varied binding proteins that recognize either the acetylated histone H4 or the acetylated histone H3 to tether different enzymatic activities onto chromatin. The communication is mechanically regulated by varieties of factors, which are much under investigated, although some proteins have been reported to potentially bind with modified histones, for example Rsc4 binding to acetylated histone H3 and Brd2 binding to acetylated histone H4. However, the related research is undergoing and the functions of the binding proteins haven't been clearly revealed {Kouzarides *et al.* 2007}. In addition to the changes of histone modification, RNA polymerase II recruitment to the promoter is significantly reduced. We also observed a slight increase of methylation of H3K9, although the result is not statistically significant.

In summary, maternal protein restriction causes a down-regulation of p16 (CDKN2A) transcription and protein levels in offspring mammary glands. It demonstrates that a histone code modification of p16 (CDKN2A) is an epigenetic outcome of maternal dietary low-protein intake. Although DNA methylation was also reported to regulate p16 (CDKN2A) gene expression in mammary cells of women with high risk of breast cancer incidence {Bean *et al.* 2007}, other literature of *in vivo* studies showed DNA methylation-independent regulation of p16 {Tsellou *et al.* 2008}. Similarly, we did not observe any changes to DNA methyltransferase (Dnmt1 or Dnmt3a) expression or CpG island methylation status (Figure 5.3) in our animal model, suggesting it is not a DNA methylation-dependent regulation. A further elucidation of the signaling pathway leading to histone modification of p16 (CDKN2A) and other tumor suppressor genes will contribute to the understanding of breast cancer prevention through maternal nutrition.

5.6 Tables & Figures

Table 5.1 Offspring Vital Results

A.	C (4) ¹	LP (4) ¹	
Pups delivered before noon (%) ¹	0	43	
Pups delivered after noon (%) ¹	100	57	
Litter size	11.5 ± 0.65	10.3 ± 0.86	
Litter sex distribution M:F	1.11	1.25	
B.	Body weight (g) ³		
Age (days)	C (4)	LP (4)	<i>p</i>
Birth (day 0)	6.32 ± 0.19	5.92 ± 0.09	0.11
7	16.55 ± 0.69	14.89 ± 0.46	0.10
15	35.06 ± 1.14	30.73 ± 0.70	0.02
22	59.37 ± 1.40	49.98 ± 1.63	0.006
35	138.56 ± 2.58	125.21 ± 3.54	0.006

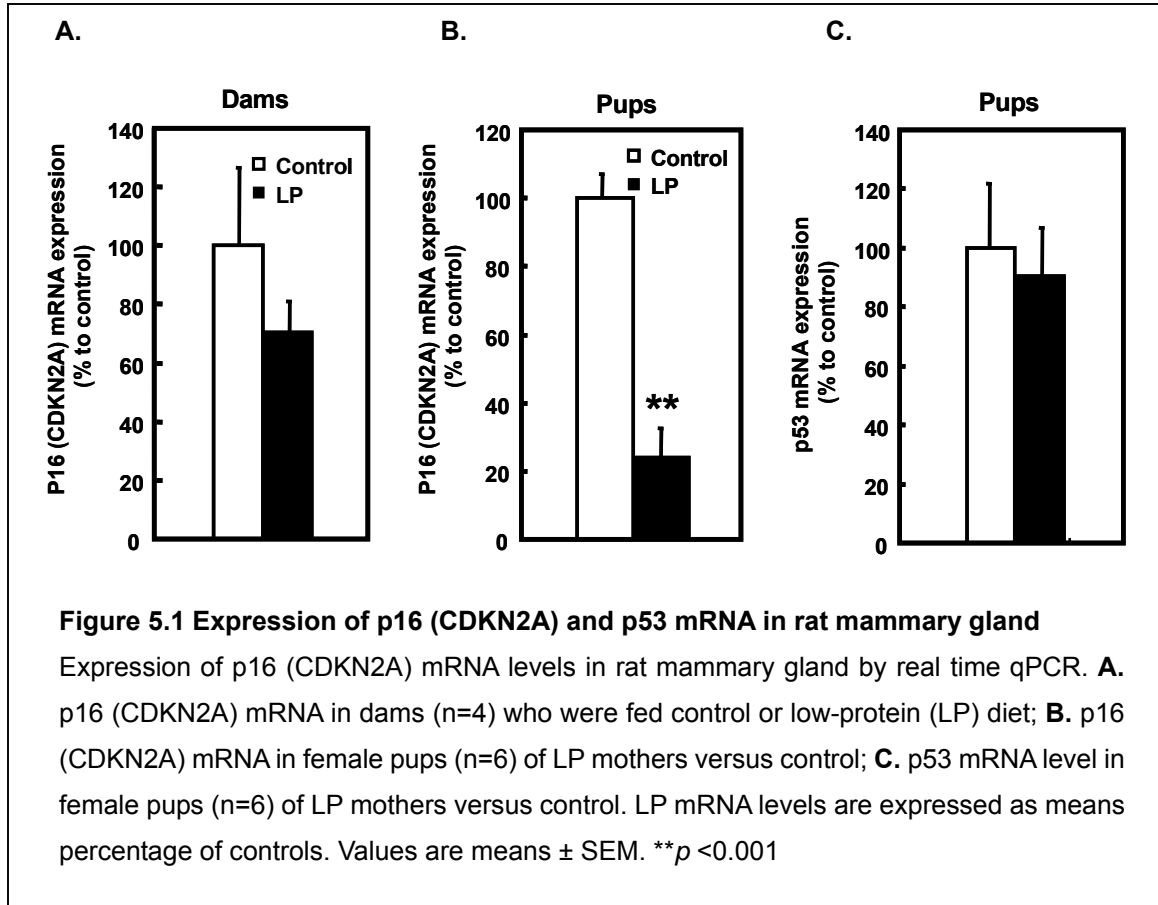
¹Number of litters shown in parentheses.

²Timing of delivery by the dams

³Body weight was shown as the average value at the specific day. Data are means with ± S.E.M. Significance testing was set at $p < 0.05$ derived from two-tail t-test.

Table 5.2 Primer sequence used in qPCR and ChIP

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Analysis of mRNA expression		
p16	TCCGAGAGGAAGGCCGAATC	GCTGCCCTGGCTAGTCTATCTG
p53	CTTACCATCATCACGCTGGAAGAC	GCACAAACACGAACCTCAAAGC
Analysis of CpG island		
p16	ACTGGGCGGGCACTGAATCTC	TTCGGGGCGTTTGGTGAAG
Analysis of histone modification		
p16	AGCATGGAGTCCTCTGCAGATAGAC	GAAAGTGTTCCGGGGCGTTTG
Analysis of transcription rate		
p16	GGCAACGCGAGACTAGCATA	CTCCCTCCCTCTGCTAACCT



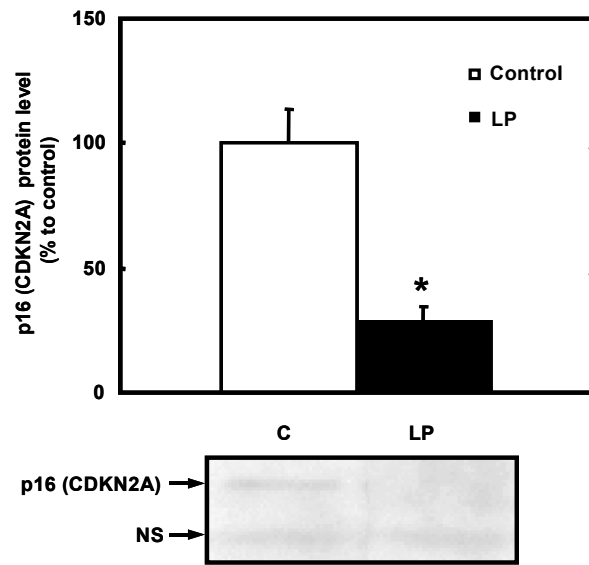
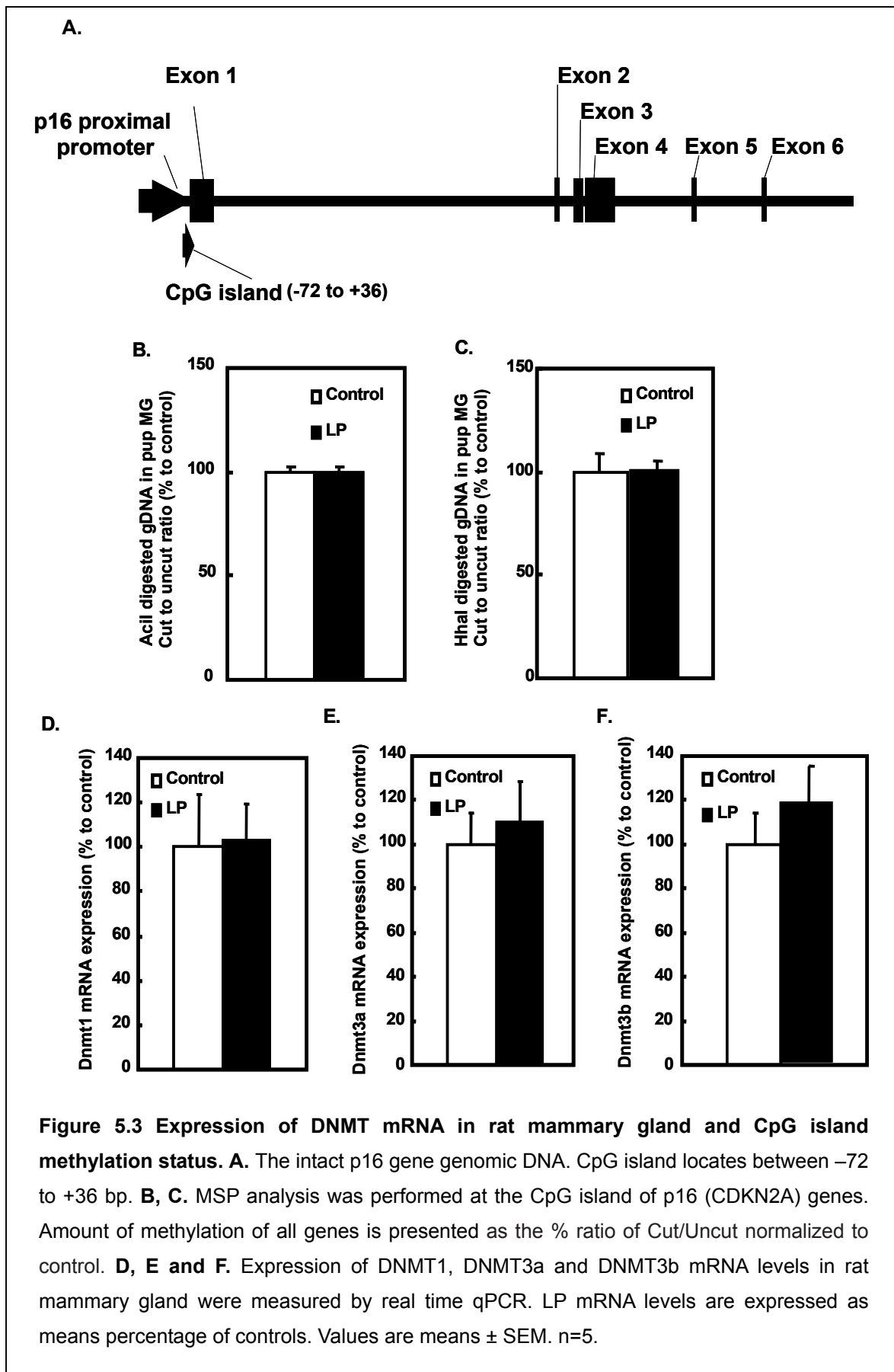
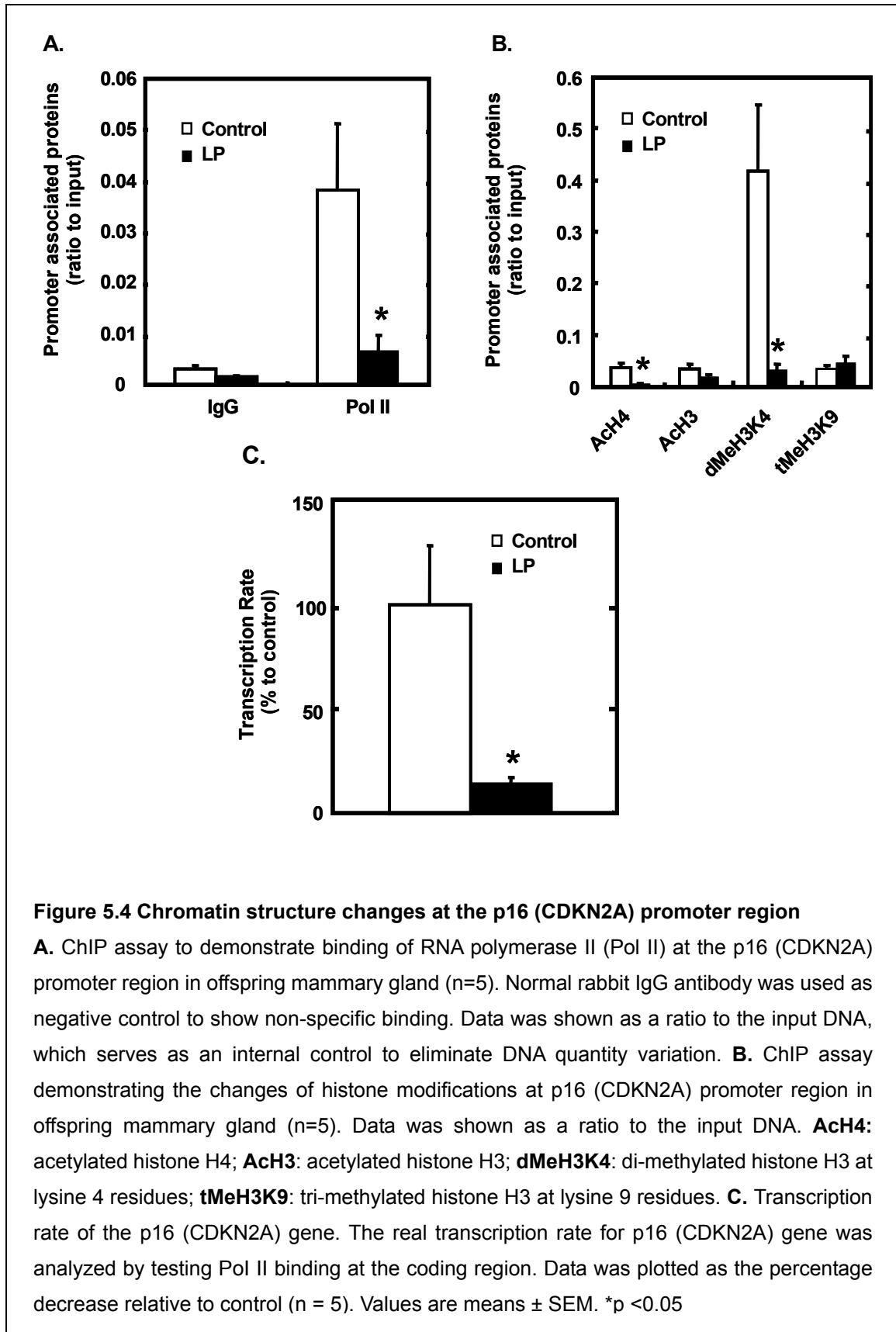


