A DISSERTATION FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN FOOD AND NUTRITION

Effects of maternal dietary protein source on liver disease development in rat offspring

어미 쥐가 섭취한 단백질 종류가 자손 쥐의 간질환 발생에 미치는 영향에 대한 연구

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Abstract

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Background: *In utero* nutrition and other environmental factors may contribute to the alteration of the fetal development process and to risks of diseases in later life. As the liver is responsible for the regulation of metabolic homeostasis, fetal liver growth programming can be affected by the maternal diet, and the latter can therefore have critical long-term health consequences. Soy proteins have been shown to alleviate metabolic diseases by lowering the triacylglycerol and cholesterol levels. In particular, certain amino acid profiles and isoflavones have been suggested to contribute to the beneficial effects of soy protein isolates on the lipid metabolism. However, very few studies have examined the role of the maternal soy protein isolate diet in association with the early liver development and later risk of disease in offspring.

Objectives: The present study investigated the short- and long-term effects of maternal dietary protein source on early liver development and the risks of diseases later in life. To this, the study model was designed to compare the effect of soy protein isolate *per se* with those of a casein (CAS) as a different protein source or a casein supplemented

with genistein. **Study 1** and **2** (focusing on the short-term effects) investigated whether the maternal consumption of a low-isoflavone soy protein isolate (SPI) diet or a casein plus genistein (250 mg/kg, GEN) diet would alter the liver development of male rat offspring at 3 weeks of age and examined the changes in the gene expression profiles and epigenetic modification. **Study 3** (focusing on the long-term effects) investigated whether the adaptive response of the offspring to chronic ethanol consumption would affect the development of liver disease and cholesterol metabolism later in life.

Methods: Female Sprague Dawley rats were fed either a CAS diet (200 g/kg), an SPI diet (200 g/kg), or a GEN diet for two weeks before mating, as well as during pregnancy and lactation. Male offspring were studied at 3 weeks of age (**Study 1** and **2** groups: CAS, SPI, and GEN) and at 15 weeks of age (**Study 3** groups: CAS/CON, CAS/EtOH, SPI/CON, and SPI/EtOH). For the **Study 3**, male offspring from the dams receiving either a CAS or SPI diet were fed with the standard chow diet from weaning to 8 weeks of age, and pairs of male offspring originated from the same dam were then fed either an ethanol or pair-fed control liquid diet for 6 weeks.

Results: In **Study 1**, offspring of the SPI group had a significantly lower body weight and body fat mass than those of the CAS and GEN groups at 3 weeks of age, whereas the relative liver weight was higher in the SPI group than in the CAS and GEN groups. In the microarray analysis, the gene expression profiles were distinctly altered in the SPI group as compared to the CAS and GEN groups. Interestingly, the most statistically significant genes involved in xenobiotic and drug metabolism were altered by the SPI group compared to the CAS and GEN groups. Moreover, a significant

correlation was found between the relative liver weigh and the gene expression related to xenobiotic and drug metabolism. The SPI group showed a significantly higher serum homocysteine level and lower global DNA methylation than the CAS group. The levels of glycine *N*-methyltransferase and 5-methyltetrahydrofolate-homocysteine methyltransferase related to one-carbon metabolism were significantly lower in the SPI group than in the CAS group. In addition, significantly lower histone H3-Lysine 9 (H3K9) trimethylation and higher H3K9 acetylation levels were observed in the SPI group than in the CAS and GEN groups. In **Study 2**, serum and hepatic triacylglycerol and cholesterol levels were significantly lowered in dams fed the SPI diet as compared to those fed the CAS diet. Similarly, the SPI group showed lower serum triacylglycerol and cholesterol levels than the CAS group. The specific gene expression responses altered by the SPI diet were associated with the peroxisome proliferator-activated receptor alpha signaling that contributes to the hypolipidemic effect. Interestingly, these gene expression changes were associated with the relative liver weight of the offspring. In **Study 3**, relatively severe liver damage was observed later in life in the SPI/EtOH group compared to the CAS/EtOH group. The activities of the aminotransferases were higher in the SPI/EtOH group than in the CAS/EtOH group. The expression of the genes involved in the endoplasmic reticulum stress and inflammatory response was higher in the SPI/EtOH group. Moreover, the one-carbon metabolism was more affected in the SPI/EtOH group. Interestingly, serum cholesterol level was lower in the SPI/EtOH group than in the CAS/EtOH group, and that there was a significant correlation between liver damage indicators and the gene expression

related to HDL-cholesterol metabolism. However, the lipid-lowering effect observed in the 3-week-old offspring of the SPI group disappeared in the control group at 15 weeks of age.

Conclusion: Liver development and growth process of male offspring at 3 weeks of age were affected by the maternal SPI diet. When male offspring were exposed to ethanol later in life, the potential effect of the maternal diet was sustainable, so that offspring from dams fed the SPI diet developed severe liver damage in comparison with offspring from dams fed the CAS diet. Taken together, this study showed that maternal dietary protein source may be responsible for the retarded liver development and growth in early life, which may influence the offspring's susceptibility to the development of liver disease in later life.

Key words: chronic ethanol consumption, liver damage, liver development, maternal dietary protein source, rat offspring, soy protein isolate

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List of Abbreviations

AAA	Aromatic amino acid
BCAA	Branched chain amino acid
BHMT	Betaine-homocysteine methyltransferase
CBS	Cystathionine beta-synthase
CHOP	DNA-damage inducible transcript 3
CYP	Cytochrome P450
DEGs	Differentially expressed genes
DNMT	DNA methyltransferase
FFA	Free fatty acid
GADD45b	Growth arrest and DNA damage-inducible 45 beta
GNMT	Glycine *N*-methyltransferase
GOT	Glutamic-oxaloacetic transaminase
GPT	Glutamic-pyruvic transaminase
H3K9Ac	Histone H3 acetyl-lysine 9
H3K9Me3	Histone H3 trimethyl-lysine 9
Hcy	Homocysteine
LCAT	Lecithin cholesterol acyltransferase
MAT	Methionine adenosyltransferase
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase
PEMT	Phosphatidylethanolamine *N*-methyltransferase
GSK3b	Glycogen synthase kinase 3b
PPARα	Peroxisome proliferator-activated receptor alpha
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
TNFα	Tumor necrosis factor alpha
Xbp-1	X-box binding protein 1

I. Introduction

1. Background

Clinical, epidemiological, and animal studies have suggested that the maternal nutritional status during pregnancy and/or lactation is extensively associated with the development and growth of offspring (Burdge et al., 2007a; Godfrey and Barker, 2001; Symonds, 2007). Maternal under- or over-feeding alters the physiological and structural phenotypes of offspring. Fetal life is an important period to develop the organs, so the manipulation of the availability of the dietary components during pregnancy and lactation may determine the organ plasticity. During this critical time, fetus is likely to adapt the abnormal environment undergoing the cell proliferation (Barker, 1997). In the late gestation, the rates of the cell division and fetal growth are decreased, and this phenomenon is consistently remained after birth, and the low body weight gain during infancy has a relation with a cardiovascular risk in later life. This mechanism is described by the concept of “fetal origins hypothesis” or “fetal programming”.

Liver is the largest organ and plays an pivotal role in the various adaptation of metabolic homeostasis and detoxification (Hyatt et al., 2008). In particular, fetal and prenatal liver growth and development may be influenced by the placental environment or maternal nutrition status during pregnancy and lactation. Early liver growth and development is also associated with the multiple organ organogenesis and has a different adaptive stage (Desai et al., 1996; Latini et al., 2004; Morrison et al., 2010). Liver size and growth are more sensitively response to the utero-perturbation during early critical life period to protect and compensate for the development of other organs,

such as brain and heart (Hyatt et al., 2008). Liver development is greatly retarded under poor maternal nutrition, and this compromised response may contribute to induce a rapid hepatic cellular proliferation in late gestation and early postnatal life (Morrison et al., 2010).

Microarray technique has extensively been used for revealing the gene expression modified by the nutrients. Hepatic gene expression throughout the lifetime may be influenced by the diet and age difference. In particular, gene expression changes in early liver development are different from those of adult period. So, gene expression profiles are useful for monitoring liver development. Genes involved in cell cycle and steroid and xenobiotic metabolism are dominantly changed in pre- and early postnatal liver compared to the adult liver. Previous study reports hepatic gene expression changes occurred during early development phase to adulthood (Lee et al., 2012). Fetal liver shows a dramatic gene expression related to cell proliferation and cell signaling compared to the adult liver, and more differentially regulated genes are identified in earlier stages. Gene expressions related to the xenobiotic metabolism are sorted into three phase: cytochrome P450s; conjugating enzymes; and transporters, and those gene changes critically regulated by the age dependence may be a reflection of the delayed liver growth rate and functions. These different gene patterns may contribute to the regulation of detoxification in adulthood.

The previous studies have shown the beneficial effects of soy protein on lipid metabolism compared to any other animal protein source, such as casein or casein-related source, in human and animal studies (Carroll and Kurowska, 1995; Orgaard and

Jensen, 2008; Torres et al., 2006). These effects may be associated with the phytochemicals, such as isoflavones, saponins, and polyphenols, found in soy protein may contribute to the reduction of the triacylglycerol or cholesterol levels. The different amino acid compositions of soy protein from the animal protein source may also contribute to lipid-lowering response as a potential beneficial component. In rats, soy protein with low isoflavone is associated with the reduction of serum triacylglycerol and cholesterol levels, suggesting that amino acid composition rather than isoflavone *per se* may act as a key regulator in hypolipidemic effect (Fukui et al., 2002; Shukla et al., 2007). Different protein sources may affect the hepatic gene expression. In adult rats, soy protein isolate diet not only shows a lipid-lowering effect but also changes the gene expression related to the lipid and xenobiotics metabolism compared to casein diet (Tachibana et al., 2005). Previous study reports that the expression of the genes involved in lipid metabolism and cell proliferation regulation is higher in rats fed an SPI diet with or without isoflavone compared to casein diet, suggesting that soy protein composition *per se* rather than the phytochemicals may be an influencing factor (Iqbal et al., 2002). However, limited studies have examined the role of soy protein or soy protein isolate related to maternal diet effect on metabolic changes in the adult offspring. Few studies have examined the role of maternal soy protein or soy protein isolate on the liver development and growth. As well, the adaptive responses of offspring to metabolic challenges in later life have not been well understood.

2. Study of objectives

Hypothesis

Protein sources of maternal diet provided for 2 weeks before mating and throughout pregnancy and lactation may influence liver development and its function in earlier and later life. Therefore, this study focused on two parts, (1) hepatic gene expression and liver development of 3-week-old male offspring and (2) the risk of the development of liver diseases in adult male offspring. The flow chart for the hypothetical process is shown in **Figure 1.1**.

Specific aims

The specific aims of the short-term (**Study 1** and **2**) and long-term (**Study 3**) study were:

Study 1: To investigate whether maternal consumption of a low-isoflavone soy protein isolate diet or a casein plus genistein (250 mg/kg diet) diet would alter the liver development of rat offspring at 3-week of age and the hepatic gene expression and epigenetic regulation.

Study 2: To investigate whether the component(s), either amino acid profiles or genistein, would regulate lipid metabolism of rat offspring at 3-week of age.

Study 3: To investigate whether the adaptive response of rat offspring to chronic ethanol consumption would affect the development of liver disease and cholesterol metabolism later in life.

Rationale of diet model

Several studies have evaluated the effect of genistein as one of the major components of soy protein isolate on fetal programming. However, the role of the other components, such as amino acid compositions, in early development has been less studied. Therefore, the dietary model used in this present study is designed to compare the effect of casein and soy protein isolate in early liver development and growth and to figure out the key component(s) of soy protein isolate. In the short-term study (**Study 1** and **2**), the effects of soy protein isolate with low-isoflavone (approximately 36.4 mg aglycone equivalent per kg diet) are compared with those of a casein as a different protein source or a casein supplemented with the purified genistein (250 mg per kg diet). In the long-term study (**Study 3**), after weaning, male offspring originated from the same dams used in the short-term study are fed the standard chow diet to remove the influence of maternal diet. To induce the liver damage, adult offspring are fed a liquid diet with 36% of the calories derived from ethanol, which is mainly metabolized in liver and is good for dealing with the moderate to severe damage.