Figure 5.2 p16 (CDKN2A) protein content in offspring mammary gland

Expression of p16 (CDKN2A) protein content in female offspring mammary gland by Western blot (n=4). The bands presented the average level of p16 (CDKN2A) protein in control and LP groups. NS: non-specific binding. LP protein levels were expressed as means percentage to control. The values represented the mean \pm S.E.M., * $p < 0.05$.





5.7 References

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Chapter 6. Chromatin Modifications Cause Transcriptional Repression of the p21 Gene in Mammary Gland in Offspring of Gestational Protein-Restricted Rats

6.1 Abstract

Maternal exposure to environmental agents throughout pregnancy may change certain metabolic processes during the offspring's mammary gland growth and alter the epigenome. This may predispose the offspring's mammary gland to breast cancer later in life. The purpose of this study is to examine the effect of maternal protein restriction on the regulation of p21 gene expression in the mammary gland of rat offspring. Timed-pregnant Sprague-Dawley rats were fed one of the two isocaloric diets, control (C, 18% casein) or low protein (LP, 9% casein), during the gestation period. Compared to control, LP offspring showed a decrease of p21 in the mammary gland at both the mRNA and protein levels. Chromatin immunoprecipitation (ChIP) assay demonstrated that the down-regulation of p21 transcription in LP offspring was associated with reduced acetylation of histone H3 and dimethylation of H3K4 within the p21 promoter region, but was not associated with acetylation of histone H4 or histone methylation. DNA methylation analysis using bisulfite sequencing did not detect a differing pattern at the p21 promoter between the offspring of control and LP groups. We conclude that maternal protein restriction inhibits p21 gene expression in the mammary gland of offspring through histone modifications within the promoter region of the p21 gene.

6.2 Introduction

Maternal nutrient supplementation, or exposure to environmental agents, may change metabolic processes in the mammary gland and alter the epigenome, which predisposes the offspring to an increased risk of developing cancers in the mammary gland {Hilakivi-Clarke *et al.* 2006}. A maternal low protein diet has been associated with an increased risk of a number of adult diseases {McArdle *et al.* 2006}. Low birth weight, which results from protein restriction during the gestation period, has been shown to be associated with breast cancer risk {Mellemkjaer *et al.* 2003}. Ozanne *et al.* have shown,

in rats, that maternal exposure to a low protein diet increases mammary tumorigenesis in offspring {Fernandez-Twinn *et al.* 2007}. However, a poor understanding of the molecular mechanism of cancer in the mammary gland hinders our further determination of the role of maternal nutrition in cancer prevention. Epigenetic modifications, including cytosine methylation of DNA and post-translational modification of chromosomal structural histones, can change gene function and phenotype without altering the DNA sequence. Such epigenetic modifications affect cell proliferation during prenatal and postnatal development. A maternal low protein diet during the pre-implantation period in rodents induced a transient reduction in concentrations of maternal serum essential amino acid, which is associated with a reduction in embryo cell numbers. This led to various abnormal postnatal phenotype changes even though the offspring were fed a normal diet {Kwong *et al.* 2000}.

As a potential tumor suppressor gene in mammary gland development, the cycline-dependent kinase inhibitor p21 is a key factor responsible for tumor suppressor p53-dependent cell cycle arrest, and may contribute to the regulation of cell proliferation, differentiation, and tumorigenesis. p21 has been accepted as a prognostic marker of breast cancer. The loss of expression of two G1-checkpoint CDK inhibitors, p21 and p27, has been implicated in the genesis or progression of many human malignancies {Cariou *et al.* 2000}. A recent long-term study indicates that p21 knockout mice developed spontaneous tumors at an average age of 16 months, in comparison to 20 months for wild-type animals, revealing the importance of p21 in tumor suppression {Martin-Caballero *et al.* 2001}. Given the crucial function of cell cycle control to cancer development, a problem in the regulation of a potent cell cycle regulator such as p21 is clearly a contributing determinant of the unrestrained proliferation typical of cancer cells. Low levels of p21 expression have been identified in patients with both early stage and later stage breast cancers {Van *et al.* 2002; Paik *et al.* 2004; Somlo *et al.* 2008}. Little is known regarding a dietary contribution to the epigenetic modifications of p21 gene regulation in mammary gland. Although a recent publication reported that isoflavone-deprived soy peptide can induce expression of p21 and suppresses mammary tumorigenesis in female rats {Park *et al.* 2009}, no studies examine the regulation of p21

through generations in mammary gland development through maternal dietary factors.

The purpose of this study is to identify how maternal protein restriction affects the expression of the p21 gene in the mammary gland of offspring. We hypothesized that maternal dietary low protein modulates p21 gene expression in offspring through an epigenetic modification-associated mechanism at its transcriptional regulatory regions.

6.3 Material and Methods

Animals and treatment

Timed-pregnant Sprague Dawley rats (65 days old; Charles River Laboratories) were obtained on day 2 of gestation and weight matched into one of two isocaloric diets, control (180 g/kg casein) or low protein (LP, 90 g/kg casein). The diets were in the form of premade pellets, which were adapted according to a modified AIN-76 diet formula (Table 6.1). Each group contained 4 dams. Both groups had free access to rat chow and drinking water. Animals were individually housed in standard polycarbonate cages with corncob bedding and were maintained in a temperature and humidity controlled colony room on a 12 hr light-dark cycle. Twenty-four hours after birth, 6 pups (3 female and 3 male) were selected and used in the litters to minimize variation in pups' nutritional status during suckling. Pups body weights were measured once a week after birth. Mothers were switched to standard rodent diet through lactation. At day 24, in both experimental groups, the pups were weaned from the mother to a standard rat chow. Female pups were sacrificed when they were 38 days old. The fourth abdominal mammary gland was collected, snap-frozen in liquid nitrogen, and stored at -70°C. All of the other tissues, pancreas, kidney, liver, brain (hypothalamus), heart, muscle (gastrocnemius), adipose (visceral), colon, and blood in this study were also collected from female offspring. Tissue samples of 5 to 6 female pups from each treatment group were randomly chosen for the following experiments.

RNA isolation and cDNA synthesis

Frozen tissue samples from 6 offspring of each group were individually ground with a

mortar and pestle in liquid nitrogen (around 100 mg of mammary tissue per animal), and total RNA was isolated from each sample with TRI reagent (Sigma, St. Louis, MO). Following isopropanol (Fisher Scientific, Fair Lawn, NJ) precipitation, RNA was re-suspended and quantified by spectrophotometry (BIO-RAD Smart Spec Plus, Hercules, CA) at A260/A280. A high-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used for reverse transcription of 2 µg of total RNA. All samples to be compared were reverse transcribed from the same reaction mixture at the same time. A template tube without RNA was used as the negative control. The whole procedure was performed in a DNA 2720 Thermal Cycler (Applied Biosystems). The samples, at the 20 µL reaction scale, were heated at 37°C for 2 hr for the reverse transcription procedure. Following this procedure, the samples were heated to 85°C for 5 seconds to inactive reverse transcriptase and terminate the reaction. The final 20 µL of cDNA was diluted to 400 µL by nuclease free water and stored at -20°C.

Real-time quantitative RT-PCR (qPCR)

In order to measure the relative amount of mRNA, cDNA samples were run in a 96-well plate on 7300 Real-Time PCR Systems (Applied Biosystem, Foster City, CA)), detected by iTag SYBR Green Supermix with ROX (Bio Red, Hercules, CA), which is a dye that binds to the dsDNA generated during amplification. The reaction mixtures were activated at 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. The volume of mRNA was quantified according to a fluorescence signal by comparing a cDNA standard, with a specific concentration gradient, to experimental Ct values. The cDNA standards were made using the same RNA samples for each type of frozen tissue. The PCR primers amplified these standard samples in a linear fashion, and the Ct values of the experimental samples were within the Ct values of the standard samples for each type of tissue. The origin of the standards was a standard curve performed for mRNA analyzed in a specific tissue. Ribosomal protein L7a mRNA level was measured at the same time to be used as an internal control. We also tested other housekeeping genes, for example 18s and actin in this study. The reason that L7a was selected as an internal control is because that its amplification curve in real-time qPCR has the most similar trend as that of p21. After PCR, a dissociation curve was generated through a stepwise

increase of the temperature from 55°C to 95°C to ensure that a unique product was amplified. Primers for the qPCR were designed using Vector NTI software (InforMax Inc., Frederick, MD) to amplify the transcriptional region of the p21 gene (Table 6.4).

Protein isolation and Western blotting

Protein samples were isolated from 25 mg of each frozen mammary gland tissue, which was ground in liquid nitrogen and put into 500 µL of protein sample buffer (0.125 mol/L Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 1% SDS, 20% glycerol, 0.4% bromophenol blue, protease inhibitor). Protein was sonicated (Fisher Scientific model 100 Sonic Dismembrator, Pittsburgh, PA) on ice with 25 pulses at power setting 2. The Lowry assay was used to determine protein content, and samples containing 20 µg of protein were resolved by SDS-PAGE. An electrotransfer of the protein to a polyvinylidene fluoride membrane (0.2 µm, Bio-Rad) using a wet transfer protocol was performed. The membrane was blocked with 5% milk in TBS/T (30 mmol/L Tris base pH 7.6, 200 mmol/L NaCl and 0.1% Tween 20) for 1 hour at room temperature, and then incubated with rabbit polyclonal antibody against p21 (Table 6.5) in 5% BSA at a 1:1000 dilution at 4°C overnight. The next day the membrane was washed five times for 5 min each with TBS/T on a shaker and then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in 1% milk at a 1:10,000 dilution for 1 hr at room temperature. The membrane was washed again five times, for 5 minutes each time. The bound secondary antibody was detected using a SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL) and western blot images were captured and analyzed by a Chemi Doc system (Bio-Rad).