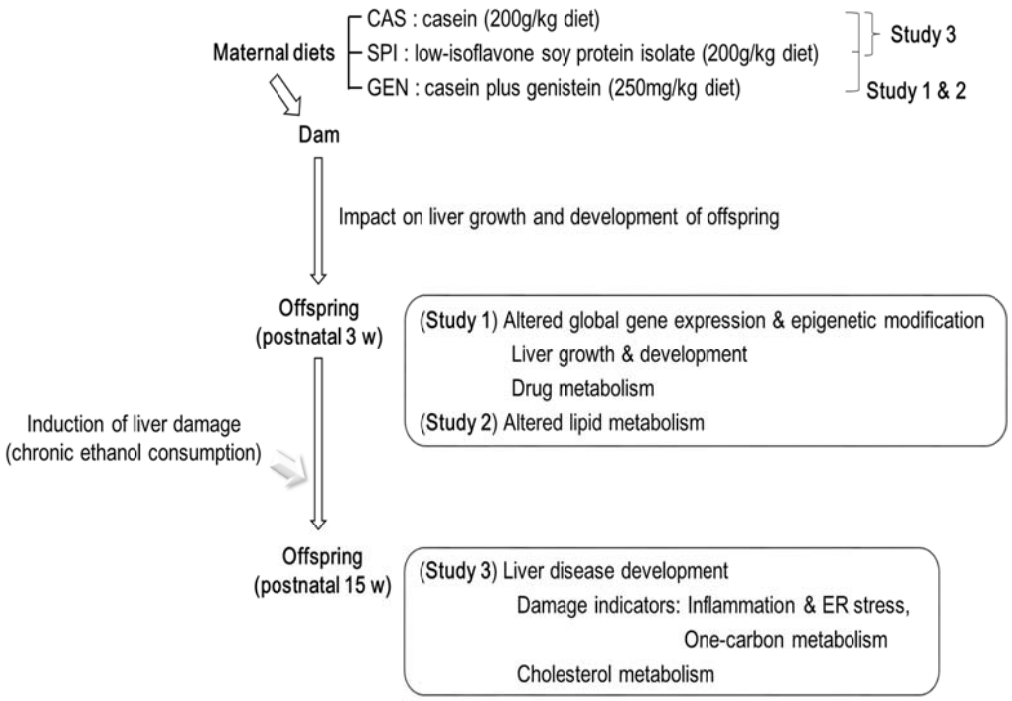


Figure 1.1. Flow chart for the hypothesis to determine the effect of maternal diet on early and later life of offspring.

II. Literature review

1. Maternal nutrition and offspring health

Prenatal maternal exposure to the specific nutrients, toxic materials, or stress condition may alter the physiological and structural phenotypes of the offspring. The manipulation of the availability of the nutrients during the gestation and lactation has been used to determine the organ plasticity. Fetal life is critical period to develop the tissue and organ of the body, and at this critical time, the fetus is likely to adapt the abnormal environment undergoing the cell proliferation (Barker, 1997). In the late gestation, the rates of the cell division and fetal growth are decreased. This phenomenon is consistently remained after birth, and the low body weight gain during infancy has a relation with a cardiovascular risk in later life. Barker and colleagues have extensively reported the effect of the growth rate on the cardiovascular disease (Barker et al., 1989), hypertension (Barker et al., 1990), insulin sensitivity (Eriksson et al., 2002), and Type II diabetes (Pulizzi et al., 2009). In addition, the fetal growth and the development of the type II diabetes in adult life are associated with the maternal malnutrition contributing to the fetal malnutrition (Hales and Barker, 2013). Among the nutritional components, the amino acid supply plays a key role in the malfunction of the organs, such as liver, beta-cell, and so on. The beta-cell malfunction leads to fetal growth retardation consequent to the adaptation of the adult beta-cell function. This mechanism is described by the concept of “fetal origins hypothesis” or “fetal programming” (**Figure 2.1**). The effect of the suboptimal offspring condition mediated by the maternal nutrition manipulation including protein restriction, calorie restriction, intrauterine growth restriction (IUGR), and methyl donor deficient diet on the

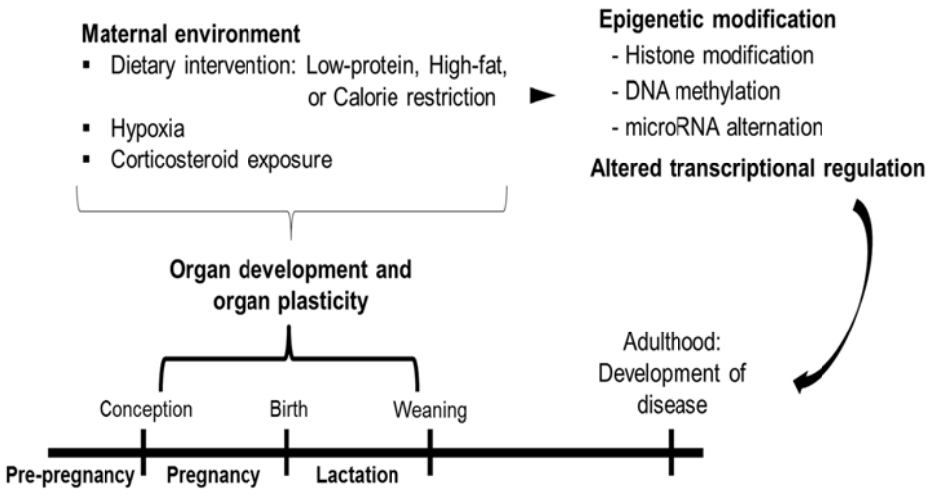


Figure 2.1. Fetal programming and the development of diseases in adulthood. The specific maternal environmental conditions contribute to the development of the organ in the early life through the epigenetic modification, related to the disease susceptibility in the adulthood. (modified from (Vo and Hardy, 2012))

organogenesis, the development of the endocrine system, and homeostasis mechanism involved in glucose and lipid is studied in various animal models. In particular, the physiological changes of the offspring exposed to the maternal protein restriction (isoenergetic) diet during pregnancy and lactation have extensively been published in rat offspring (**Table 2.1**).

2. Epigenetic regulation

Specific dietary intervention during the periconceptional period may modify the methionine-homocysteine cycle and DNA methylation consequent health problem in adult life (Sinclair et al., 2007). **Table 2.2** shows the effect of the maternal dietary intervention during pregnancy and lactation on the epigenetic modification in offspring liver.

2.1 One-carbon metabolism

Homocysteine (Hcy) and methyl group metabolism play a pivotal role in the improvement of health. *S*-adenosylmethionine (SAM) derived from methionine is a major methyl donor and is responsible for the various transmethylation reactions such as DNA biosynthesis (Nieman and Schalinske, 2011). Particularly, the methyl group metabolism via SAM is involved in the epigenetic modification. As shown in **Figure 2.2**, there are various methyltransferases acting as a key regulator responsible for the SAM-dependent transfer of the methyl group to *S*-adenosylhomocysteine (SAH), and the SAM/SAH ratio is an indicator of the methylation capacity. Methionine is obtained

Table 2.1. Protein restriction and its general physiological changes in rat

Exposure condition	Sample	General physiological change	Ref
Pregnancy & PND 10: Protein (20% or 10%: RP)	Rat Dam & Offspring (PND 21 & 70)	↓ body weight & food intake at lactation in RP dam ↑ corticosterone, testosterone, estradiol at gestation in RP dam ↓ body weight, LH, testosterone at PND in RP offspring	(Zambrano et al., 2005)
Pregnancy & PND 21: Protein (20% or 10%: RP, after weaning 20%; RC)	Rat Offspring (birth, PND 21 & 110)	No body weight difference in RP male offspring at birth ↓ body weight in RP female offspring at birth ↓ body weight in RP male offspring on PND 21 ↑ body weight in RC male offspring after weaning ↑ serum TG & CHOL in RC male offspring on PND 110	(Zambrano et al., 2006)
Pregnancy: Protein (20% or 8%: LP, after birth 20%)	Rat Offspring (embryonic D19 & PND 4m)	↑ glucose tolerance & AUC in LP offspring No *Lxrα* mRNA difference, ↓ *G6Pase* & *11bHsd1* mRNA in LP offspring at embryonic D19 ↓ *Lxrα*, ↑ *G6Pase* & *11bHsd1* mRNA & protein in LP offspring at PND 4m	(Vo et al., 2013)
Pregnancy & postnatal: Protein (20% or 8%: LP1 postnatal & LP2 weaning)	Rat Offspring (PND 130)	↓ body weight in LP offspring ↑ *Cyp3a23*, *Cyp2c11*, *Cyp2b1* mRNA in LP2 offspring No *Cyp3a23*, *Cyp3a2*, *Cyp2c11*, *Cyp2b1* mRNA in LP1 offspring ↑ *CAR* mRNA in LP2 offspring ↑ testosterone enzyme kinetic in LP2 offspring	(Sohi et al., 2014)
Pregnancy: Protein (20% or 5%: LP)	Rat Dam & Fetus	↓ body weight in LP dam & fetus ↓ number of fetus in LP dam ↓ liver weight in LP fetus ↑ mean area of total hepatocyte & cytoplasm of liver in LP fetus No difference in the mean area of nuclei of hepatocyte ↑ apoptosis, steatosis, ↓ proliferation ↑ glycogen cons, ↓ protein cons in LP fetus liver	(Ramadan et al., 2013)
Pregnancy & lactation: Protein (19% or 8%: LP, after PND 28 normal chow diet)	Rat Offspring (PND 65 & 150)	↓ body weight in LP offspring at birth & postnatal period ↓ liver weight in LP offspring, but no relative liver weight No serum TG & FFA, hepatic glycogen ↓ hepatic TG in LP male offspring at PND 65 & 150	(Qasem et al., 2010)

		↓ hepatic CHOL in LP male offspring at PND 150	
Pregnancy: Protein (24% or 6%: LP)	Rat Dam & Fetus	No body weight difference between dams ↑ plasma TG in LP dam ↑ α-amino nitrogen, & NEAA in LP dam at G18 & G21 ↓ BCAA & EAA in LP dam at G10 No difference in the translation initiation factor in dam ↓ placenta weight & fetus weight No difference in the litter number ↓ liver weight in LP fetus without the change of brain & heart ↑ lysine, glycine & serine, ↓ histidine in LP dam & fetus	(Parimi et al., 2004)
Pregnancy & lactation: Protein (170g or 60g: LP)	Rat Lactating or non-lactating dam & offspring	↓ body weight in LP dam & offspring ↓ liver weight, mammary gland in LP dam ↓ serum total protein, albumin, glucose, insulin, glucagon, prolactin in LP dam ↓ milk production in LP dam No difference in lipogenic enzymes except ↑ malic enzyme in LP dam, ↓ in non-lactating LP dam	(Moretto et al., 2011)
Pregnancy: Protein (17% or 6%: LP, after birth 21.6%)	Rat Offspring (PND 30 or PND 45 – 300)	↓ body weight in LP offspring at birth & No difference at PND 30 No difference in liver weight in LP offspring at PND 30 ↑ hepatic glycogen in LP offspring at PND 30 No difference in serum CHOL, TG, total protein & hepatic CHOL ↓ Ca^{2+}-induced mitochondrial swelling in LP offspring	(Moraes et al., 2014)
Pregnancy: Protein (180g or 90g; LP), 1) after birth, transfer to standard chow diet 2) self-selected food (fat, protein, carbohydrate)	Rat Offspring (PND 12w or 30w)	Food preference study: female showed more sensitive response for food type: at 12w offspring exposure to LP during fetal development self-selected a mainly fat-based diet After transferring to standard chow diet, no difference of body weight between control and LP at 12w ↑ Gonadal fat in LP at 12w	(Bellinger et al., 2004)
Pre & pregnancy & PND 12: Protein (20% or 10%: LP)	Rat Offspring (PND 32w)	↓ body weight in LP offspring at birth & 32w ↑ insulin sensitivity in LP offspring at 32w	(Lim et al., 2011)