Genomic DNA extraction from mammary gland

Frozen mammary gland tissues from 6 offspring of each group were individually cut into pieces on ice (around 100 mg of mammary tissue per animal). Proteinase K (100 µL of 10 mg/mL) and 240 µL of 10% SDS were then added to the samples and incubated in a 45oC water bath overnight. The next day, 2.4 mL of phenol was added to the system, shaken completely for 10 min, and spun at 3000 rpm for 5 min at 10oC. The supernatant

was collected, and 1.2 mL phenol and 1.2 mL chloroform/isoamyl alcohol (24:1) were added. This mixture was shaken by hand for 5 min, spun at 3000 rpm for 5 min at 10°C, and then the supernatant was transferred into a fresh tube. Both 25 µL of 3 mol/L sodium acetate (pH 5.2) and 5 mL of ethanol were added to the supernatant, shaken gently for DNA precipitation, and spun at 16,000 g for 20 min at 4°C. After discarding the supernatant, the DNA pellet was washed with 70% ethanol. The pellet was air-dried, suspended with 300 µL of TE, and stored at -20°C.

Bisulfite sequencing

Genomic DNA (1 µg) was utilized for bisulfite conversion with the EZ DNA Methylation-Gold Kit (ZYMO), following the manufacturer's instructions. Briefly, DNA samples were treated with sodium bisulfite at 64°C for 2.5 hours. They were then desalted, desulfonated, and eluted into 40 µL of TE. Bisulfite-modified DNA (25 ng) was then subjected to PCR amplification of the p21 CpG islands using the bisulfite primer sets, designed online by the tool Methprimer (<http://www.urogene.org/methprimer/index.html>). The PCR products obtained were cloned into pCR4.0 TOPO vectors (Invitrogen, Carlsbad, CA). We tested 6 animals from each group. Ten colonies of each animal were sequenced for each specimen in order to obtain a sufficient sampling of allele-specific methylation patterns.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed according to a modified protocol {Chen *et al.* 2004}. Briefly, 200 mg of frozen tissue was ground in liquid nitrogen, re-suspended in PBS and cross-linked in 1% formaldehyde for 10 min at room temperature. The tissue pellet was re-suspended in nuclei swelling buffer (5 mmol/L Pipes [NaOH] pH 8.0, 85 mmol/L KCl, 0.5 % NP40) containing protease inhibitor. The separated nuclei were lysed in SDS lysis buffer (50 mol/L Tris-HCl pH 8.1, 10 mmol/L EDTA, 1% SDS) containing protease inhibitors. The resultant chromatin was sonicated (Fisher Scientific model 100 Sonic Dismembrator, Pittsburgh, PA) on ice with 8 bursts for 40 s at power setting 5, with a 2 min cooling interval between each burst. The average length of sonicated chromatin was determined by resolving it on a 1.6% agarose gel, and was found to be around 200-500 bp.

The sample was then centrifuged at 13,000 rpm for 10 min at 4°C to remove cell debris from the crude chromatin lysate. One milliliter of sheared chromatin was diluted in 10 mL of ChIP dilution buffer. Ten percent of the diluted lysate was subsequently incubated overnight on a hematology mixer (346, Fisher Scientific) with 2 µg of primary antibodies (Table 6.5) at 4°C. Normal rabbit IgG antibody was used as the negative control to demonstrate non-specific binding. Antibodies were considered negative for binding if the resulting value was equal to or less than the IgG value (ratio to input). Pre-blocked salmon sperm DNA/protein A agarose beads (60 µL, 50% slurry; Upstate Biotechnology, Lake Placid, NY) were then added to the chromatin for 2 hr, followed by centrifugation at 2000 rpm for 1 min at 4°C. The supernatant of normal rabbit IgG was saved as the input control for PCR after clean up. The pellets containing immunoprecipitated complexes were washed sequentially with 1 mL of low salt solution (0.1% SDS, 1% triton X-100, 2 mmol/L EDTA, 20 mmol/L pH 8.0 Tris-HCl, 150 mmol/L NaCl), high salt solution (0.1% SDS, 1% triton X-100, 2 mmol/L EDTA, 20 mmol/L pH 8.0 Tris-HCl, 500 mmol/L NaCl), LiCl solution (0.25 mol/L LiCl, 1% NP40, 1% sodium deoxycholate, 1 mmol/L EDTA, 10 mmol/L pH 8.0 Tris-HCl) and twice with TE (pH 8.0). Antibody/protein/DNA complexes were eluted from Protein A agarose beads by adding 250 µL of the elution buffer (50 mmol/L NaHCO₃ and 1% SDS) two times, followed by 15 minutes of shaking at 300rpm at 37°C and finally by flash spinning the samples down at room temperature. The combined supernatants were incubated at 65°C for 4-5 hr, after the addition of 20 µL of 5 mol/L NaCl and 1 µg of RNase A to reverse the formaldehyde cross-linking and release the DNA fragments. Samples were then treated with proteinase K at 37°C for 1 hr to remove any protein, and DNA was purified with a Wizard SV Gel and PCR Clean up System (promega). Each real time PCR reaction used 5% of immunoprecipitated DNA. The binding of Pol II and modified histones were expressed as ratios to the input. The standards and the samples were simultaneously amplified using the same reaction master mixture at the 25 µL scale. Primers were used to amplify genomic sequences at the promoter region of p21 (see Table 6.4).

Statistical analysis

Results are expressed as the mean ± SEM. Data comparison between control and low

protein groups were performed by a two-tailed t-test. Significance testing was set at the $p < 0.05$ level. Sample size was calculated by statistical power analysis based on a 95% confidence interval using SAS.

6.4 Results

Mothers and offspring observations

Pregnant dams were monitored beginning at day 2 of gestation. Table 6.2 shows mothers' daily food intake and body weight during the gestation period. Body weights of the LP dams during pregnancy were reduced by 13% both at day 16 and at day 21 ($p < 0.05$). There were no differences in the daily food intake between the control and low protein dam groups ($p > 0.05$), indicating that the quantity of food intake should not affect experimental results. At birth, the control pups weighed 6.32 ± 0.19 g, which was similar to the weight of the LP pups at 5.92 ± 0.09 g. As the pups grew to 15 days, the average body weight was significantly lower in the LP pups than in the control ($p < 0.02$). Table 3B provides the average offspring body weight at birth and at day 7, 15, 22 and 35 of postnatal life. The average weight of the LP offspring was reduced by 12% at day 15, 16% at day 22 and 13% at day 35 (Table 6.3; $p < 0.05$) compared to the control pups. Control dams delivered all pups after noon. Percentages of pups delivered by the LP dams before noon and after noon were 43% and 57% respectively (Table 6.2A), suggesting a preterm risk, which has been known for frequent occurring in low birth weight infant {Sluncheva *et al.* 2010}. There were no differences in the litter size or litter sex distribution between control and LP mothers (Table 6.3A).

Maternal low protein diet specifically repressed p21 gene expression in offspring mammary glands

The effect of a maternal low-protein diet on offspring gene expression was examined through real-time qPCR. Figure 6.1A shows the tissue distribution of p21 mRNA expression in female offspring rats. The mRNA of the p21 gene in the mammary gland of the LP pups was decreased by 38% relative to the control pups ($p < 0.05$), but it did not differ in any other tissue. Therefore, we concentrated our study on the regulatory

mechanisms for the p21 gene in only the female offspring mammary gland. Expression of p53 was not affected by maternal low protein (data not shown).

p21 protein content was decreased by maternal low-protein regime

To examine if the altered p21 mRNA produces a change in protein content in offspring mammary gland, we performed Western Blot analysis using an anti-p21 antibody. p21 protein was reduced by 60% ($p < 0.05$) with a maternal low-protein diet in female offspring mammary gland (Figure 6.2). Significantly lower p21 protein content in LP pups' mammary gland tissue was consistent with the trend observed in its mRNA expression. An anti-actin antibody was used as an internal control to show the equality of protein levels loaded.

Maternal low-protein diet did not change p21 gene methylation pattern in offspring

To determine whether CpG islands at the p21 promoter exhibited DNA methylation related to its mRNA expression, two pairs of bisulfite primer sets were designed at the p21 promoter region. These primer sets span from -481 to +7 bp and -16 to +386 bp, containing 26 and 30 CpG sites, respectively (Figure 6.3). Figure 3A represents the positions of the p21 gene promoter and its CpG islands on the intact genomic sequence. In this study, the p21 methylation status was assessed by bisulfite sequencing analysis (Figure 6.3B and C). CpG methylation levels were similar between offspring of both control and low-protein dams. Therefore, the reduced p21 mRNA expression in offspring was DNA methylation-independent under the maternal protein restriction.

Chromatin modifications at the p21 promoter were associated with the maternal diet

Changes in chromatin structure caused by chemical modifications of histone proteins, such as methylation and acetylation, regulate gene transcription by affecting the ability of eukaryotic RNA polymerase II (Pol II) to transcribe DNA in order to synthesize precursors of mRNA and most snRNA {Roeder *et al.* 1969; Conaway *et al.* 1997}. ChIP assays were performed to investigate Pol II binding status at the p21 promoter in offspring mammary gland tissue. Normal rabbit IgG antibody was used as the negative control, indicating non-specific binding. Antibodies were considered negative for binding

if the resulting value was equal or less than the IgG value (≤ 0.002 ratio to input). Female offspring liver was used as a negative control to test the changes of the same set of modified histones at the p21 promoter, which is used to support the result specificity in mammary gland (Figure 6.4A). Recruitment of PoI II to the p21 promoter was decreased in LP pup mammary gland compared to control (Figure 6.4B; $p < 0.05$).

To further determine if the altered transcriptional level of p21 was regulated by the changes of chromatin structure at the p21 promoter, antibodies to either methylated histone or acetylated histone were utilized in the ChIP assay. Figure 6.4C shows the histone modifications at the p21 promoter in the mammary gland of offspring rats. A 33% decrease in acetylated H3 (H3Ac) ($p < 0.05$) and a 34% decrease of methylation at H3K4 (H3K4Me2) ($p < 0.05$) were detected in the LP pups, correlating to the 38% transcriptional repression (Figure 6.1B). No difference was detected in histone H4 acetylation (H4Ac) or methylation at lysine 9 residues (H3K9Me3). This data suggests that the altered p21 gene transcription might be regulated by the changes in histone modification at its promoter region, specifically histone H3 acetylation as well as histone H3 methylation at lysine 4 (Figure 6.5).

6.5 Discussion

The mechanism of p21 regulation during mammary gland development, specifically in regards to maternal dietary factors, may provide new approaches to reducing breast cancer risk. In the current study, we used a gestational low protein diet with 9% of protein to investigate the maternal protein restriction that highly likely occurs in teen mothers in the US. Similar animal model has been used by many researchers to study the effect of maternal protein restriction on offspring gene regulation {Fernandez-Twinn *et al.* 2007; Lillycrop *et al.* 2005 11 /id}, while some other research groups used a more restricted protein diet to trigger more straightforward consequences {Buffat *et al.* 2007}. According to Acceptable Macronutrient Distribution Ranges (AMDR), 10%-35% of energy is from protein. 9% of protein we used in this study is right close to the range. One goal of our designed study is to mimic a protein-restricted status that teen mothers usually experience

in reality. We presented several results demonstrating that a maternal low protein diet can down-regulate p21 expression in offspring mammary glands in a rat model. We also revealed that the suppression of p21 is caused by histone modifications, but not DNA methylation, within the p21 promoter region.

p21 alone represents an important tumor suppressor gene. As a prognostic marker in cancer, p21 has been extensively investigated. When the susceptibility of p21-deficient mice to the colon carcinogen azoxymethane was studied, enhanced formation of aberrant crypt foci in p21-deficient mice suggests a tumor suppressor function for p21 in the colon {Poole *et al.* 2004}. Similarly, loss of p21 allowed proliferation of cells with DNA damage and resulted in cancer development in liver and kidneys of fumarylacetoacetate hydrolase-deficient mice {Willenbring *et al.* 2008}. Ozanne *et al.* have shown, in rats, that maternal exposure to a low protein diet increases mammary tumorigenesis among the offspring. Changes in p21 expression have also been reported in the pancreas, in the low protein exposed offspring. Also, clinical data suggests that loss of p21 expression in patients marks the switch from hepatocyte dysplasia to HCC {Plentz *et al.* 2007}. These results suggest that p21 may represent an important tumor suppressor that is inactivated in liver and renal cancer. However, little is known about the role of repressed p21 in mammary gland growth.