Pre & pregnancy: Protein (18% or 9%: LP)	Rat Offspring (PND 15w)	No body weight difference in LP male offspring at birth ↑ systolic blood pressure in LP at 15w Impaired acetylcholine-induced vasodilatation in LP at 15w ↓ endothelial NO synthase mRNA in LP at 15w	(Torrens et al., 2006)
Pregnancy: Protein (20% or 10%: LP)	Rat Dam & Fetus	↓ body fat, hepatic lipids in LP dam No difference in body weight & liver weight ↑ insulin & leptin in LP dam No difference in serum TG, CHOL, HDL, LDL, VLDL ↑ stearic, ↓ oleic, arachidic, arachidonic, docosahexaenoic in LP dam ↓ desaturase & elongase in LP dam ↓ body weight, liver weight, liver lipids, brain lipids, DHA in LP fetus No difference in brain weight in fetus Inverse relationship between maternal insulin and fetal body weight	(Torres et al., 2010)

PND, postnatal day; RP, restricted protein; LH, luteinizing hormone; RC, restricted protein-casein; TG, triacylglycerol; CHOL, cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; LP, low-protein; AUC, area under the curve; FFA, free fatty acid; NEAA, *G6Pase*, glucose 6-phosphatase; *11bHsd1*, 11β-hydroxysteroid dehydrogenase type 1; *Lxrα*, Liver X receptor alpha; *CAR*, constitutive androstane receptor; G, gestation; non-essential amino acid; BCAA, branched chain amino acid; EAA, essential amino acid; DHA, docosahexaenoic acid; ↑: increased; ↓: decreased

Table 2.2. Maternal nutrient manipulation and epigenetic modification in rodent model

Nutrient or diet	Exposure condition	Tissue	Epigenetic change	Ref
Protein restriction	18% or 9%	Rat liver (PND 38)	↑ H3K9Me3 at ASN promoter region in LP	(Zhou and Pan, 2011)
	18%, 9%, or 8%+threonine	Rat liver, heart, kidney (fetal d21)	↑ DNA methylation in liver of LP	(Rees et al., 2000)
	20% or 8%	Rat liver (embryonic d19, PND 21 & 130)	↑ H3K9/14Ac in *Cyp7a1* promoter region: embryonic d19 in LP ↓ H3K9/14Ac & ↑ H3K9Me3 in *Cyp7a1* promoter region: PND 21 & 130 in LP	(Sohi et al., 2011)
	180g, 90g, or 90g+5 mg folic acid	Rat liver (PND 34 & 80)	↓ methylation of mean *Pparα* promoter via specific reduction at CpG dinucleotides in LP both 34 & 80d	(Lillycrop et al., 2008)
	180g, 90g, or 90g+5 mg folic acid	Rat liver (PND 34)	↓ methylation of GR_{10} promoter in LP ↑ H3K9Ac, H4K9Ac, H3K4Me & ↓ H3K9Me2 at GR_{10} promoter in LP ↓ *Dnmt1* & binding of *Dnmt1* at GR_{10} promoter in LP	(Lillycrop et al., 2007)
	180g, 90g, or 90g+5 mg folic acid	Rat liver (PND 34)	↓ methylation of *Pparα* & GR_{10} promoter in LP	(Lillycrop et al., 2005)
	180g, 90g, or 90g+3mg folic acid	Rat liver (PND 0)	↑ DNA methylation of *Igf2*/*H19* locus in LP ↑ *Dnmt1*, *Dnmt3a*, *Mbd2* in LP	(Gong et al., 2010)
	180g or 90g	Mouse liver (19.5d of gestation)	↑ DNA methylation of *Lxrα* promoter in LP	(van Straten et al., 2010)
	180g or 90g	Rat liver (PND 80 in F1 & F2)	↓ methylation of *Pparα* & GR_{10} promoter in F1 & F2 of LP	(Burdge et al., 2007b)
IUGR : Calorie restriction	Hypoxia	Rat liver (PND 4 & 12m)	↑ H3K9Me3 in *G6Pase* promoter in hypoxia	(Osumek et al., 2014)
	Uterine arteries' ligation	Rat liver (PND 0 & 21)	↑ H3K9Ac, H3K14Ac, H3K18Ac in IUGR at PND 0 & PND 21 male, not female ↑ H3K9Ac in *Pgc1* & *Cpt1* promoter region	(Fu et al., 2004)
	Uterine arteries'	Rat liver (PND 0 & 21)	↓ genomic DNA methylation ↑ H3Ac in IUGR at PND 0 & 21,	(MacLennan et al., 2004)

	ligation		but no difference in H4Ac ↓ *Dnmt1* in IUGR at PND 0, not at PND 21	
	Food restriction (50%)	Rat liver (PND 1 & 9m)	↓ H3K4Me2 in FR at Igf at PND 1 & 9m No difference in H3K4me3	(Tosh et al., 2010)
Folate deficient	0g: PND 3w-folate deficiency-> 8w-folate adequate-> 30w)	Rat liver (PND 30w)	↑ genomic DNA methylation: early folate deficiency	(Kotsopoulos et al., 2008)
Methyl deficient: choline, methionine	Low-methionine (0.18% vs. 0.4%) & no choline & folic acid for 36 & 54 w	Rat liver	↓ H4K20Me3 & ↑H3K9Me3: nodules & tumors	(Pogribny et al., 2006)
	Choline (0, 8, or 36 mmol/kg) at embryonic days (E17)	Rat fetal liver & brain	↓ H3K9Me2 & H3K27Me3 in choline deficiency ↑ H3K4Me2 in choline deficiency	(Davison et al., 2009)
	Choline (0, 8, or 36 mmol/kg) at embryonic days (E17)	Rat fetal liver & brain	↑ genomic DNA methylation in choline deficiency ↑ DNA methylation of *Igf*2 Dmr2 in choline deficiency ↑ *Dnmt1* & *Dnmt3a* in choline deficiency ↓ methylation of *Dnmt1* in choline deficiency	(Kovacheva et al., 2007)

PND, postnatal day; LP, low-protein; *Cyp7α1*, cholesterol 7 alpha-hydroxylase; *Pparα*, peroxisome proliferator-activated receptor alpha; GR_{10}, glucocorticoid receptor 10; H4K9Ac, histone H3 acetyl-lysine 9; H3K4Me, histone H3 methyl-lysine 4; H3K4Me2, histone H3 dimethyl-lysine 4; H3K9Me2, histone H3 dimethyl-lysine 9; H3K9Me3, histone H3 trimethyl-lysine 9; *Dnmt1*, DNA methyltransferase 1 ; *Dnmt3a*, DNA methyltransferase 3a; *Dnmt3b*, DNA methyltransferase 3b; *Igf2*, insulin-like growth factor 2; *Lxrα*, liver X receptor alpha; *G6Pase*, glucose 6-phosphatase; Mbd2, methyl-CpG binding domain 2; H3K18Ac, histone H3 acetyl-lysine 18; IUGR, intrauterine growth restriction; *Pgc1*, peroxisome proliferator-activated receptor gamma coactivator 1; *Cpt1*, carnitine palmitoyltransferase I; H3Ac, histone H3 acetylation; H4K20Me3, histone H4 trimethyl-lysine 20; H3K27Me3, histone H3 trimethyl-lysine 27 ; Dmr2, differentially methylated region 2 ; ↑: increased; ↓: decreased

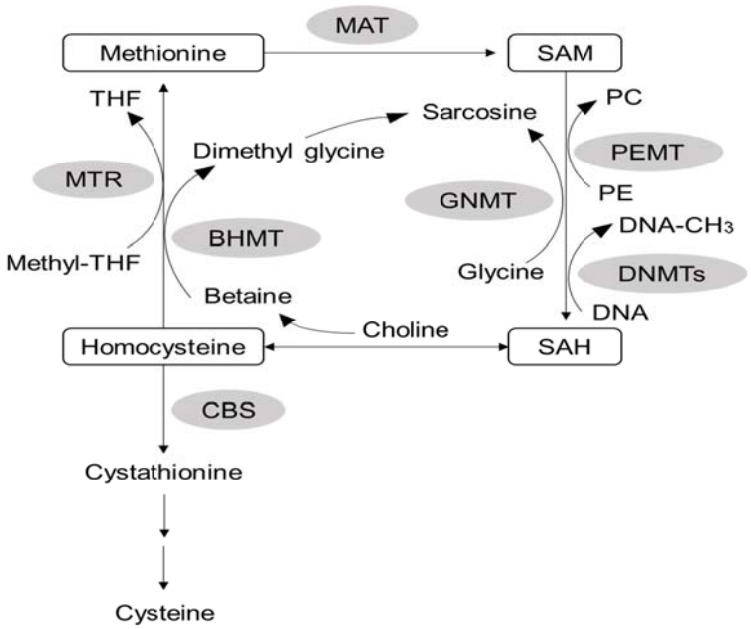


Figure 2.2. Overview of the methionine-homocysteine cycle. BHMT, betaine homocysteine methyltransferase; CBS, cystathionine beta-synthase; DNMTs, DNA methyltransferases; GNMT, glycine *N*-methyltransferase; MAT, methionine adenosyltransferase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltranserase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate

from the dietary source. Methionine adenosyltransferase (MAT) enzymes, mainly MAT1A and MAT2A, are responsible for converting methionine to SAM, and the deficiencies in these enzymes are known to increase methionine (Lu et al., 2001) suggesting that impairment of these enzymes could be responsible for the increase in methionine levels. Glycine *N*-methyltransferase (GNMT) and phosphatidylethanolamine *N*-methyltransferase (PEMT) are also associated with the regulation of the translation reactions. The GNMT is a cytosolic protein in liver and acts as a key regulatory protein for the conversion of glycine to sarcosine (Schalinske and Nieman, 2005). In the liver, phosphatidylcholine (PC) can be generated by two pathways, either Kennedy pathway or PEMT pathway (Obeid and Herrmann, 2009). Kennedy pathway is related to choline, the precursor of betaine. Betaine is the methyl donor and involved in the Hcy metabolism via betaine homocysteine methyltransferase (BHMT). The PEMT is abundant in liver and catalyzes the conversion of phosphatidylethanolamine (PE) to PC. So, the inhibition of these pathways may affect the phospholipid production. The Hcy is converted to SAH via SAH hydrolase and is metabolized either by methionine synthase (MS, or 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR)) or BHMT to methionine or by cystathionine β-synthase (CBS) to cystathionine. Moreover, the Hcy is involved in the induction of lipoprotein lipase (Lpl) via protein kinase C activation (Beauchamp and Renier, 2002). Lpl is the major lipolytic enzyme and involved in the hydrolysis of triacylglycerol in lipoproteins. This potentially leads to the triacylglycerol accumulation in liver. Most of all, the Hcy levels influence on the apoptosis and inflammation response linking to the

c-Myc signaling and endoplasmic reticulum (ER) stress.

2.2 DNA methylation

Epigenetics refers to the study of the heritable changes, which include DNA methylation and posttranslational modification of histones, in gene expression without change of DNA sequence (Dolinoy et al., 2007b). These changes play a pivotal role in the regulation of gene expression during early development. Particularly, the change of global DNA methylation may be regulated by the methyltransferases. SAM is a major methyl donor for DNA methyltransferases (DNMTs) involving in methylation reaction and being a metabolite of the Hcy-methionine cycle (Delage and Dashwood, 2008). There are three DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b. DNMT1 is responsible for the maintenance of global methylation patterns on DNA, whereas DNMT3a and DNMT3b are the de novo methyltransferases in fetal and postnatal organs and establish methylation patterns at the unmethylated CpGs (Dolinoy et al., 2007b) and show a catalytic function in global DNA methylation during mouse embryonic development (Auclair et al., 2014). Partial global hypomethylation was induced by the decrease in DNMT3a or DNMT3b, and the reduction of one enzyme can be compensated for by the other. Diet enriched in the methyl donors can enhance the methylation of CpGs (Dolinoy et al., 2007b). Phytochemicals can interfere with the Hcy-methionine cycle involved in the alteration of the SAM/SAH ratio and DNA methylation (Dolinoy et al., 2006). The most critical time points for the epigenetic reprogramming are the fetal period and the early postnatal development, and its altered

epigenome is heritable and maintained (Junien, 2006). DNA methylation of CpG-cytosine is conducted by DNMTs, which are expressed in most dividing cells (Schaefer et al., 2007). Global DNA hypomethylation in the DNMT3a/3b knockout embryonic stem cells suppresses the cell differentiation associated with apoptosis (Jackson et al., 2004).