Mammary glands develop all through different growth cycles. They exist during the embryonic stage, but form only a rudimentary duct tree at birth. At this stage, mammary gland development is systemic hormone independent {Watson *et al.* 2008}. Under the regulation of paracrine communication between neighboring epithelial and mesenchymal cells, a primary duct tree is formed before puberty. Extensive proliferation of mammary tissue occurs in females in response to circulating ovarian hormones from puberty {Wysolmerski *et al.* 1998}. In terms of the SD rats that we used in this study, their puberty period is within 6 to 8 weeks after birth, but the area of abdominal mammary has a dramatic increase between day 35 and day 40 {Sinha *et al.* 1966}. In order to avoid the impact of extensive mammary gland development during puberty, but still have enough mammary gland tissue to perform a variety of experiments, we chose day-38 offspring to

investigate the changes of p21 gene expression in neonatal mammary. Another animal study in our laboratory is currently ongoing and focuses on more time points in adult rats to further investigate the effect of maternal protein restriction on offspring mammary gland development during and after puberty.

Results regarding DNA methylation in this study did not differ within the p21 promoter, which extends from around -500 to +400 regions on the p21 genome. This has been known as the most important region, which includes 8 CpG islands on the p21 promoter and also contains cumulative binding sites for its transcription factors. There are two additional CpG islands on the p21 genome. One is proximally located around -3000 and the other is approximately at the +5000 region. We cannot deny the possibility that those two proximal regions might be methylated, but the regulatory role of that has never been reported.

Histone acetylation patterns and methylation patterns were analyzed by ChIP. Histone acetylation is associated with increased gene transcription, and therefore, with transcriptionally active chromatin domains. As a posttranslational modification, histone acetylation is maintained by a dynamic balance between the activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). The acetylation of lysine residues on histones H3 and H4 represents the active or open state of chromatin, which allows various transcription factors access to the promoters of target genes. In contrast, shifting the promoter occupancy from HATs to HDACs to deacetylate lysine residues will result in chromatin compaction and transcriptional repression. Recruitment of HDACs by Sp1/Sp3 sites at the p21 promoter, which in turn represses p21 transcription, is a potential model for p21 down-regulation by a maternal diet. We determined a decrease in acetylated H3 ($p < 0.05$), which may indicate increased binding of HDACs to the p21 promoter through certain machinery. Histone H3 hypoacetylation has been reported to be associated with reduced p21 expression by gastric carcinoma, but there is no association between p21 expression and histone H4 acetylation status in the p21 promoter {Mitani *et al.* 2005}. Similar results were found in our study. It is histone H3 hypoacetylation, but not H4 acetylation, that is associated with reduced p21 expression in offspring mammary

gland through maternal protein restriction. Distinct roles for acetylation of histones H3 and H4 have been reported in yeast {Wan *et al.* 1995}. Although a number of proteins have been identified that are recruited to specific histone code modifications, further studies in mammalian also need to reveal the functional significance of histones H3 and H4.

With regard to histone methylation, we found reduced di-methylation of H3K4 ($p < 0.05$), but unchanged tri-methylation of H3K9, at the p21 promoter in LP offspring mammary gland. Histone methylation appears to have multiple effects on chromatin function in a system- and site-specific manner. Methylation of H3 on K4 is implicated in the activation of transcription. In budding yeast, H3K4Me3 localizes to the 5' end of the target gene and is found to be associated with the initiation of RNA Pol II {Kouzarides *et al.* 2007}. Methylation of H3 on K9, on the other hand, is largely associated with silencing and repression in many species {Bartova *et al.* 2008}. Our results indicate that methylation of H3K9 in the p21 promoter region is not an absolute requirement for p21 repression.

The regulation of p21 gene transcription is controlled by both p53-dependent and -independent mechanisms. For instance, p53 mutant mouse p53R172P lost its ability to induce apoptosis, but retained its capacity to induce p21. When these mice were crossed onto a p21-null background, loss of p21 entirely eliminated the cell cycle arrest function of p53R172P {Barboza *et al.* 2006}. Other p53-independent pathways in regulating p21 are also reported. MYC represses p21 transcription by binding to and inhibiting SP1 {Gartel *et al.* 2003}. This can be alleviated through the binding of the ligand-independent nuclear receptor hepatocyte nuclear factor 4 α 1 (HNF4 α 1) to SP1 {Hwang-Verslues *et al.* 2008}. In response to DNA damage, MYC is recruited to the p21 promoter by MIZ1, and forms a ternary complex with the DNA methyltransferase DNMT3a, which represses p21 transcription {Brenner *et al.* 2005}. Additionally, AP4, a basic helix-loop-helix protein and a transcriptional target of MYC, represses the p21 promoter through binding to four proximal E-box motifs independently of MIZ1, SP1 or SP3 {Jung *et al.* 2008}. Taken together, these mechanisms could potentially provide several lines of evidence for further investigation of the altered p21 expression by a maternal low protein diet.

In summary, we documented here that maternal dietary low protein modulates p21 gene expression and histone modifications within its promoter in the mammary gland of offspring rats (Figure 6.5). The findings suggest that a maternal protein-restricted diet during pregnancy may potentially alter cell cycle control pathways, thereby predisposing offspring rats to the risk of developing breast cancer later in life.

6.6 Tables & Figures

Table 6.1 Composition of the two isocaloric diets

Ingredients	C, g/kg	LP, g/kg
Casein	180	90
Folic acid	0.001	0.001
Cornstarch	405	465
Sucrose	213	243
Choline	2	2
Cellulose	50	50
Corn oil	100	100
D,L-Methionine	5	5
Vitamin mix (AIN-76A)*	10	10
Mineral mix (AIN-76A)	35	35
Total energy, Kcal/g	3.868	3.868

* Vitamin mix (AIN-76A) is folic acid free

Table 6.2 Mother observations

A.	C (4) ¹	LP (4) ¹	
Pups delivered before noon (%)	0	43	
Pups delivered after noon (%) ²	100	57	

B.	Body weight (g) ³		<i>p</i> value
Days during pregnancy	C (4)	LP (4)	
2	195.78 ± 8.33	180.12 ± 2.93	0.09
9	240.08 ± 12.49	217 ± 2	0.08
16	290.38 ± 13.32	253.1 ± 5.93	0.03
21	362.88 ± 18.92	315.36 ± 6.89	0.04

C.	Daily food intake (g) ³		<i>p</i> value
Days during pregnancy	C (4)	LP (4)	
3	18.75 ± 2.1	17.22 ± 0.67	0.47
9	19.73 ± 0.89	17.2 ± 0.96	0.1
16	22.25 ± 1.32	20.5 ± 0.58	0.23
21	21.53 ± 1.75	19.24 ± 1.29	0.32

¹Number of dams shown in parentheses. Data are means with ± S.E.M.

²Timing of delivery by the dams

³Body weight and food intake are shown as the average value at the specific day. Data are means with ± S.E.M. Significance testing was set at $p < 0.05$ derived from two-tail t-test.

Table 6.3 Offspring observations

A.			
	C (4) ¹	LP (4) ¹	
Litter size	11.5 ± 0.65	10.3 ± 0.86	
Litter sex distribution male to female	1.11	1.25	
B.			
	Body weight (g) ²		p value
Age (days)	C (4)	LP (4)	
Birth	6.32 ± 0.19	5.92 ± 0.09	0.11
7	16.55 ± 0.69	14.89 ± 0.46	0.10
15	35.06 ± 1.14	30.73 ± 0.70	0.02
22	59.37 ± 1.40	49.98 ± 1.63	0.006
35	138.56 ± 2.58	125.21 ± 3.54	0.006

¹ Number of litters shown in parentheses.

² Body weight is shown as the average value at the specific day.

Data are means with ± S.E.M. Significance testing was set at p<0.05 derived from two-tail t-test.

Table 6.4 p21 primer sequence

Gene (Ensembl ID)	Forward primer (5' → 3') Reversed primer (5' → 3')	Purpose
<i>p21</i> (ENSRNOG00000000521)	(+208) CCGAGAACGGTGGAACCTTTGAC (+278) GAACACGCTCCCAGACGTAGTTG (-259) CTGGTGGGCTGCGTGACAAG (-110) GGACCAGCCGCTCCTTCAC (-481) GAATTTTTATGTTTAGTTTATGGGA (+7) AACACTATAACAACCTCACACCTCTC (-16) GAGGTGTGAGTTGTTATAGTGTT (+386) AACTAAAAAATACCTACCATCC	mRNA expression Histone modifications at gene promoter Bisulfite sequencing

Table 6.5 Antibodies used in Western blot and ChIP assay

Antibody	Introduction & company	Purpose
p21	sc-397, Santa Cruz Biotechnology, Santa Cruz, CA	Protein analysis by Western
H3Ac	Anti-acetylated histone 3 at lysine 9, 14 residues; 06-599, Upstate, Temecula, CA	Analysis of histone modifications
H4Ac	Anti-acetylated histone 4 at lysine 5, 8, 12 and 16 residues; 06-866, Upstate, Temecula, CA	
H3K4Me2	Anti-di-methylated histone 3 at lysine 4 residues; 07-030, Upstate, Temecula, CA	
H3K9Me3	Anti-tri-methylated histone 3 at lysine 9 residues; cs200604, Upstate, Temecula, CA	
IgG	sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA	
Pol II	sc-899, Santa Cruz Biotechnology, Santa Cruz, CA	RNA polymerase II analysis

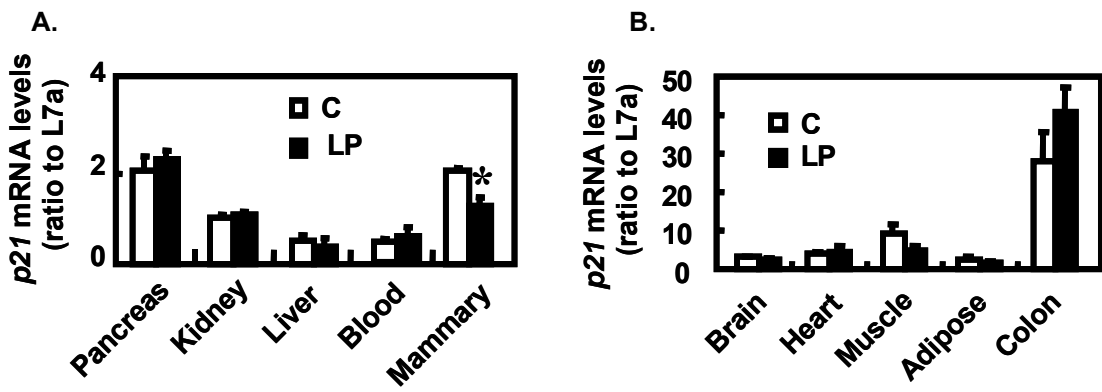


Figure 6.1 *p21* mRNA tissue distribution in female offspring

Expression of *p21* mRNA in female offspring pancreas, kidney, liver, blood, mammary gland, brain, heart, muscle, adipose, and colon of control mothers versus LP mothers by qPCR (n=6, samples from 6 animals were tested in each of the two conditions). mRNA levels are shown as the ratio to *L7a* housekeeping gene, which acts as an internal control to normalize total RNA variation. The bar values represent the mean \pm S.E.M.; *indicates comparison of *p21* mRNA in pups of control and LP groups, $p < 0.05$.

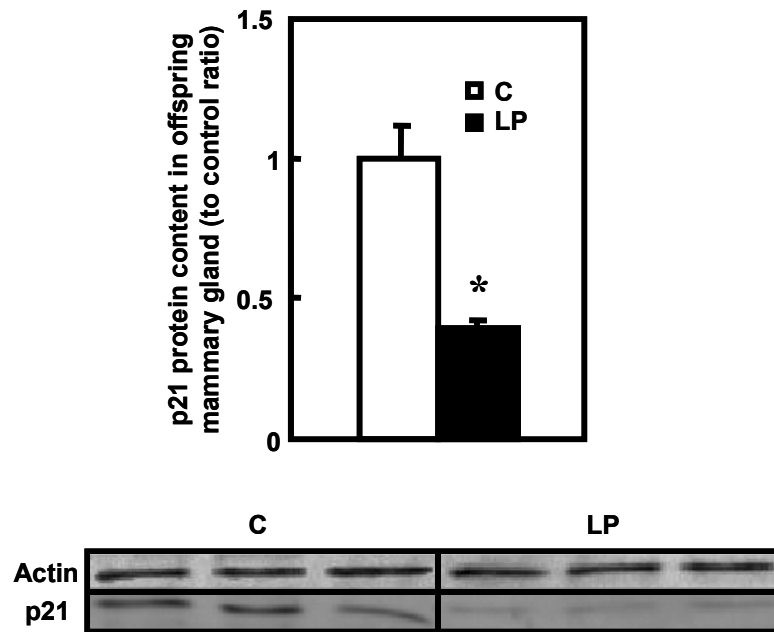


Figure 6.2 p21 protein content in pup mammary gland by Western blot

Expression of p21 protein in female offspring mammary gland from LP mothers versus control. LP protein levels were expressed as mean ratio to control. The values represent the mean \pm S.E.M., * $p < 0.05$.

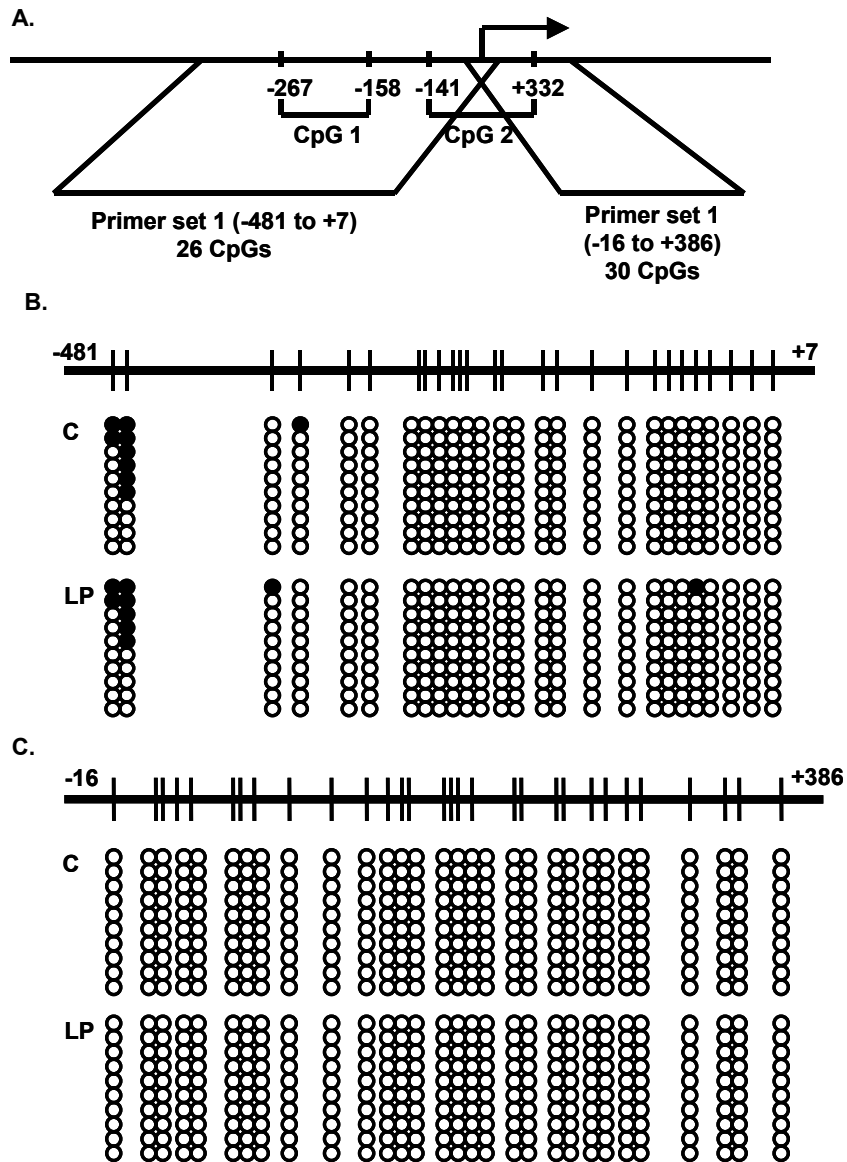
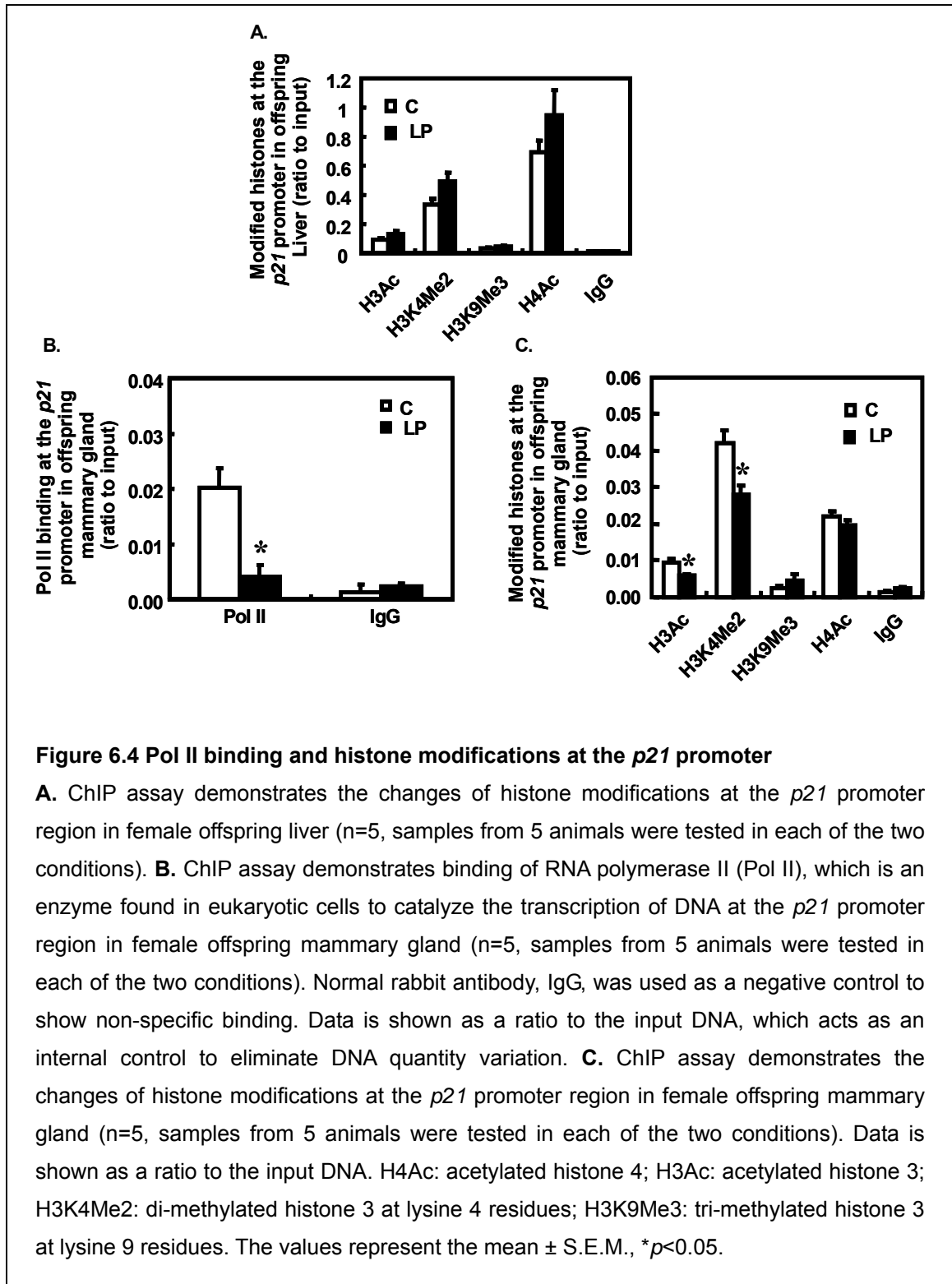


Figure 6.3 DNA methylation status within the *p21* promoter in offspring mammary gland

A. Rat *p21* gene promoter includes two major CpG islands, which were amplified by two sets of bisulfite sequencing primers, respectively. **B.** Bisulfite sequencing results amplified by the primers, which cover -481 to +7 at the *p21* promoter. **C.** Bisulfite sequencing results amplified by the primers, which cover -16 to +386 at the *p21* promoter. In Figure 3B and 3C, each solid bar on the top line represents a CpG site at the *p21* promoter. 10 clones were picked from each animal and 6 animals were involved for each group, control versus low protein group. Each set of 10 circles represents the average level of DNA methylation



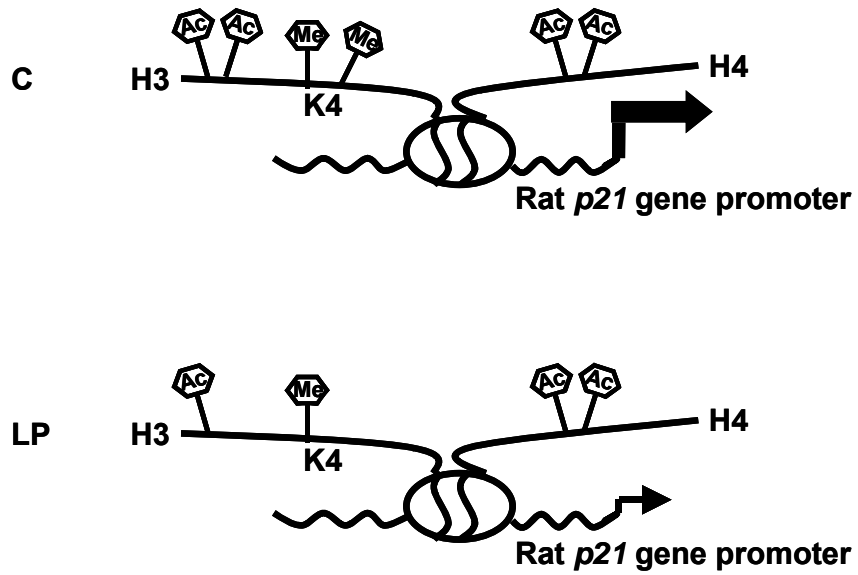


Figure 6.5 A summary scheme to show the changes of acetylation of H3 and methylation of H3K4 within the *p21* promoter in offspring mammary gland, comparing control and low protein groups.

6.7 References

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Chapter 7. Future Directions

7.1 Background

Breast cancer is partially caused by the disruption in the normal pathways involved in mammary gland development and differentiation. Maternal nutrient supplementation or exposure to environmental agents may change a host of metabolic processes in mammary gland and the accompanied alteration in epigenome thereby predisposing mammary gland to the development of cancers later in life. The responses may relate to changes in hormonal and growth factor status and modifications to the chromatin structure and epigenome regulation. All these alterations may change gene expression during development that could lead to either regulatory malfunction later in life and predispose to breast cancer development or long-term protection against breast cancer by modulating fetal ductal development in mammary gland.

Transcriptional regulation of cancer genes, particularly tumor suppressor genes, during cancer development is governed by epigenetic mechanisms, which makes it an important area of investigation. Our contribution here is to clarify the specific mechanism(s) by which a maternal low protein diet participates in regulation of p21, a potential breast tumor suppressor gene.

7.2 Significance

This contribution is significant because it is expected to expand our understanding of epigenetic control of tumor suppressor gene expression by maternal nutrition and also provide valuable insight into new avenues for clinical intervention and treatment. Once such strategies become available, novel therapeutic approaches targeting this level of gene regulation will undoubtedly emerge.