2.3 Histone modification

Global DNA hypomethylation and the reduced DNMTs may be associated with histone modification. Hypomethylation of histone H3 lysine 9 (H3K9) and histone H3 lysine 4 (H3K4) and hyperacetylation of H3K9 have shown to be related to DNA hypomethylation (Delage and Dashwood, 2008). This indicates that H3K9 may have antagonistic response between the histone H3 acetyl-lysine 9 (H3K9Ac) and histone H3 methyl-lysine 9 (H3K9Me). Previous study showed that the age-associated with the site specific histone modification in liver (Kawakami et al., 2009). Acetylation of H3K9 is affected by aging related to cell proliferation, so its expression has been decreased following by aging rat liver. Other studies (Pogribny and James, 2002; Pogribny et al., 2004; Pogribny et al., 2007a) have showed that methyl deficient diets induce global and regional DNA hypomethylation and lead to the progressive decrease of histone H3 trimethyl-lysine 9 (H3K9Me3) and increase of H3K9Ac in liver during the early stage of carcinogenesis.

3. Liver development and function

3.1 Liver development

Liver is the largest organ and responsible for the various adaptation of metabolic homeostasis and detoxification (Hyatt et al., 2008). These functions in fetal and prenatal liver growth and development are closely associated with placental and/or maternal nutrition in specific gestation stage. Critical windows of the developmental plasticity are likely to involve in programming mechanism (Chmurzynska, 2010; McMillen and Robinson, 2005). In rat model, the fetal programming metabolism is corresponded to the developmental plasticity based on the following periods: in the early gestation, embryonic phase of development (day 0~7 of pregnancy) is associated with the epigenetic regulation of gene expression; in the mid-gestation, the period of organogenesis (day 8~14 of pregnancy) is associated with the cell cycle regulation; in the late gestation, the period of rapid fetal growth and development of integrated systems (day 15~22 of pregnancy) is associated with the adaptive metabolic responses. Liver growth is induced by the combination of hepatocyte hypertrophy and proliferation. Growth of the multiple organs of offspring shows a different adaptive pattern (Desai et al., 1996; Latini et al., 2004; Morrison et al., 2010). Among the organogenesis, liver size and growth are more sensitively affected by utero-perturbation during the critical period of the development to protect and compensate for other organs' function (Hyatt et al., 2008). Under poor maternal nutrition status, liver growth is greatly decreased compared to other organs like brain and heart, which are hardly influenced. This compromised response may contribute to induce a rapid

hepatic cellular proliferation in late gestation and early postnatal life and unfavorable effect on glucose and lipid metabolism (Morrison et al., 2010). After birth, the pattern of liver development is unique. During the postnatal period, the maturation processes of liver include the different achievement of the adult gene expression, the increase in liver size, and the alteration of the mechanisms related to cell proliferation and differentiation (Septer et al., 2012). Maternal low protein diet may affect the structure and proliferative capacity of fetal liver in rats (Ramadan et al., 2013). In liver cell proliferation analysis, hepatocyte proliferation of fetal from dams fed a low protein diet was decreased. Therefore, fetal liver growth programming is a pivotal role to improve offspring survival and long-lasting effect on maintaining metabolic homeostasis in later health (**Figure 2.3**).

3.2 Xenobiotic metabolizing enzymes

Liver growth and development is associated with the induction of xenobiotic metabolizing enzymes. In developing female rat, the relationship between Cyp3a1 expression and low birth weight of offspring is observed with the relative liver weight change at different ages (Zhu et al., 2013). Expression of Cyp3a1 is peak at PND 21 in offspring with low birth weight, whereas that of Cyp3a1 in the control group is peak at PND 3 and 14 then decreased after PND 56. The increased relative liver weight of offspring from the undernourished maternal diet group shows a relation from the increase in hepatic metabolic activity regarded as an essential requirement for fetal development, indicating that the increased Cyp3a1 expression and relative liver weight

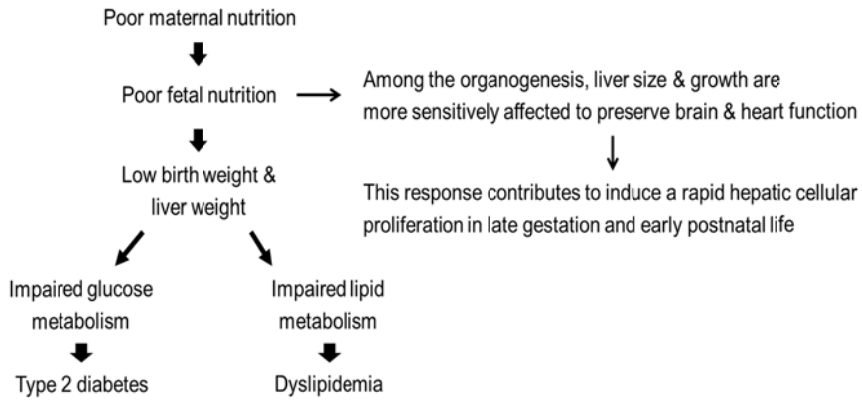


Figure 2.3. The association between the maternal malnutrition and liver function.

may be beneficial in response to early fetal survival. These CYPs are regulated by the nuclear receptor, and these response patterns are closely associated with the epigenetic modification. The constitutive androstane receptor (CAR, NR3I1), which is located in the cytoplasm, is one of the nuclear receptor superfamily and regulates the drug metabolism, energy homeostasis, and cell proliferation (Yang and Wang, 2014). The CYPs, particularly, Cyp2b and Cyp3a families, induced by the CAR may be along with the increase of the liver weight and DNA synthesis (Wei et al., 2000). Growth arrest and DNA-damage-inducible, beta (Gadd45b), another CAR-induced gene, is also involved in liver hyperplasia through the increase in cell proliferation (Tian et al., 2011). Gadd45b binds to CAR as a coactivator, and this anti-apoptotic gene is induced in the exposure to xenobiotic, such as 1,4-bis[2-(3,5)-dichoropyridyloxy] benzene (TCPOBOP). Under the administration of TCPOBOP, which induces hepatic drug-metabolizing enzymes and rapid liver growth, the *Gadd45b*-/- mice show a significantly decreased liver mass. In addition, Gadd45b expression is more strongly dependent on the CAR activation rather than tumor necrosis factor alpha (TNFα)-NFκB signaling (Columbano et al., 2005). In particular, Cyp2b10 gene, a strong target gene of CAR in mice, is upregulated by Gadd45b. Furthermore, CAR activation in neonatal period affects the epigenetic modification (Chen et al., 2012b). The increased histone H3 trimethyl-lysine 4 is associated with Cyp2b10 locus, whereas H3K9Me3 is decreased in mouse liver. During liver regeneration, caspase-3 protein level in *Gadd45b*-/- mice exhibiting the impaired hepatocyte proliferation is increased compared to the wild-type mice, whereas the proliferating cell nuclear antigen (PCNA)

protein level is suppressed in *Gadd45b*-/- mice (Papa et al., 2008). Under the cellular stress response, the increased Gadd45b gene protects from the apoptosis (Gupta et al., 2005) and interacts with PCNA (Liebermann and Hoffman, 2008). CAR activation involves the induction of hepatocyte proliferation (Huang et al., 2005), suppression of apoptosis (Hasmall and Roberts, 1999), and hepatic hyperplasia via the increased gene expression of c-Myc and forkhead box protein M1 (FoxM1) (Blanco-Bose et al., 2008). In addition, CAR activation induces the suppression of gluconeogenesis through the pathway involving the hepatocyte nuclear factor 4 alpha (HNF4α) and forkhead box protein O1 (FoxO1) (Kodama et al., 2004; Miao et al., 2006). In lipid homeostasis, CAR activation mediated by the exposure of phenobarbital reduces serum triacylglycerol (Kiyosawa et al., 2004; Roth et al., 2008) and high density lipoprotein (HDL) level (Masson et al., 2008), but it increases serum and hepatic cholesterol level (Kiyosawa et al., 2004). As well, bilirubin clearance is stimulated by the UDP glucuronosyltransferase 1 family polypeptide A1 medicated by CAR activation (Huang et al., 2003). However, TCPOBOP treatment shows the different pattern. CAR activation by TCPOBOP treatment reduces serum total cholesterol and HDL-cholesterol levels in the normal chow through the regulation of the cholesterol uptake genes (Rezen et al., 2009). Unexpectedly, hepatic cholesterol level is also lowered by the TCPOBOP treatment thanks to the increased bile acid synthesis via the alternative pathway except cholesterol 7 alpha-hydroxylase (Cyp7a1) and the activation of bile efflux. In microarray result, CAR activation increases hepatic export system of bile acids transporters. However, CAR activation by the TCPOBOP treatment inhibits

hepatic lipogenic genes, very low density lipoprotein (VLDL) secretion, and hepatic steatosis resulting in the lower-triacylglycerol level in high-fat diet-fed mice (Gao et al., 2009). As well, CAR activation by the TCPOBOP treatment indices the incomplete fatty acid overloading. Another major nuclear receptor, Pregnane X receptor (PXR, NR1I2), is also involved in the induction of the xenobiotics and regulator of lipid metabolism (Lopez-Velazquez et al., 2012). PXR is highly expressed in the liver, intestine, and kidney (Ihunnah et al., 2011). Particularly, PXR is responsible for the induction of CYP enzymes, such as Cyp3a4 Cyp3a23, Cyp3a11, Cyp2b6, and so on, in rodents and humans. As well, PXR can regulate the UDP-glucuronosyltransferases, sulfotransferases, and glutathione *S*-transferases. In addition, PXR can regulate triacylglycerol and cholesterol metabolism regardless of the sterol regulatory element-binding protein (Zhou et al., 2006). PXR increases lipogenic enzymes, such as cluster of differentiation 36 and stearoyl-CoA desaturase-1, whereas the carnitine palmitoyltransferase 1A and 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 enzymes responsible for β-oxidation are decreased (Lopez-Velazquez et al., 2012). These results lead to hepatic steatosis via increasing hepatic triacylglycerol, cholesterol, and phospholipid levels and increase in the relative liver weight. The relationship between PXR and HDL-cholesterol metabolism is observed in rodent model (Bachmann et al., 2004). PXR agonist induces the activity of Cyp3a, and there is a strong positive correlation between the Cyp3a and apolipoprotein A1 and HDL-cholesterol levels.

3.3 Cholesterol metabolism in the liver

Cholesterol is indispensable component of the plasma membrane and the precursor of the steroid hormones and bile acids. In animals and clinical studies, the disturbance of cholesterol metabolism may be associated with various disease conditions, such as cholesterol gallstone, atherosclerosis, and neurological disorders (Bjorkhem et al., 2010; Marschall and Einarsson, 2007; McNamara, 2000). Liver is a key organ in cholesterol metabolism responsible for cholesterol synthesis and disposal of cholesterol (Rai et al., 2013). Major hepatic cholesterol sources are originated from the intestinal absorption of dietary cholesterol via chylomicron remnants, *de novo* synthesis in the liver, and cholesterol synthesized in the extrahepatic tissues. Major routes by which cholesterol leaves from the liver are the VLDL secretion, free cholesterol secretion via ATP-binding cassette sub-family G member 5/8 transporter (Abcg5/8), and the conversion to bile acids/salts. In plasma, cholesterol is carried by the various lipoprotein fractions. According to the density, there are four lipoprotein classes, VLDL, intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and HDL. In addition, cholesterol is required for cell proliferation and mitosis completion (Fernandez et al., 2004). Cholesterol deficiency in the cells may reduce the DNA ploidy. During early pregnancy in the rat, maternal cholesterol transfers into the fetus and maternal cholesterol contributes substantially to fetal cholesterol requiring for organogenesis (Baardman et al., 2013). Maternal total cholesterol levels are increased by 30-50% from the enhanced cholesterol synthesis in liver. Maternal low cholesterol level may be related to the regulation of the lipid metabolism in the fetal. Maternal

protein restriction reduces the gene expression related to cholesterol metabolism and fatty acid synthesis in the fetal liver (van Straten et al., 2010).