Nutritional environment likely directly or indirectly alters epigenetic modifications such as DNA methylation and histone modifications, and thereby affects gene transcription,

gene expression and ultimately the development of disease. However, nowadays whether maternal low protein affects the incidence of breast cancer in offspring is undocumented. In addition, the study of maternal protein intake in the contribution to the transgenic epigenetic modifications for genes regulation in mammary gland development will assist the determination of optimal low costing maternal nutrition in breast cancer preventions.

The cycline-dependent kinase inhibitor p21 is a key responsible factor regulating cell proliferation, differentiation, and tumor genesis. Low level of p21 expression has been identified as a molecular marker for patients with both early stage and higher stage breast cancers³⁰. Little is known about the dietary contribution to the epigenetic modification for p21 regulation in mammary gland. Although one report did show that isoflavone-deprived soy peptide can induce expression of p21 and suppresses mammary tumorigenesis in female rats {Park K *et al.* 2009}, there is no study to date to examine the regulation of p21 through generations in mammary gland development by maternal dietary factors. We are interested in effects of maternal low protein in the regulation of p21 and the associated molecular mechanisms in mammary gland. We are hoping that an improved understanding of the influence of a maternal low protein diet on offspring gene expression will allow us to identify the specific molecular target that could provide new opportunities for significantly lowering breast cancer risk.

7.3 Research Proposal for Future Study

This section is written to correspond to the outline in the specific aims section, and each series of experiments has an experimental design paragraph followed by a rationale paragraph. The experiments will test the hypothesis that maternal low protein diet modulates p21 gene expression in offspring through an epigenetic mechanism in its transcriptional regulatory regions. The experiments have been integrated so that often data from more than one scientific approach will be used in a complementary fashion to provide multiple lines of evidence to test the hypothesis. We believe that the proposed experiments test important hypotheses and that they will generate valuable new information regarding the mechanisms by which offspring senses and responds to

changes in maternal nutrient availability.

Two of our main goals of this project are firstly to identify the target genes in the epigenetic programming and secondly to unfold related mechanism at molecular level thereby providing us meaningful guide to develop useful approaches to prevent breast cancer for women. We have identified that maternal dietary low protein modulates p21 gene expression in offspring rats. In most cases of breast cancer, p21, a negative regulator of the cell cycle, is generally down-regulated. Therefore, we plan to start investigating the mechanisms that control p21 expression transcriptionally and how deregulation of these mechanisms may lead to tumorigenesis.

Understanding of the role of epigenetic information in the development of breast cancer and its significance as predictive markers for fetal origin of tumor genesis can potentially offer new approaches for breast cancer preventions. Deacetylation/acetylation of histone is one of the best-known epigenetic events in mammalian cells and is involved in the complex mechanism that regulates promoter transcription. The exact interplay of these factors in transcriptional repression activity is not yet well understood. Histone acetylation pattern is maintained by a dynamic balance between the activities of histone acetyltransferases (HAT) and histone deacetylases (HDAC). The acetylation of lysine amino acids on histones H3 and H4 represent the active or open state of chromatin, which allow various transcription factors access to the promoters of target genes. In contrast, shifting the promoter occupancy from HAT to HDAC to deacetylate lysine amino acids will result in chromatin compaction and transcriptional repression. Recruitment of HDACs at the p21 promoter, which in turn represses p21 transcription, is a potential model for p21 down-regulation by maternal diet.

HDAC1 and HDAC6 are over-expressed in breast cancer samples, while HDAC2 and HDAC3 are commonly found in colon cancers {Bolden JE *et al.* 2006}. Some evidence correlating HDAC binding status with p21 expression has been reported. Dissociation of HDAC1 from the C terminus of Sp1 reduces the binding of HDAC1 at the p21 promoter and induces p21⁴³. The growth factor independent 1 (GFI1) is a transcriptional regulator

and has ability to recruit HDAC1 on the promoter of p21. As the result, down-regulation of p21 was observed {Duan Z *et al.* 2005}. HDAC inhibitors (HDACi) induce p21 expression mainly by activating the Sp1/Sp3 pathway independent of p53. Upon HDACi treatment *in vitro*, HDAC1 is released from the Sp1-binding site at the p21 promoter leading to a loss of repression and an induction of transcription {Ocker M *et al.* 2007}. Trichostatin A, one of HDACi's, effectively induces p21 expression and cell cycle arrest in human gastric and oral carcinoma cell lines {Suzuki T *et al.* 2000}. The HDACi butyrate induces p21 and apoptosis in colorectal cancer cells {Mahyar-Roemer M *et al.* 2001}. Another HDACi, SAHA, induces nine-fold increase in p21 mRNA and protein in T24 bladder cancer cells due to the enhancement of acetylation of histones H3 and H4 around the promoter region {Richon VM *et al.* 2000}.

Histon acetyltransferases (HATs) transfer an acetyl group from acetyl-coenzyme A (acetyl-CoA) to certain lysine side chains of histone, which neutralizes part of a *N*-tail region's positive charge and weakens histone-DNA interactions. This signals a chromatin conformational change and gives transcription activators more access to a genetic locus, which is associated with states of transcriptional activation. GCN5 (general control nonderepressible-5) is the best-characterized HAT in yeast, both structurally and functionally *in vivo* and *in vitro*. In mammals, the GCN5 subclass is represented by two closely related proteins, GCN5 and PCAF (p300/CREB-binding protein-associated factor). These proteins share about 70% identity and 80% similarity throughout their sequences {Xu W *et al.* 1998}. The substrate specificity of GCN5 is particularly interesting to us. *In vitro*, recombinant GCN5 or PCAF were found to acetylate primarily on lysine-14 of histone H3, and more weakly on lysine-8 of histone H4 {Schiltz RL *et al.* 1999}.

In previous study, compared to control, offspring rats from the LP group showed a decrease of p21 expression in both mRNA and protein levels in mammary gland. ChIP assay demonstrated that the down-regulation of p21 transcription in LP offspring was associated with reduced acetylation of histone H3 and di-methylation of H3K4 within the p21 promoter region, but not acetylation of histone H4 or tri-methylation of H3K9. DNA

methylation was also tested using bisulfite sequencing. However, it did not indicate any difference at the p21 promoter between offsprings of control and LP groups.

7.3.1 Specific Aim I: determine the extent to which expression of HDACs in offspring mammary is associated with decreased histone acetylation at the p21 gene promoter

The *objective* of this aim is to determine whether the decreased histone acetylation observed within the p21 gene promoter in offspring mammary is accompanied by the changes of HDAC1 or HDAC6 expression and their interactions within the p21 promoter. Our preliminary data has shown that maternal protein restriction down-regulated histone 3 acetylation at the p21 promoter in offspring mammary gland. We therefore *hypothesize* that the altered histone acetylation could be affected by HDAC1 or HDAC6 expression level and their interactions with the p21 promoter.

7.3.1a Aim IA: identify whether maternal protein restriction affects HDAC1 or HDAC6 expression in offspring mammary gland

In addition to p21 gene we have tested, this initial experiment will test the expression of HDAC1 and HDAC6 in offspring mammary gland.

Experiment Design: mRNA and protein expressions of HDAC1 or HDAC6

Briefly, frozen mammary will be randomly chosen from six animals (offspring) of each group, control versus low protein group, for sampling. Total RNA will be isolated using Tri-reagent (Sigma, St. Louis, MO). cDNA will be synthesized in a 20 ul reaction volume using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and a thermal cycler (Applied Biosystems), with the following program: 25°C for 10 min, 37°C for 120 min, 85°C for 5 s, and a 4°C hold. To measure the relative amount of mRNA, Real-Time qPCR will be performed using 25 ng cDNA as the template and SYBR Green PCR Master Mix (Applied Biosystems) in the 7300 Real-Time PCR System (Applied Biosystems), with the following program: 95°C for 10 mins, 95°C for 15 s, 60°C

for 1 min, 95°C for 15 s, 55°C for 1 min, 95°C for 15 s, with 40 cycles of step 2 and 3.

For isolating protein, frozen mammary sample will be ground in liquid nitrogen and put into protein sample buffer (0.125 M Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 1% SDS, 20% glycerol, 0.4% bromophenol blue, protease inhibitor). Samples will be then sonicated (Fisher Scientific model 100 Sonic Dismembrator, Pittsburgh, PA) on ice with 25 pulses at power setting 2. Lowry assay will be used to determine protein content and samples containing 20-30 µg of protein were resolved by SDS-PAGE. After electrotransfer to a polyvinylidene fluoride membrane (0.2 µm, Bio-Rad) using a wet transfer protocol, 5-10% milk in TBS/T (30 mM Tris base pH 7.6, 200 mM NaCl and 0.1% Tween 20) will be performed to block the membrane for 1 hr at room temperature and then the membrane will be incubated with antibody against HDAC1 or HDAC6 in 5% BSA at 4 °C overnight. The membrane will be washed five times for 5 min with TBS/T on a shaker the next day and then incubated with secondary antibody in 1-5% milk for 1 hr at room temperature. The membrane will be then washed again for 5 min by five times. The bound secondary antibody will be detected using a SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL) and western blot images will be captured and analyzed by a Chemi Doc system (Bio-Rad).

Rationale and expected results

This series of experiments is designed to perform a concrete expression analysis of HDAC1 and HDAC6, which are an important class of enzymes in mammary that remove acetyl groups from a ϵ -N-acetyl lysine amino acid on a histone. Real-time qPCR is an efficient assay to analyze gene mRNA expression. A housekeeping gene L7a and a set of standard cDNA will be used to control the validation of the experiment. Western blot analysis will further reveal the functional change of HDAC1 and HDAC6 from protein aspect. We expect that maternal protein restriction will affect the expression of HDAC1 or HDAC6. Thus, the result will potentially contribute to the declined histone acetylation.

7.3.1b Aim IB: determine the interaction of HDAC1 or HDAC6 within the p21 gene promoter in offspring mammary gland

In order to determine whether HDAC1 or HDAC6 is playing a role in the regulation of p21 transcription, the interaction of HDAC1 or HDAC6 within the p21 promoter will be investigated *in vivo*.

Experiment Design: the interaction of HDAC1 or HDAC6 within the p21 promoter

Cross-linked chromatin immunoprecipitation (xChIP) assays will be performed according to our previously published protocol to determine the promoter binding of HDAC1 or HDAC6. Briefly, 200 mg of frozen mammary tissue will be homogenized and cell protein will be cross-linked using formaldehyde. Nuclear extract will be isolated and chromatin will be sonicated into around 500 bp fragments. Aliquots of diluted sample will then be incubated overnight with 2 ug of antibody against HDAC1 or HDAC6. After incubation, the immune complex will be captured by incubating with Protein G Sepharose beads. An antibody to normal IgG will be used as a negative control, indicating non-specific binding. All samples will then be washed sequentially with low salt buffer, high salt buffer, LiCl buffer and TE. Immunoprecipitated DNA will be eluted and reverse cross-linked. Proteinase K will be added into samples to remove protein and DNA will be purified with a Wizard SV Gel and PCR Clean up System (promega). Real-time qPCR will then be carried to test the amount of immunoprecipitated DNA using p21 promoter primers, which reflecting the binding of HDAC1 or HDAC6 to the p21 promoter region.

Rationale and expected results

This experiment will investigate the interaction of HDAC1 or HDAC6 within the promoter region of p21 gene in order to reveal the potential mechanism that resulting in the alteration of chromatin remodeling. In the consideration of the role of HDACs, which may be recruited by other transcription factor to the p21 gene promoter, xChIP is designed to cross-link all the interacting factors, including those that are not directly bound with DNA sequence. Recruitment of HDACs by Sp1/Sp3 sites at the p21 promoter, which in turn represses p21 transcription, is a potential model for p21 down-regulation by maternal diet.

7.3.1c Aim IC: determine whether HDAC1 or HDAC6 is necessarily required for the regulation of p21 transcription

In order to confirm our hypothesis that HDACs is indispensable for the down-regulation of p21 observed in offspring mammary by maternal diet, we will isolate normal mammary cell from living rats to explore, when HDACs is inhibited, how p21 transcription will change *in vitro*.

Experiment Design: the change of p21 expression in vitro under HDAC-inhibited condition

Primary Culture of Mammary Epithelial Organoids

Female Sprague Dawley rats (Charles River Laboratories) at 56-66 days of age will be used for primary culture of rat mammary epithelial cell. All animals will receive care according to the National Institute of Health guidelines for the care and use of laboratory animals. The animals will be anesthetized with ether and operated upon using an aseptic technique under laminar airflow. The mammary gland will be removed, minced, and digested with collagenase III (Worthington Biochemical Corp., Lakewood, NJ) at 172.8 U/ml and with dispase (Roche Applied Science, Mannheim, Germany) at 1.2 mg/ml for 12 h to isolate mammary epithelial organoids. After enrichment, the rat organoids will be embedded within an Engelbreth-Holm-Swarm (EHS)-derived reconstituted basement membrane (RBM) and cultured in an EGF-free serum-free medium (SFM) consisting of phenol red-free DMEM-F12 (50:50, vol:vol) with 10 ug/ml insulin, 1 ug/ml progesterone, 1 ug/ml hydrocortisone, 1 ug/ml prolactin, 5 ug/ml transferrin, 5 uM ascorbic acid, 1 mg/ml fatty acid-free BSA, and 50 ug/ml gentamycin. Cells will be then placed in a CO₂ stove (Nuaire) during 24 hours at 37° C. The SFM medium will be again changed on the 4th and 5th day {Zhang J *et al.* 2007}.

TSA Treatment

The cells will then be seeded in 12-well dishes (Costar, Cambridge, MA) at 3-10×10⁴ cells/well and cultured in complete minimal essential medium (MEM) with L-glutamine (pH 7.4, SCS Cell Media Facility, University of Illinois at Urbana-Champaign, Urbana, IL), following supplementation of 10% fetal bovine serum (Cellgro, Herndon, VA) and

1% penicillin (Cellgro, Herndon, VA). Cells will be maintained at 37°C in a 5% CO₂ incubator and be replenished with fresh MEM 12 hr prior to initiating TSA treatment. TSA will be purchased from Wako Biochemicals (Osaka, Japan). A stock solution of TSA at the concentration of 1 mg/ml will be prepared in ethanol. The cells will be exposed to medium containing 0, 10, 50, 100, 500 and 1,000 ng/ml TSA for 72 hr and RNA will be extracted using Tri-reagent. The same amount of ethanol will be added to the MEM as the control experiment. All experiments will be performed in duplicate and repeated 3 times.

Small Interfering RNA Assay

Purified primary mammary cells (around 2×10^5 cells) will be transfected with 165 nM of small interfering RNA against HDAC1 or HDAC6 (Applied Biosystems/Ambion, Austin, TX) using lipofectamine according to the manufacturer's instruction. Scramble siRNA will be used as control. After four hours post-transfection, cells will be washed once in HBSS buffer and resuspended in culture medium in order to avoid cellular toxicity due to siRNA transfection. Twenty-four hours post-transfection, cells will then be stimulated as described and cell-free supernatants will be harvested and tested for the presence of p21 by ELISA or cells will be lysed for Western blot analysis {Michaud F *et al.* 2010}

Rationale and expected results

Primary cell cultures closely mimic the *in vivo* state and generate more physiologically relevant data. We will absolutely confirm the importance of HDACs in the regulation of p21 transcription by using HDAC inhibitor treated, primary cultured, rat mammary cell. We believe that being able to visualize results from primary cultured mammary cell will provide stronger evidence to show HDACs is indispensably involved in the down-regulation of p21 gene expression. Small interfering RNA assay will be carried out as a backup option to inhibit HDAC expressions in primary cell in case the TSA treatment does not work.

7.3.2 Specific Aim II: determine the extent to which expression of HATs in offspring mammary is associated with decreased histone acetylation at the p21 gene promoter

The ***objective*** of this aim is to determine whether the HATs is also playing a role that contributing to the down-regulation of histone acetylation observed within the p21 gene promoter in offspring mammary gland. We previously detected the decreased acetylation of histone 3, but not histone 4. This selective acetylation of histone during chromatin remodeling might be associated with specific target sites of different members in HATs. We ***hypothesize*** that the altered histone 3 acetylation could be affected by GCN5 or PCAF, which are specifically function on lysine sites in histone 3, but not histone 4.

7.3.2a Aim IIA: identify whether maternal protein restriction affects HATs expression in offspring mammary gland

It will provide more useful information about how a maternal low protein diet affects the balance of HATs versus HDACs in the chromatin remodeling to the p21 gene promoter.

Experiment Design: mRNA and protein expressions of HATs

The mRNA expression of HATs, particularly GCN5, will be tested using cDNA samples generated from offspring mammary gland. Protein level will be then tested by Western Bolt assay. The detailed procedure will be the same as described in Aim IA.

Rationale and expected results

This series of experiments is designed to perform a concrete expression analysis of HATs, which are an important class of enzymes that acetylate conserved lysine amino acids on histone proteins by transferring an acetyl group from acetyl CoA to form ϵ -N-acetyl lysine. Although we previously observed decreased acetylation of histone 3, this may be also resulted from the effect of the decreased HATs expression by maternal diet. Moreover, the selectivity of HATs could potentially reveal the mechanism that why the alteration of acetylation occurred on histone 3 only.

7.3.2b Aim IIB: determine the interaction of HATs within the p21 gene promoter in offspring mammary gland

In order to determine whether HATs is also playing a role in the regulation of p21 transcription, the interaction of HATs within p21 promoter will be investigated *in vivo*.

Experiment Design: the interaction of HATs within p21 promoter

Cross-linked chromatin immunoprecipitation (xChIP) assays will also be performed to determine the promoter binding of HATs as described in Aim IB.

Rationale and expected results

This experiment will investigate the interaction of HATs within the promoter region of p21 gene in order to reveal the potential mechanism that resulting in the alteration of chromatin remodeling. In the consideration of the role of GCN5, which only acetylate lysine amino acids on histone 3, we expect the decrease binding level of GCN5 at the p21 promoter, which in turn represses p21 transcription, is another potential model for p21 down-regulation by maternal diet.

7.3.2c Aim IIC: determine whether GCN5 is necessarily required for the regulation of p21 transcription

In order to examine whether or not GCN5 is also indispensable for the down-regulation of p21 by a maternal protein-restricted diet, we will use primary rat mammary cell as described in Aim IC to explore, when GCN5 is inhibited, how p21 transcription will change *in vitro*.

Experiment Design: the change of p21 expression *in vitro* under GCN5-inhibited condition

α -Methylene- γ -Butyrolactones Treatment

Rat primary mammary cells will be isolated from 56-66 days old Female Sprague Dawley rats and then be seeded in 12-well dishes (Costar, Cambridge, MA) at $3-10 \times 10^4$ cells/well and cultured in complete minimal essential medium (MEM) with L-glutamine (pH 7.4, SCS Cell Media Facility, University of Illinois at Urbana-Champaign, Urbana,

IL), following supplementation of 10% fetal bovine serum (Cellgro, Herndon, VA) and 1% penicillin (Cellgro, Herndon, VA). Cells will be maintained at 37°C in a 5% CO₂ incubator and be replenished with fresh MEM 12 hr prior to initiating α -methylene- γ -butyrolactones treatment. The cells will be exposed to medium containing 0, 5 and 10 mM of α -methylene- γ -butyrolactones {Biel M *et al.* 2004} and RNA will be extracted using Tri-reagent. All experiments will be performed in duplicate and repeated 3 times.

Small Interfering RNA Assay

Purified primary mammary cells (around 2×10^5 cells) will be transfected with 165 nM of small interfering RNA against GCN5 (Applied Biosystems/Ambion, Austin, TX) using lipofectamine according to the manufacturer's instruction. Scramble siRNA will be used as control. After four hours post-transfection, cells will be washed once in HBSS buffer and resuspended in culture medium in order to avoid cellular toxicity due to siRNA transfection. Twenty-four hours post-transfection, cells will then be stimulated as described and cell-free supernatants will be harvested and tested for the presence of p21 by ELISA or cells will be lysed for Western blot analysis {Michaud F *et al.* 2010}

Rationale and expected results

α -Methylene- γ -butyrolactone is the only compound that we have known to display an inhibition against GCN5. The proposed experiment is important to identify the role of GCN5 in the regulation of p21 transcription by using GCN5 inhibitor treated primary cell. This will be powerful way for analyzing whether or not GCN5 is indispensably involved in the down-regulation of p21 gene expression. Small interfering RNA assay will also be carried out as a backup option to inhibit GCN5 expression in case the α -methylene- γ -butyrolactones treatment does not work.

7.3.3 Specific Aim III: determine the role of sp1 and sp3 in the regulation of p21 promoter

The *objective* of this aim is to determine whether Sp1 or Sp3 acts as a transcription factor

regulating p21 gene expression in our maternal protein-restricted animal model. Our preliminary data has shown that restricted maternal protein decreases p21 expression and acetylation of H3 as well as di-methylation of H3K4. The changed histone modification could potentially create less-opened chromatin structure, thus, blocking the bindings of transcription factors. We hypothesize that Sp1 or Sp3 can affect p21 gene transcription initiation frequency through direct binding to the p21 promoter, following a dose dependent manner.

7.3.3a Aim IIIA: identify whether maternal protein restriction affects the direct binding of Sp1/Sp3 transcription factor within p21 promoter in offspring mammary gland

The direct binding of Sp1 or Sp3 transcription factor at p21 promoter will be tested to explore further mechanism that resulting in the repressed p21 expression under maternal protein restriction in vivo.

Experiment Design: direct binding of Sp1/Sp3 at p21 promoter in vivo

This series of experiments will investigate the direct binding of Sp1/Sp3 within the promoter of p21 gene. We will perform native chromatin immunoprecipitation (nChIP) experiments. In brief, 300mg of mammary gland samples will be ground in liquid nitrogen and intact nuclei will be isolated. Nuclei (0.3 mg) will be digested with 25 U of micrococcal nuclease at 37°C for 6 min in 1 ml of digestion buffer (50mM NaCl, 20 mM Tris-HCl pH 7.5, 3.0 mM MgCl₂, 1.0 mM CaCl₂, 10 mM sodium butyrate, 0.1 mM PMSF). The digestion will be terminated by the addition of Na₂EDTA to 0.5 mM and the salt-soluble chromatin will be isolated and concentrated using a microcon centrifugal filter (Amicon Inc., Bedford, PA). 250 µg of this chromatin (input) will be incubated with 10 µl each of antisera specific for the acetylated forms of histone H3 or H4 (Upstate Biotech., Lake Placid, NY) in a total volume of 500 µl. Protein A-Sepharose (Amersham Pharmacia Biotech) precipitates will be generated, washed, and DNA will be purified from the pellets (bound) and supernatants (unbound). DNA samples from the input, bound and unbound fractions will be analyzed by electrophoresis on 1% agarose gels to

determine the size distribution of the resulting oligonucleosomes. Equal masses of DNA (100 ng) from input, unbound and antibody-bound DNA from the nChIP fractions are conveniently and accurately determined by real-time qPCR. Determine the enrichment of a given target sequence achieved by the antibody against sp1 by the ratio of the sequence content of the bound to input fractions. All ratios will be verified in a minimum of two separate experiments

Rationale and expected results

This series of experiments is designed to perform ChIP on native chromatin prepared from offspring mammary gland tissues, in order to analyze whether maternal protein restriction changes the direct binding of Sp1/Sp3 at p21 promoter. The key features of a native ChIP (nChIP) experiment are the fragmentation of chromatin in the nuclei by micrococcal nuclease and the immunoselection of chromatin fragments bearing the modified protein of interest using an appropriate antibody. Formaldehyde crosslinking is not used at any point during the preparation. It is particularly suited for proteins such as core histones that are tightly bound to DNA. nChIP will reveal nucleosomes bearing transcription factor-directly bound genomic DNA, which would not be interfered by other proteins in crossed-linked ChIP (xChIP). We expect to reveal the role of Sp1 or Sp3 within p21 promoter related to maternal dietary regulation.

7.3.3b Aim IIIB: determine how different doses of Sp1 transcription factor regulates p21 promoter activity in primary cultured mammary cells

This application is to determine the essential role of sp1 regulating p21 promoter activity *in vitro*. We will use reporter gene luciferase assay and perform cell transfection to identify how dose dependent Sp1 protein can affect p21 gene promoter activity.