HDL-cholesterol originates from enterocyte, hepatocyte, and a minor portion (Davidson and Toth, 2007), and HDL-cholesterol biosynthesis is very complicated process. Various key enzymes synthesized in the liver are responsible for HDL production and the assembly. The scavenger receptor B type 1 (Srb1) is primarily expressed in hepatic and steroidognic cells and leads to selective uptake of cholesterol ester from the lipid rich (Van Eck et al., 2003). Srb1 deficiency leads to the impaired biliary cholesterol secretion in liver, whereas in the arterial wall, Srb1 deficiency is associated with a severe dysregulation of cholesterol homeostasis and induction of inflammation and atherosclerosis. HDL is comprised of two main apolipoproteins (Apo), Apoa1 and Apoa2. Apoa1 is the primary structural HDL (approximately 70% of protein) and produced by the liver and intestine, whereas Apoa2 is synthesized in liver and frequently combined with Apoa1 (Davidson and Toth, 2007). In reverse cholesterol transport, the released Apoa1 into the plasma binds phospholipids to form nascent discoidal or pre-β-HDL and stimulates the activity of the ATP-binding cassette, sub-family A, member 1 (Abca1) and lecithin cholesterol acyl transferase (Lcat). Later, HDL-cholesterol binds to Srb1 on the hepatocyte surface, and this interaction is mediated by Apoa1. Apoa2 deficiency is related to the reduction of HDL-cholesterol level. Serum Hcy level reduces the hepatic expression of Apoa1 followed by plasma Apoa1 and HDL-cholesterol (Obeid and Herrmann, 2009). HDL assembly is initially involved in the cell surface Abca1-mediated the transfer of cellular phospholipid and

cholesterol to the extracellular lipid-poor Apoa1 (Zannis et al., 2006). Because liver is the major organ of lipoprotein synthesis and catabolism, liver dysfunction is likely to regulate plasma lipids. Patients with acute or chronic liver injury shows the decreased level in total cholesterol, HDL-cholesterol, and so on, indicating that lipid and lipoprotein levels can be regarded as the indicator of the degree of liver damage (Luo et al., 2010; Mandal et al., 2013). Particularly, circulating HDL-cholesterol level and its function may act as a key factor for anti-inflammation and consequent liver damage. Chronic inflammation is likely to reduce HDL-cholesterol level and its function, suggesting that low HDL-cholesterol level may be an indicator for the severity of inflammation (Lim et al., 2007). Interestingly, the activity of Lcat, one of HDL-cholesterol regulating factors, has been considered as the liver damage indicator. In liver disease, Lcat deficiency is associated with the failure to cholesterol esterification leading various cellular and metabolic disorders. Hepatic Lcat mRNA level is lowered compared to other secreted hepatic proteins (Bingle et al., 1991). Blood cholesterol levels are changed by the liver injury, and the low cholesteryl ester level is attributed to parenchymal liver damage (McIntyre, 1978). The degree of parenchymal liver damage is related to the range of Lcat activity, and Lcat deficiency increases serum free cholesterol. Abnormal HDL of liver disease also links to the disturbance of humoral and cellular immunity. In lipopolysaccharides (LPS) or TNFα-induced liver damage animal model, HDL-cholesterol and cholesterol levels are reduced (Ly et al., 1995). LPS and TNFα administration inhibits hepatic Lcat mRNA level contributing to decrease in plasma Lcat activity. Methyl enzymes may serve as a marker for HDL-

cholesterol metabolism. Abca1, Lcat, and Srb1 are linked to phospholipid metabolism (Lewis and Rader, 2005; Yancey et al., 2003), so a decrease in Pemt mRNA expression may contribute to the reduction of phospholipid production. Other methyl enzyme, Gnmt, is also involved in the reverse cholesterol transport. Recent study shows that Gnmt deficiency is attributable to the reduction of Abca1, Srb1, and Abcg5/8 and accelerates the inflammation markers (Chen et al., 2012a).

3.4 Ethanol-induced liver injury

Alcohol is mainly metabolized in liver and a major contributor to liver-related injury in both early and later life (Keegan et al., 1995; Meyers et al., 2002). Previous clinical study suggests that the association between birth size and adult mortality from liver damage is potentially related to alcohol intake (Andersen and Osler, 2004). Liver is the only location for ethanol metabolism, but its metabolic response is not fully understood. Several possible hypotheses suggest that ethanol exposure can be linked to the redox shifts generated by the increased production of nicotinamide adenine dinucleotide (NADH). In liver, alcohol converts to acetaldehyde and acetate by alcohol dehydrogenase and aldehyde dehydrogenase, respectively (Lieber, 2000). This major process produces more reduced NADH from nicotinamide adenine dinucleotide (NAD). The increased NADH production inhibits the activity of the citric acid cycle and fatty acid oxidation and then contributes to the inhibition of gluconeogenesis. Under the chronic ethanol exposure, the second major pathway uses the microsomal ethanol-oxidizing system catalyzed by cytochrome P450 2E1 (Cyp2e1), which contributes to

ethanol degradation as a major risk factor (Lieber, 2000). The activation of the Cyp2e1 induced by ethanol exposure produces the reactive oxygen species, which enhance lipid peroxidation and change the signal transduction pathways. The metabolic consequences of hepatic ethanol exposure may develop the progression of alcoholic liver injury including fatty liver, hepatocyte necrosis, inflammation, fibrosis, and cirrhosis (Lieber, 2000). Break of the balance of liver homeostasis in chronic ethanol exposure causes an increase in the Hcy level associated with the disturbance of methylation capacity (Ji, 2012; Sozio and Crabb, 2008). The perturbation of the ER homeostasis stimulated by the Hcy is also a key factor in the pathogenesis of ethanol related liver damage (Godfrey and Barker, 2001; Ji, 2012). Hcy and ER stress response up-regulated by ethanol feeding contributes to dysregulate lipid metabolism (Chen et al., 2012a). Although there is a controversial issue related to HDL-cholesterol level in ethanol intake, chronic ethanol exposure may exacerbate HDL-cholesterol metabolism through the down-regulation of Srb1, Abca1, and Lcat levels. Chronic alcoholic intoxication also alters liver metabolism. A significant decrease in HDL-cholesterol level and Lcat activity are observed in cirrhotic patients (Lemberg et al., 2007). In other study group showing the similar association between HDL-cholesterol-mediated by Lcat and alcoholic liver damage, an elevated plasma HDL-cholesterol level is observed among alcoholics without liver disease, but not in alcoholics with liver disease (Athukorala et al., 1988). Particularly, serious liver dysfunction in alcohol consumption suppresses hepatic production of nascent HDL. HDL-cholesterol level in alcoholics without liver disease is positively associated with a rise in HDL_3-cholesterol

rather than in HDL_2-cholesterol. Apoa1 and Apoa2 levels are also stimulated by alcoholics without liver damage, but gradually reduced by alcoholics with cirrhosis (Duhamel et al., 1984). Consistently, the amount of HDL_3-cholesterol in alcohol consumption is markedly reduced with progressive liver injury.

4. Soy protein

4.1 Components of soy protein

Soybean contains up to 50% protein, of which 90% is consist of the storage globulins, such as 11S globulin (glycinin) and 7S globulin (β-conglycinin) (Gianazza et al., 2003). Soy protein contains various bioactive components contributing to the beneficial effects, such as isoflavones, phytic acid, and saponins (Friedman and Brandon, 2001). Isoflavones are a subclass of flavonoids, and major isoflavones found in soy protein are genistein, daidzein, and glycitein. Soy protein isolate is obtained from the purified process of soy protein to increase protein content (more than 90% protein). **Table 3** shows the general composition and amino acid contents between casein (Reeves, 1997) and soy protein isolate (Kalman, 2014). Comparison with casein protein source, methionine content is lower in soybean, but higher cystine content is found.

4.2 Role of soy protein in health and diseases

In natural, animal protein sources show an increased cholesterol levels compared to the plant protein sources. In particular, soy protein is shown to lipid-lowering effect compared with any other animal protein sources in human and animal studies

Table 2.3. Amino acid contents of casein and soy protein isolate

Amino acid (g/kg)	Casein[1]	Soy protein isolate[2]
Alanine	22.6	35.9
Arginine	31.3	66.7
Aspartic acid	56.6	102.0
Cystine	3.5	10.5
Glutamic acid	181.0	174.5
Glycine	15.7	36.0
Histidine	22.6	23.0
Isoleucine	41.8	42.5
Leucine	76.6	67.8
Lysine	64.4	53.3
Methionine	22.6	11.3
Phenylalanine	43.5	45.9
Proline	101.8	49.6
Serine	47.0	45.9
Threonine	33.1	31.4
Tryptophan	10.4	11.1
Tyrosine	46.1	32.2
Valine	49.6	41.0

[1] and [2] data are derived from (Reeves, 1997) and (Kalman, 2014), respectively.

(Carroll and Kurowska, 1995; Orgaard and Jensen, 2008; Torres et al., 2006). Its beneficial effects on cholesterol reduction may be accomplished by the phytochemicals, mainly isoflavones, or different amino acid profiles, although there has a still controversial. Original studies related to amino acid compositions in soy protein have found there is a lower amounts of lysine and methionine and higher amounts of arginine and glycine compared to casein or casein-related sources. These different profiles affect the growth rate and regulation of insulin/glucagon ratio, so insulin can more stimulate lipid synthesis by casein intake compared to soy protein. In rats, soy protein with low isoflavone reduces serum triacylglycerol and cholesterol levels, suggesting that amino acid composition rather than isoflavone *per se* may be a key regulator in hypolipidemic effect (Fukui et al., 2002; Shukla et al., 2007). In addition, soy protein hydrolysis or peptides may be associated with the cholesterol-lowering effect. The storage proteins, such as 7S globulin and 11S globulin, reduce the cholesterol level in rats fed with hypercholesterol diet (Ferreira Ede et al., 2011). Cholesterol level is also reduced by the peptides digested from 7S globulin without containing isoflavones (Lovati et al., 2000). There are different features and concerns between cow's milk- and soy protein-based infant formula. Soy proteins do not only contain the same protein contents as cow's milk and lactose but also contain the isoflavone and phytochemicals (Merritt and Jenks, 2004; Society, 2009). To prevent the concern for protein quality and isoflavone, the additional amino acids are fortified in the commercial infant formula and soy protein isolate with low isoflavone is used. In recent decades, the relationship between soy infant formula and infant growth rate has

been studied, but their correlation effect is not clearly understood and a limited experiment condition is available such as protein or energy restriction and intrauterine (Bhasin et al., 2009; Metges, 2001). Sole protein source may play a pivotal role in body growth and tissue development, particularly in liver.

Maternal diet composition may contribute to the alteration of the lipid levels of both dam and offspring, and maternal serum free amino acid levels may be associated with the biochemical properties and the growth rate of the offspring. The previous study reports the differences in body weight and fat pad weight between offspring of dams fed a soy protein diet with isoflavone and offspring of dams fed a casein plus genistein diet, although similar levels of serum genistein are observed between two groups (Simmen et al., 2010). Soy protein isolate contains higher amounts of several amino acids including cysteine, arginine, and glycine than casein. Its low proportion of methionine among the total sulfur-containing amino acids has been shown to be involved in the beneficial effect of dietary soy protein on lipid metabolism (Sugiyama et al., 1986). Exposure to dietary soy protein isolate with isoflavone throughout the *in utero*, neonatal, and adult periods reduces hepatosteatosis in offspring of A^{vy} mice but do not change coat color proportions (Badger et al., 2008). Recently, maternal phytosterol consumption during pregnancy and lactation shows a lipid-lowering effect in offspring through the lowered maternal serum lipid and lipoprotein levels (Rideout et al., 2015).