Experiment Design: Sp1 regulates p21 promoter activity

Cell Culture

Primary cells isolated from rat mammary gland will be used as described in Aim IC. Cells

will be cultured in complete minimal essential medium (MEM) with L-glutamine (pH 7.4, SCS Cell Media Facility, University of Illinois at Urbana-Champaign, Urbana, IL), following supplementation of 10% fetal bovine serum (Cellgro, Herndon, VA) and 1% penicillin (Cellgro, Herndon, VA). Cells will be maintained at 37°C in a 5% CO₂ incubator and be replenished with fresh MEM 12h prior to initiating all treatments.

Plasmid Construction

For expression of Sp1 protein, rat Sp1 cDNA will be generated by PCR and subcloned into pcDNA3.1/zeo (Invitrogen) to generate the Sp1 expression vector driven by the cytomegalovirus promoter (rSp1/pcDNA3.1-zeo). It then will be used to express the corresponding protein in rat breast cancer cells. The reporter plasmid (p21^{-481/+7}/luciferase) was made by inserting a p21 promoter fragment (nucleotides -481/+7) upstream of the Firefly luciferase reporter gene using the HindIII site of the pGL3 plasmid (Promega, Madison, WI).

Transient Transfection & Luciferase Analysis

Cells (0.2×10^6 cells/well) will be seeded on 24-well plates 18-24 hr before transfection with Superfect reagent (Qiagen) at a ratio of 6 ul of Superfect to 1 ug of DNA. For each transfection, 0.5 ug of p21^{-481/+7}/luciferase plasmid will be used along with the different amounts of the Sp1 transcription factor expression plasmids. The total amount of transfected DNA will be kept constant among experimental groups by the addition of empty pcDNA3.1 plasmid. At 12 hr following transfection, the cells will be transferred to fresh complete MEM for 12 h before all treatments. At 36 hr following transfection, cellular extracts will be prepared for analysis of luciferase activity, by washing the cells with phosphate-buffered saline (PBS) and incubating in 300 ul of Passive lysis buffer (Promega). The lysates will be collected and stored at -80 °C until use. A 10 ul aliquot will be used for Firefly luciferase assays using the luciferase reporter assay system (Promega, Madison, WI). The luciferase values will be normalized to protein content for each sample. For each experimental condition, three assays will be performed for each transfection.

Rationale and expected results

The proposed experiments are used to explore how p21 promoter activity is affected by Sp1 protein expression. To analyze the activity of a promoter, the putative promoter sequence of p21 gene must be inserted upstream of the reporter gene. The proteins of transcriptional factor sp1 will be incorporated into the experiments as translated from an expression vector. Through transfection with both p21 promoter inserted reporter gene and Sp1 inserted expression vector into the breast cancer cells, we will be able to reveal whether or not the p21 promoter activity is regulated by Sp1 protein in a dose dependent manner.

7.4 Discussion

Deregulated cell cycle progression, driven by activation of growth-stimulating oncogenes, is one of the primary characteristics of cancer cells. Understanding how dietary components regulate proliferation and cell survival could play a critical role in development of new agents that can prevent and/or treat cancer with low cost. Following successful completion of the aims outlined in this project, elucidating the mechanism by which maternal low protein regulates the cell cycle gene in offspring, we will further characterize the interaction of maternal protein and offspring cell cycle signaling pathway. More extensive studies would address in vitro cell culture and in vivo animal to evaluate whether regulation of p21 expression associates with the risk of cancer development programmed by maternal fetal nutrition.

We are hoping that an improved understanding of the influence of maternal low protein diet on offspring gene expression will allow us to identify the specific molecular target that could provide new opportunities for significantly lowering breast cancer risk. We are excited about our laboratory's research to contribute to the understanding of how the availability of maternal nutrients controls and programs gene expression and chromatin modifications in offspring.

7.5 References

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CURRICULUM VITAE

SHASHA ZHENG

EDUCATION

- Ph.D., Food Science and Human Nutrition** **2011**
University of Illinois at Urbana-Champaign, Urbana, Illinois (Cumulative GPA: 3.93/4.0)
- M.A., Medical Nutrition Sciences** **2007**
Boston University School of Medicine, Boston, Massachusetts (GPA: 3.78/4.0)
- B.E., Food Sciences and Engineering** **2002**
Southern Yangtze University, Wu Xi, China

RESEARCH & PROFESSIONAL EXPERIENCE

University of Illinois at Urbana-Champaign, Urbana, Illinois **2007-2011**
Graduate Research Fellow – Department of Food Science and Human Nutrition

Advisor: Professor Yuan-Xiang Pan

- Explored role of maternal nutrition in modulating gene expression and disease progression in offspring via animal and cell line models, with focus on epigenetic mechanisms.
- Revealed alterations of important gene expressions in glucose metabolism, amino acid homeostasis, and molecular markers in breast cancer risk by maternal protein restriction model.
- Researched related publications that discussed fetal and neonatal development of body structure and function, as well as disease risks later in life.
- Conducted testing and experiments using nucleotides and protein isolation, real-time PCR, Western blotting, chromatin immunoprecipitation, luciferase reporter assay, cell transfection, gene cloning, bisulfite sequencing, methylation sensitive PCR, methylation specific PCR, MeDIP, microarray, cell proliferation and invasion assay, animal surgery and tissue collection, primary cell culture,

ELISA, H & E staining histology, siRNA and LC-MS/MS.

- Supervised three undergraduate students and two visiting scholars in lab; trained them in experimental technologies and elements of nutrition genetics; guided new graduate students in laboratory skills and rules.
- Selected as sole member of group to assist advisor on conducting experiment and writing proposal for successful NIH grant application (CA139557).

Boston Medical Center, Boston, Massachusetts

2005-2007

Graduate Research Assistant – Department of Endocrinology, Diabetes, and Nutrition

Advisor: Professor Tai C. Chen

- Examined biological effects of vitamin D₃ analogues on prostate cancer prevention and treatment using cancer cell line models and patient samples.

Part-time Research Assistant – Steroid Hormone Assay Laboratory

- Tested serum samples of normal adults and children from Framingham Heart Study through LC-MS/MS assay to establish national standards for testosterone and estradiol.

Boston University School of Medicine, Boston, Massachusetts

2005-2007

Graduate Research Assistant – Medical Nutrition Sciences

Advisor: Professor Barbara E. Millen

- Evaluated contemporary literatures and wrote review article on nutrition and prevention of type II diabetes.

Advisor: Elizabeth Krall Kaye

- Analyzed NHANES III (Third National Health and Nutrition Examination Survey) database and determined relationship between fruit intake and blood pressure regulation.

International Life Sciences Institute (ILSI)-Focal Point in China, Beijing 2003-2004

Chief Executive Assistant

Advisors: Professor Chun-Ming Chen and Professor Jun-Shi Chen

- Participated in national intervention study of NaFeEDTA-fortified soy sauce for reduction of iron deficiency anemia in China (funded by Global Alliance for Improved Nutrition).
- Coordinated construction of Chinese BMI model to study obesity in Chinese adults and children.
- Organized all events for Conference on Obesity of Chinese Children and Adolescents, sponsored by Chinese Center for Disease Control and Prevention, held at Beijing in November 2003.

Southern Yangtze University, Wu Xi, China

1998-2002

Undergraduate Research Assistant – School of Food Science and Engineering

Advisor: Professor Zhang Wang

- Developed novel food processing technique for manufacturing natural lemon oolong tea powder.
- Optimized digestive ratio of pectinase to cellulase and created efficient way to extract soluble component from tea broth.
- Interned in dairy, meat, other general food supply companies, and beer factories.
- Conducted chip and cola sensory evaluations on behalf of Pepsi and Coca-Cola.

TEACHING & RELEVANT EXPERIENCE

University of Illinois at Urbana-Champaign

Fellowship Evaluation Assistant

2010

- Chosen as one of department's advanced graduate students to serve on Fellowship Board Block Grant Area Committees on behalf of the Graduate College; helped to evaluate the quality of the graduate programs for university Block Grant applications.

Teaching Assistant – Nutritional Biochemistry (FSHN 426)

2009

- Lectured on lipoproteins; directed review sections for students before exams; and prepared questions for all quizzes and exams, and graded exams.

Delivered lecture during PepsiCo Chicago visit on behalf of advisor; interfaced with company's senior managers.

PROFESSIONAL MEMBERSHIPS

American Association for the Advancement of Science (AAAS)

American Society for Nutrition (ASN)

HONORS & RECOGNITION

Philip L. and Juanita Fitzer Francis Fellowship in Health and Wellness	2010
Graduate College Conference Travel Grant	2009
Henry D. and Donna E. Strunk Fellowship Merit Award	2007-2010
Jeannette Chu and Winston Y. Lo Fellowship	2007-2010
Southern Yangtze University Scholarship	1998-2001

PUBLICATIONS

Peer-Reviewed Papers

S Zheng, M Rollet, Y-X Pan. Maternal Protein Restriction during Pregnancy Induces CCAAT/enhancer-binding protein (C/EBP β) Expression through the Regulation of Histone Modification at Its Promoter Region in Female Offspring Rat Skeletal Muscle. *Epigenetics* 2011; 6: 161-170.

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19-nor-2alpha-(3-hydroxypropyl)-1alpha, 25-dihydroxyvitamin D₃ as a therapeutic agent for androgen-dependent prostate cancer. *Anticancer Research* 2009; 29: 3547-53.

TC Chen, KS Persons, **S Zheng**, J Mathieu, MF Holick, YF Lee, B Bao, MA Arai, A Kittaka. Evaluation of C-2-substituted 19-nor-1a, 25-dihydroxyvitamin D₃ analogs as therapeutic agents for prostate cancer. *Journal of Steroid Biochemistry & Molecular Biology* 2007; 103:717-720.

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Manuscripts under Peer Review

S Zheng, M Rollet, Y-X Pan. Protein Restriction during Gestation Alters Histone Modifications at the Glucose Transporter 4 (GLUT4) Promoter Region and Induces GLUT4 Expression in Skeletal Muscle of Female Rat Offspring. *Journal of Nutritional Biochemistry*

S Zheng, M Rollet, KF Yang, Y-X Pan. Chromatin Modifications Cause a Methylation-Independent Transcription Repression of p21 Gene in Mammary Gland in the Offspring of Gestational Protein-Restricted Rats. *British Journal of Nutrition*

S Zheng, D Iglesias-Gato, JN Flanagan, L Jiang, T Sakaki, S Nakabayashi, A Kittaka, NK LeBrasseur, G Norstedt1, TC Chen. The C-2 substituted 19-nor-1a,25-dihydroxyvitamin D₃ analog, MART-10, has enhanced chemotherapeutic potency in

PC-3 prostate cancer cells. *Journal of Steroid Biochemistry & Molecular Biology*

S Zheng, Q Li, R Borhan, Z Balluff, Y Zhang, H Chen and Y-X Pan. Maternal high fat exposure during pregnancy causes transcriptional repression of the p16INK4a gene in offspring mammary gland via epigenetic modulations. *Epigenetics*

Q Li, **S Zheng**, Y-X Pan and H Chen. Maternal exposure to a high fat diet programs the microRNA expression in offspring rats. *Epigenetics*

L Gong, **S Zheng**, H Chen, Y-X Pan. Amino acid response pathway is programmed differently by protein restriction during gestation versus whole lifelong in male offspring rats. *Nucleic Acids Research*

KF Yang, **S Zheng**, Y-X Pan and Wei Cai. Early Life Protein Restriction Represses Wnt Signaling Through Epigenetic Modification in Skeletal Muscle of Rat Offspring. *British Journal of Nutrition*

Abstracts

S Zheng, Y-X Pan. Epigenetic Modifications Cause Transcriptional Repression of p21 Gene in Mammary Gland in the Offspring of Protein-Restricted Rats. Experimental Biology 2010 (oral presentation). *FASEB J.* 2010 24:344.5

S Zheng, M Unander, Y-X Pan. Maternal Protein Restriction Alters Histone Modifications and Glucose Transporter 4 Expression in Female Rat Skeletal Muscle during Postnatal Development. Experimental Biology 2009 (poster presentation). *FASEB J.* 2009 23:724.2

S Zheng, Y-X Pan. Oral intake of genistein changes DNA methylation at the promoters of rat colon genes. Experimental Biology 2008 (poster presentation). *FASEB J.* 2008 22:885.6

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