III. Study 1

Effects of maternal low-isoflavone soy protein isolate consumption on hepatic gene expression and liver development in rat offspring

1. Abstract

Several studies in humans and experimental animals have demonstrated that *in utero* environment may have an impact on fetal developmental processes. Therefore, this study investigated whether maternal consumption of a low-isoflavone soy protein isolate (SPI) diet and a casein supplemented with genistein (250 mg/kg diet, GEN) diet would alter hepatic gene expression and liver development of rat offspring. Female Sprague Dawley rats were fed a casein (CAS) diet, an SPI diet, or a GEN diet for 2 weeks before mating and throughout pregnancy and lactation. Male offspring were studied at 3 weeks of age. Hepatic gene expression profiles from the offspring were determined by microarray analysis. The role of one-carbon metabolism in epigenetic regulation was also assessed. Male offspring of the SPI group had a significantly lower body weight and body fat mass than those of the CAS and GEN groups, whereas the relative liver weight was higher in the SPI group than the CAS and GEN groups. In microarray analysis, a total of 965 genes was differentially expressed in response to the maternal diet. Interestingly, the most statistically significant genes involved in xenobiotic and drug metabolism were altered by the SPI group compared to the CAS and GEN groups. Moreover, a significant correlation was found between the relative liver weigh and the gene expression related to xenobiotic and drug metabolism. A higher serum homocysteine level and global DNA hypomethylation were observed in the SPI group compared to the CAS group. According to the response in DNA methylation, expression levels of the reduced histone H3-Lysine 9 (H3K9) trimethylation and elevated H3K9 acetylation were observed in the SPI group. Taken

together, these results suggest that maternal SPI diet may contribute to the relatively slow development of liver in offspring and consequent changes in the hepatic gene expression profiles and epigenetic processes.

2. Introduction

In utero nutrition and other environmental factors can alter prenatal development, inducing permanent changes in the chronic disease susceptibility of humans and experimental animals (Burdge and Lillycrop, 2010; Ross and Desai, 2005). The long-term effects of maternal nutrition on the offspring's physiology and metabolism are related to modifications of major organs due to rapid cell proliferation during fetal and neonatal life (Engeham et al., 2010). The fetal liver is a key target for extracellular signals that instruct cells to proliferate, differentiate, or undergo apoptosis, which are essential processes in tissue and organ development. Because the liver has a major role in coordinating metabolism, changes in liver phenotype may have long-term metabolic consequences (McMillen and Robinson, 2005). Maternal undernutrition regulates gene expression in liver tissue in young adult male offspring rats. However, there were no significant changes in gene expression in white adipose fat or skeletal muscles, suggesting that major changes in gene expression in the liver precede those in other metabolically relevant tissues (Morris et al., 2009).

The methylation status of CpG islands in the promoter region of genes correlates with the gene expression patterns established during embryo development and cell differentiation (Zhou et al., 2011). Previous studies have shown that intrauterine nutrition can program adult disease susceptibility by altering the epigenetics of the fetal genome through processes such as DNA methylation and post-translational histone modification (MacLennan et al., 2004). Clinically relevant restrictions of vitamin B_{12}, folate, and methionine during the periconceptional period

alter the DNA methylation status of CpG islands in offspring, which may have long-term implications for adult health (Sinclair et al., 2007). A similar report was observed that the programmed changes in DNA methylation affected by lactation may be in differently response to sex-specific (Daniel et al., 2014). Conversely, feeding the epigenetically sensitive agouti viable yellow (A^{vy}) mouse a methyl-supplemented diet at conception led to increased DNA methylation at the A^{vy} locus and increased the longevity of the offspring (Waterland and Jirtle, 2003). Additionally, a high-fat maternal diet altered the DNA methylation patterns and transcription levels of cell cycle-associated genes, which may contribute to the delayed maturation of early postnatal livers (Dudley et al., 2011). While these changes may be limited to early development and not be affected in DNA methylation profiles of adult liver in spite of the sustained high-fat maternal diet effect (Cannon et al., 2014).

Exposure to dietary soy protein isolate with isoflavone throughout the *in utero*, neonatal, and adult periods reduced hepatosteatosis in offspring of A^{vy} mice but did not change coat color proportions (Badger et al., 2008). Conversely, supplementing the maternal diet with genistein promotes intracisternal A particle methylation and protects A^{vy} mouse offspring from obesity by modifying the fetal epigenome (Dolinoy et al., 2006). Moreover, maternal dietary supplementation with methyl donors or genistein counteracted the hypomethylating effect of bisphenol A on both the A^{vy} and the $Cabp^{IAP}$ metastable epialleles (Dolinoy et al., 2007a). Interestingly, *in utero* exposure to maternal diets containing soy protein isolate with isoflavone, but not genistein alone (250 mg/kg diet), protected young adult rat offspring from chemically

induced mammary tumorigenesis, suggesting the distinct fetal programming effects of soy protein isolate and genistein (Su et al., 2007a).

Although the effect of soy isoflavone in the form of the soy protein isolate component or supplementation on fetal programming has been well characterized, the effect of soy protein isolate with low levels of isoflavone in the same context has not been determined. Therefore, the present study would compare the effect of a low-isoflavone soy protein isolate (SPI) with 1) a casein as a protein source or 2) a casein protein supplemented with the purified genistein as a major isoflavone. This study investigated whether maternal nutrition could alter gene expression and development in the offspring livers. The present study reported that maternal consumption of the SPI diet contributed to epigenetic modifications in the offspring livers, which may be associated with the regulation of gene expression and the development of livers.

3. Materials and Methods

3.1 Diets

Experimental diets were prepared using the published AIN-93G formula (Reeves et al., 1993) except that soybean oil was replaced with corn oil. The composition of diets and amino acid contents of casein and soy protein isolate are shown in **Table 3.1** and **3.2**, respectively. Soy protein isolate used in the present study contained 52.42 mg isoflavone (7.47 mg genistein, 29.42 mg genistin, 2.73 mg daidzein, and 12.81 mg daidzin/200g protein/kg diet), which is 36.41 mg aglycone equivalents, as determined by HPLC. In general, total isoflavone content in commercial soy protein isolate ranges from 88 to 164 mg/100 g (Genovese et al., 2007). So, casein plus genistein diet contained casein as sole protein source to which genistein was added at 250 mg/kg diet. This level of genistein in the diet has been reported to result in serum genistein levels (~1.5 μmol/L) that were within the range of those found in humans consuming normal to high soy diets (Eason et al., 2005).

3.2 Animals

Seven-week-old virgin female Sprague-Dawley rats were obtained from the local animal facility (Orient Bio Inc., Korea) and were maintained in a temperature (22 ± 3°C) and humidity (50 ± 10%)-controlled room with a 12 h dark/light cycle. The experimental procedures used in the present study were approved by Seoul National University Institutional Animal Care and Use Committee. The study scheme is presented in **Figure 3.1**. After one week acclimation period, rats were randomly

Table 3.1. Composition of experimental diet

Composition (g/kg)	Diet		
	CAS	SPI	GEN
Casein[1]	200	-	200
Soy protein isolate[2]	-	200	-
Cornstarch	397.5	397.5	397.5
Dextrinized cornstarch	132	132	132
Sucrose	100	100	99.75
Corn oil	70	70	70
Fiber	50	50	50
Mineral mix[3]	35	35	35
Vitamin mix[4]	10	10	10
L-cystine	3	3	3
Choline bitartrate	2.5	2.5	2.5
t-Butylhydroquinone	0.014	0.014	0.014
Genistein[5]	-	-	0.25

[1] Protevit-S: Lactoprot Deutschland GmbH, Germany

[2] PRO-FAM® 974: ADM, USA

[3] AIN-93G-MX: Dyets Inc., USA

[4] AIN-93-VX: Dyets Inc., USA

[5] Chromadex Inc., USA

CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

Table 3.2. Amino acid contents of casein and soy protein isolate

Amino acid (g/100g protein)	Casein	Soy protein isolate
Alanine	3.2	4.3
Arginine	3.8	7.5
Aspartic acid	7.7	11.5
Cystine	0.45	1.2
Glutamic acid	24.4	19.2
Glycine	2.0	4.1
Histidine	3.2	2.7
Isoleucine	5.6	4.8
Leucine	10.1	8.0
Lysine	8.3	6.3
Methionine	3.2	1.4
Phenylalanine	5.4	5.2
Proline	10.2	5.2
Serine	6.6	5.5
Threonine	4.8	3.7
Tryptophan	1.3	1.1
Tyrosine	6.0	3.8
Valine	7.3	4.8

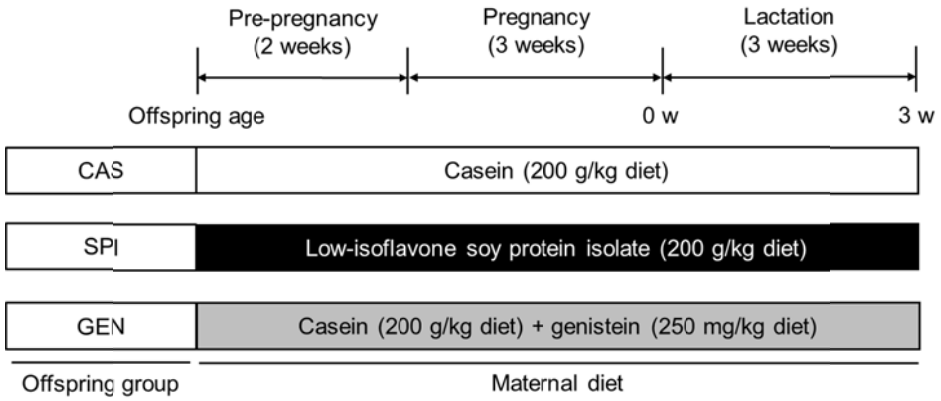


Figure 3.1. Overview of the study design.

assigned groups fed diets containing a casein (CAS) diet, an SPI diet, or a casein plus genistein diet (250 mg/kg diet, GEN). Experimental diets were provided for two weeks before mating and throughout pregnancy and lactation. Diets and water were provided *ad libitum*. Female rats were mated with proven male breeders. Mating was confirmed by detection of a vaginal plug, and this day was denoted as day 0 of gestation. Only females that were pregnant within 5 days of the introduction of the male were retained in the study. Dams were then housed individually until the end of experiment. Maternal body weight was measured every two days during pre-pregnancy period, everyday during pregnancy period, and every three day during lactation period. The litter size and pup body weight were recorded at birth, and litters were adjusted to 2 females and 6 males to normalize the growth. Male offspring were sacrificed on postnatal day 21 (3-week-old) after an overnight fast (CAS group: offspring of dams fed a CAS diet, SPI group: offspring of dams fed an SPI diet, and GEN group: offspring of dams fed a GEN diet). Blood samples were rapidly obtained by cardiac puncture. Tissues were removed, snap-frozen immediately in liquid nitrogen, and stored at –80°C until use.

3.3. Determination of serum homocysteine

The total serum homocysteine (Hcy) level was determined by HPLC method according to previously described protocol (Minniti et al., 1998). Briefly, a serum sample containing 2.5 mM acetylcysteine was reduced with tri-*n*-butylphosphine, and proteins were precipitated with 10% trichloroacetic acid as an internal control. Thiols were derivatized with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate and were

separated by reversed-phase HPLC using a 5 μm C18 bead column (Phenomex, UK). The fluorescence intensities were observed at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. The mobile phase was 0.1 M acetate buffer containing 2% methanol (pH 4.0) that was filtered through a 0.45 μm filter and was degassed under vacuum. The isocratic flow rate was 1 ml/min. Calibration standards were also prepared by the same procedure.

3.4. Determination of hepatic SAM and SAH

Hepatic *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) levels were determined using HPLC according to previously described methods (Bottiglieri, 1990). Briefly, liver samples were homogenized in 0.4 M perchloric acid and centrifuged for 20 min at 10,000 × *g* at 4°C. The supernatants were passed through a 0.2 μm filter and injected into an HPLC apparatus equipped with a 5 μm C18 bead column (Phenomex) and a UV-VIS detector operating at 254 nm. The mobile phase for eluting the SAM and SAH consisted of 0.1 M sodium acetate, 5 M heptanesulfonic acid, and 4.2% acetonitrile, adjusted to pH 4.5 with acetic acid. The mobile phase was filtered and degassed under vacuum. The isocratic flow rate was 1 ml/min. To assure standardization between the sample runs, calibration standards were interspersed at intervals during each run.

3.5. Microarray hybridization and analysis

Total RNA was prepared using RNAiso-Plus (Takara, Japan) and was purified using an

Rneasy column (Qiagen, USA). RNA purity and integrity were analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Microarray hybridization was performed with the Illumina RatRef-12 v1.0 Expression BeadChip platform (USA). Samples from each group (n = 4) were analyzed by microarray. Briefly, 550ng of total RNA was amplified and purified using the Ambion Illumina® RNA amplification kit (Ambion, USA) to prepare biotinylated cRNA. The cRNA was fragmented and 750ng of biotinylated cRNA samples was hybridized to each rat-12 expression bead array. The array signal was detected using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, UK) according to the product manual, and arrays were scanned with an Illumina bead array Reader confocal scanner. The quality of hybridization and overall chip performance were monitored by visual inspection of both the internal quality control checks and the raw scanned data. Raw data were extracted using Illumina BeadStudio v3.1.3 software (Gene Expression Module v3.3.8). All probe signals were transformed by logarithm and were normalized by the quantile method. Genes with at least one of the samples with a detection value of $p < 0.05$ were selected by filtering. The expression values in log2 scale were analyzed with one-way analysis of variance (One-way ANOVA) to identify differentially expressed genes (DEGs). The statistical significance was adjusted by the Benjamini-Hochberg method for correcting for multiple hypotheses, known as the false discovery rate method. The criteria used were a *p*-value less than 0.05. Hierarchical cluster analysis was carried out using complete linkage and Euclidean distance as a measure of similarity, and Heatmap was visualized using a PermutMatrix software (Caraux and

Pinloche, 2005). R package (www.r-project.org) was used to generate the volcano plot. The microarray data analysis and visualization of DEGs were performed using the ArrayAssist® 5.5.1 software (Stratagene, USA). The DEGs were categorized by biological process and molecular function using the Panther database (http://www.pantherdb.org). And they were annotated and biological processes were analyzed by the Database for Annotation, Visualization and Integrated Discovery (DAVID).

3.6. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from liver tissue using RNAiso-Plus (Takara, Japan). cDNA was synthesized using 2 μg of total RNA with the Superscript®II Reverse Transcriptase (Invitrogen, USA). All amplification reactions were performed using a StepOne™ Real Time PCR System (Applied Biosystems, USA). The commercially available TaqMan® Assay primers and probes were purchased from Applied Biosystems or the selective genes were designed for SYBR green analysis (**Appendix 1** and **2**, respectively). Relative gene expression was analyzed according to the manufacturer's suggested protocol for the $^{\Delta\Delta}$Ct assay.

3.7. Tissue extract preparation and immunoblotting

Liver samples were homogenized in ice-cold lysis buffer and the protein concentrations of lysates were determined using the Bio-Rad Protein Assay Reagent (USA). Equal amounts of protein were loaded into the lanes of an SDS-PAGE gel and

were separated and blotted onto a PVDF membrane (Millipore, USA). After being blocked with 5% nonfat milk or bovine serum albumin, the membrane was probed with a specific primary antibody for cleaved caspase-3 (Cell signaling, USA), β-catenin (Santa Cruz, USA), phosphorylated glycogen synthase kinase 3-β (p-GSK3β; Cell signaling), or β-actin (Sigma) and was incubated with horseradish-peroxidase-linked secondary antibody for chemiluminescent detection. The band intensities were quantified with Quantity One software (Bio-Rad, USA).

3.8. Determination of global DNA methylation

Genomic DNA was extracted using a Dneasy® Blood & Tissue Kit (Qiagen, USA). Global DNA methylation was assessed using the Imprint® Methylated DNA Quantification Kit (Sigma, USA). Percent DNA methylation was calculated by a single point assay relative to the methylated control DNA.

3.9. Histone isolation and immunoblotting

Isolation of histones was performed as previously described (Liu and Xu, 2004). Briefly, liver tissues were homogenized in a lysis buffer (10 mM Tris-HCl, pH 6.5, 50 mM sodium bisulfate, 10 mM $MgCl_2$, 10 mM sodium butyrate, 8.6% sucrose, and 1% Triton X-100). The homogenates were incubated on ice for 10 min. After centrifugation at 1,000 × *g* for 5 min at 4°C, the pellets were washed with resuspension buffer (10 mM Tris-HCl, 13 mM EDTA, pH 8.0). After a second centrifugation, the pellets were resuspended in sulfuric acid to a final concentration of 0.4 N, were

incubated on ice for 1 h, and were then centrifuged at 10,000 × *g* for 5 min at 4°C. The supernatant was mixed with 10 volumes of acetone before placing it at –20°C overnight to precipitate the histones. The mixture was then centrifuged at 10,000 × *g* for 5 min at 4°C. The pellets were dried, resuspended in water, and sonicated. Methylation and acetylation of histone proteins were investigated by immunoblotting using the following antibodies: histone H3 (H3; Cell signaling, USA), acetylated histone H3-Lysine 9 (H3K9Ac; Abcam, UK), or trimethylated H3K9 (H3K9Me3; Abcam, UK).

3.10. Statistical analysis

The results were analyzed by the one-way analysis of variance (One-way ANOVA) followed by either Duncan's multiple comparison test or an independent *t*-test using SPSS software (version 19.0). The data were expressed as means ± SEM, and differences were considered statistically significant at $p < 0.05$. Correlation between two variables was analyzed by *Pearson* correlation coefficient.

4. Results

4.1. Effects of maternal diet on the body weight change and organ weights of 3-week-old offspring

There were no significant differences in the litter sizes (CAS: 14.4 ± 0.4; SPI: 13.5 ± 0.4; GEN: 13.5 ± 1.1) or in the percentage of male pups in the litters (CAS: 53 ± 3%; SPI: 59 ± 5%; GEN: 52 ± 11%) among the groups, suggesting that the level of genistein in the maternal diet did not interfere with the mechanism of sex determination. Male offspring of the GEN group presented the significantly greater birth weights than those of the CAS group (**Table 3.3**). No significant difference in the birth weights between the SPI group and the other two groups was observed. However, the body weights of offspring of the SPI group were significantly lower than those of the other groups from PND 12. The relative epididymal fat weight was also significantly lower in the SPI group compared to those in both the CAS and GEN groups (**Table 3.3**). The relative liver weight was significantly higher in the SPI group than in the CAS group.

Table 3.3. Effects of maternal diet on the body weight change and organ weights of 3-week-old offspring

	Maternal diet		
	CAS	SPI	GEN
Birth body weight (g)	6.7 ± 0.1[b]	6.9 ± 0.1[ab]	7.1 ± 0.1[a]
Body weight at 3-week (g)	56.5 ± 1.1[a]	49.4 ± 1.2[b]	56.7 ± 2.1[a]
Organ weight (g)			
Liver	1.78 ± 0.04[ab]	1.68 ± 0.05[b]	1.86 ± 0.0.5[a]
Epididymal fat	0.19 ± 0.01[a]	0.13 ± 0.01[b]	0.19 ± 0.01[a]
Relative organ weight (g/100g body weight)			
Liver	3.14 ± 0.04[b]	3.39 ± 0.04[a]	3.32 ± 0.09[ab]
Epididymal fat	0.33 ± 0.02[a]	0.27 ± 0.01[b]	0.33 ± 0.02[a]

CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

Data are means ± SEM (n=14–17 for each group). Means in the same row with different superscript are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test).

4.2. Effects of maternal diet on the global gene expression profile in the liver of 3-week-old offspring

Global gene expression analysis was performed to identify signatures in hepatic gene expression profiles associated with the liver development of offspring. Microarray analysis showed that a total of 965 genes was differentially expressed in response to maternal diet ($p < 0.05$) (**Figure 3.2**). Among differentially expressed genes (DEGs), the expression of 590 genes was different between the CAS and SPI groups. The expression of 480 genes was different between the SPI and GEN groups. In contrast, the expression of 88 genes was different between the CAS and GEN groups. Hierarchical Cluster Analysis (HCA) was employed to describe the gene expression profiles for three groups of offspring. Two apparent clusters were revealed in HCA with the samples separating well into two clusters (SPI *vs.* CAS and GEN), indicating a clear effect of the maternal diet on the gene expression profile in the liver (**Figure 3.3A**). **Figure 3.3B** shows the volcano plot of the hepatic global gene difference. Green dots represented the significantly down-regulated genes between two groups (Criteria: less than 1.5 fold change), whereas red dots showed the significantly upregulated genes (Criteria: more than 1.5 fold change).

Appendix 3 shows a comprehensive list of 119 DEGs with a fold-change greater than 1.5 between two groups. Among 71 DEGs with a fold-change greater or less than 1.5 and p-value < 0.05 analyzed by t-test between the CAS and SPI groups, 48 genes were significantly up-regulated and 23 genes were significantly down-regulated in the SPI group compared to the CAS group (**Figure 3.3B**).

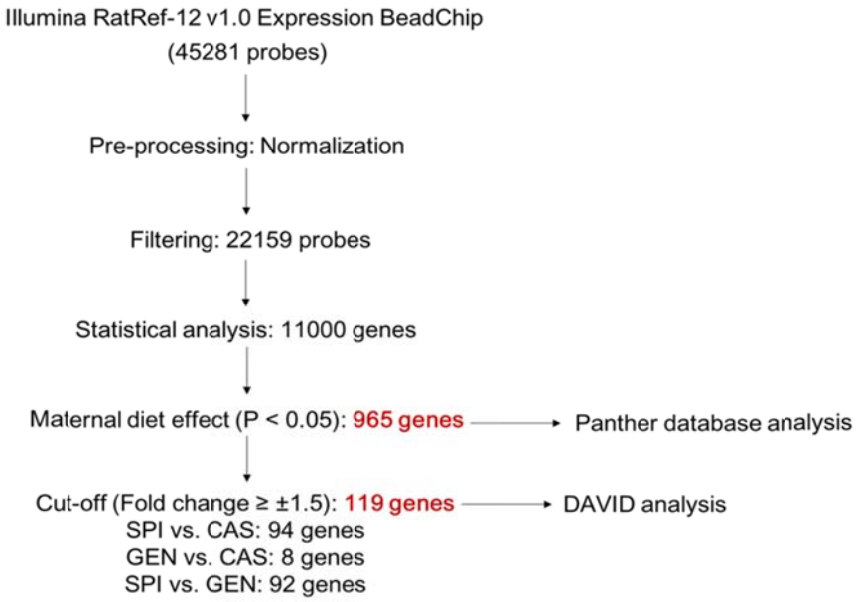
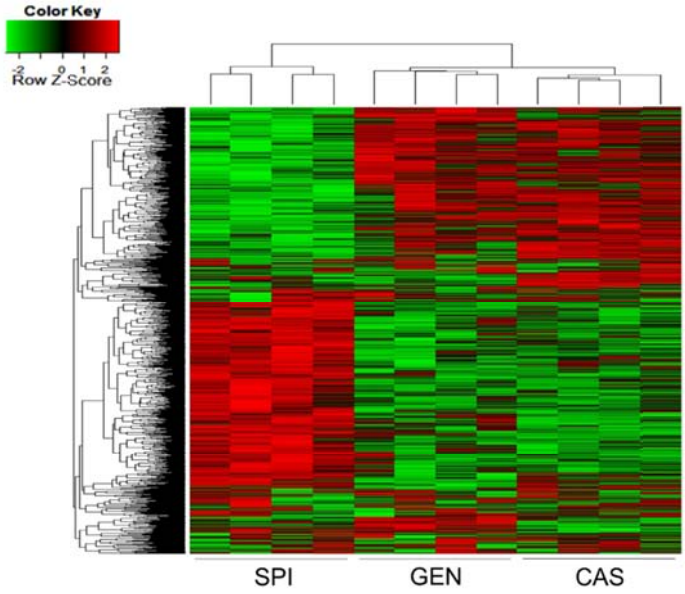


Figure 3.2. Flow chart of the microarray analysis. CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

(A)



(B)

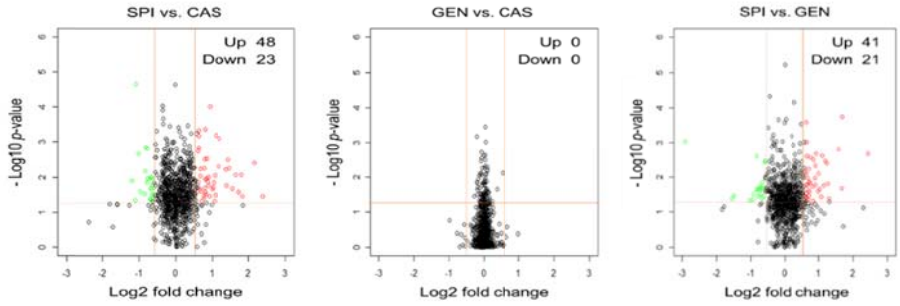


Figure 3.3. Effects of maternal diet on the global gene expression profile in the liver of 3-week-old offspring. (A) Two-dimensional hierarchical clustering of gene expression profiles. Each cell represents the individual differentially expressed gene (DEG) in each offspring liver sample (n=4) identified by One-ANOVA ($p < 0.05$). The 965 DEGs were clustered by Euclidean distance. The relationship between the samples within a gene was determined by the Z-score normalization with a color indication. Red color represents the up-regulated gene expression compared to the mean, whereas green color represents the down-regulated expression below the mean. (B) Volcano plots of 965 DEGs. Green dots represent the down-regulated genes, and red dots represent the up-regulated genes by the criteria of fold change > ±1.5 with p-value < 0.05. CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

Similar to the pattern of the CAS group, 21 genes were significantly up-regulated and 41 genes were significantly down-regulated in the SPI compared to the GEN group. So, further comparison between the SPI and GEN groups was limited due to the high similarity found between the CAS and GEN groups. According to the gene ontology through the PANTHER database, the DEGs were mainly grouped into protein metabolism & modification (166 genes), nucleoside, nucleotide & nucleic acid metabolism (125 genes), and signal transduction pathways (100 genes), suggesting that the maternal diet has a significant effect on the expression of genes involved in liver proliferation and growth (**Figure 3.4**).

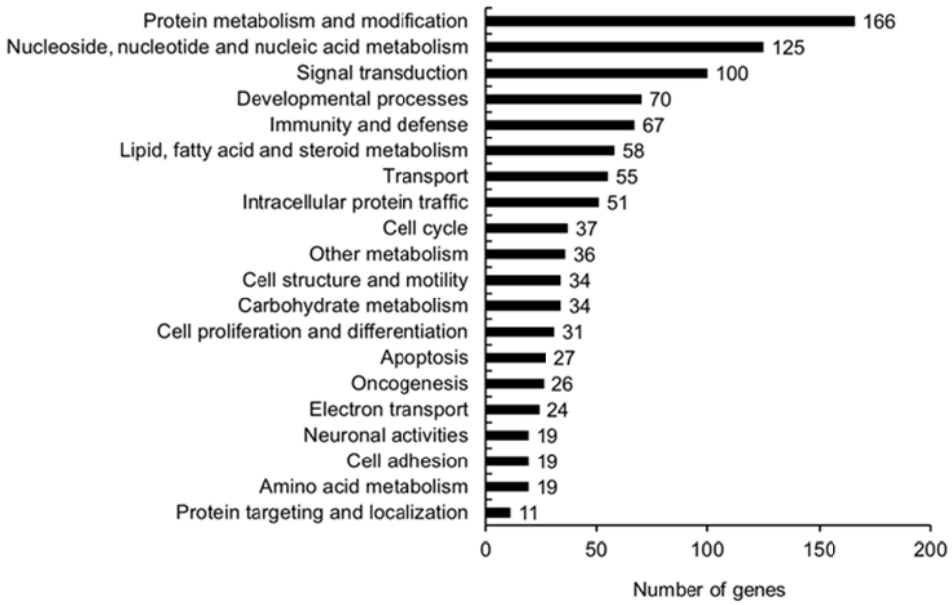
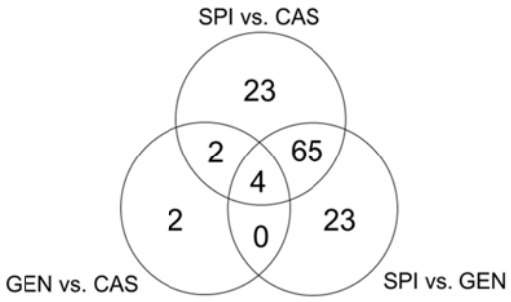


Figure 3.4. Gene ontology analysis of the biological functions affected by maternal diet in the liver of 3-week-old offspring. According to the gene ontology, the 965 DEGs identified by One-way ANOVA ($p < 0.05$) were categorized by the biological functions through the Panther database. The gene was counted once when it is categorized by more than one minor biological functions within the major biological function. Biological process including more than 10 genes was presented in the biological functions.

4.3. Effects of maternal diet on the gene expression related to detoxification in the liver of 3-week-old offspring

Among the 119 DEGs, **Figure 3.5** shows the clustering patterns of the expression of the up-regulated genes by the CAS or SPI group. Genes in Cluster I were down-regulated by the SPI group, whereas those in Cluster II were up-regulated by the SPI group. The functional category was determined by the DAVID analysis. The top 10 biological process groups (GO terms) based on the *p*-value under the highest stringency were shown in **Table 3.4**. In the KEGG analysis, the most statistical significant genes targeting the metabolism of xenobiotics by cytochrome P450, drug metabolism, and glutathione metabolism were up-regulated (**Table 3.5 and Appendix 3**). Particularly, the expression of 13 genes (*Cyp2c6*, *Cyp2c7*, *Cyp2c12*, *Ugt2b1*, *Ugt1a1*, *Gstm5*, *Gstm1*, *Gstm2*, *Adh4*, *Ces2*, *Ces3*, Gsta5, and *Ugt1a6*) involved in metabolism of xenobiotics by cytochrome P450s and drug metabolism were affected by the SPI group compared to the CAS and GEN groups (**Figure 3.6**).

(A)



(B)

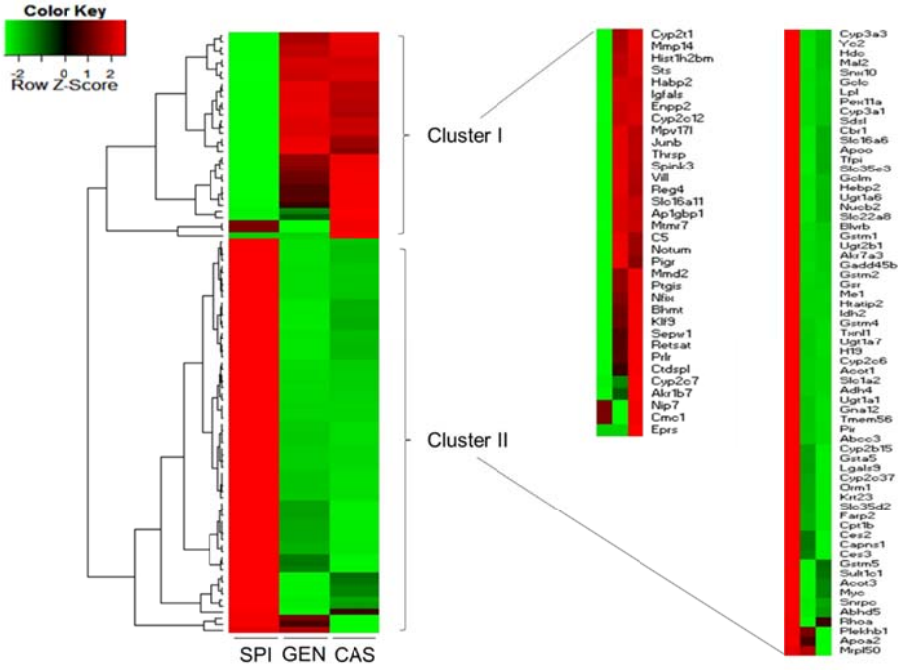


Figure 3.5. Analysis of differentially expressed genes (DEGs) affected by maternal diet in the liver of 3-week-old offspring. (A) Overlap of 119 DEGs (See in **Appendix 3**) in three different comparisons were shown using Venn diagram. (B) Clustering profiles of the identified DEGs were shown using the heat map analysis. Genes in Cluster I were down-regulated by the SPI group, whereas those in Cluster II were up-regulated by the SPI group. CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

Table 3.4. Top 10 enriched GO terms of 119 DEGs in response to maternal diet

Term	Benjamini *p*-value	Fold Enrichment	Genes
GO:0055114~oxidation reduction	1.5E-05	5.69	*Me1, Cyp2c6, Txnl1, Cyp2c37, Cyp2t1, Cyp2c7, LOC361841, Cyp2b15, Gclm, Gsr, Akr1b7, Cbr1, Adh4, Idh2, Akr7a3, Cyp2c12, Retsat*
GO:0051186~cofactor metabolic process	2.2E-03	9.13	*Gstm1, Gsr, Ugt1a6, Cbr1, Gclc, Adh4, Idh2, Acot1, Gclm, Ugt1a1*
GO:0009719~response to endogenous stimulus	4.1E-03	4.65	*Me1, Sts, Gclc, Cyp2c7, Mmp14, Ugt1a1, Junb, H19, Gstm1, Ugt1a6, Apoa2, Slc1a2, Rhoa, Abcc3*
GO:0010033~response to organic substance	4.9E-03	3.53	*Me1, Sts, Gclc, Slc22a8, Cyp2c7, Mmp14, Ugt1a1, Junb, H19, Gstm1, Ugt1a6, Gstm2, Apoa2, Slc1a2, Abcc3, Rhoa, Myc*
GO:0042493~response to drug	4.1E-03	6.27	*Cyp2c6, Lpl, Ugt1a6, Slc1a2, Apa2, Cyp2c7, Rhoa, Abcc3, Gclm, Ugt1a1, Junb*
GO:0009725~response to hormone stimulus	2.0E-02	4.42	*Me1, Ugt1a6, Apoa2, Sts, Gclc, Cyp2c7, Rhoa, Abcc3, Mmp14, Ugt1a1, Junb, H19*
GO:0006575~cellular amino acid derivative metabolic process	1.8E-02	8.54	*Gstm1, Gsr, Apoa2, Gclc, Hdc, Akr7a3, Gclm*
GO:0009410~response to xenobiotic stimulus	3.1E-02	30.36	*Ugt1a6, Gstm2, Gclc, Ugt1a1, LOC494499(Gsta5)*
GO:0006749~glutathione metabolic process	3.4E-02	28.27	*Gstm1, Gsr, Gclc, Gclm*
GO:0048545~response to steroid hormone stimulus	4.0E-02	5.63	*Ugt1a6, Apoa2, Sts, Rhoa, Abcc3, Mmp14, Ugt1a1, Junb, H19*

GO analysis was performed using DAVID Bioinformatics Resources. Shown are GO biological process terms that significantly overrepresented (Benjamini $p < 0.05$) in response to maternal diet

Table 3.5. Significantly over-represented pathways of 119 DEGs in response to maternal diet (KEGG pathway)

Term	Benjamini *p*-value	Fold Enrichment	Genes
rno00980:Metabolism of xenobiotics by cytochrome P450	1.3E-08	22.72	*Gstm1, Cyp2c6, Ugt1a6, Gstm2, Adh4, Ugt2b1, Cyp2c7, Cyp2c12, Gstm5, Ugt1a1, LOC494499(Gsta5)*
rno00982:Drug metabolism (cytochrome P450)	3.6E-08	18.94	*Gstm1, Cyp2c6, Ugt1a6, Gstm2, Adh4, Ugt2b1, Cyp2c7, Cyp2c12, Gstm5, Ugt1a1, LOC494499(Gsta5)*
rno00480:Glutathione metabolism	9.1E-07	21.81	*Gstm1, Gsr, Gstm2, Gclc, Idh2, Gstm5, LOC494499(Gsta5), Gclm*
rno00830:Retinol metabolism	4.8E-05	16.18	*Cyp2c6, Ugt1a6, Adh4, Ugt2b1, Cyp2c7, Cyp2c12, Ugt1a1, Retsat*
rno00590:Arachidonic acid metabolism	1.8E-02	9.74	*Cyp2c6, Cbr1, Ptgis, Cyp2c7, Cyp2c12*
rno00983:Drug metabolism (other enzymes)	3.7E-02	12.39	*Ces3, Ugt1a6, Ces2, Ugt2b1, Ugt1a1*

GO analysis was performed using DAVID Bioinformatics Resources. Shown are KEGG pathway terms that significantly overrepresented (Benjamini $p < 0.05$) in response to maternal diet

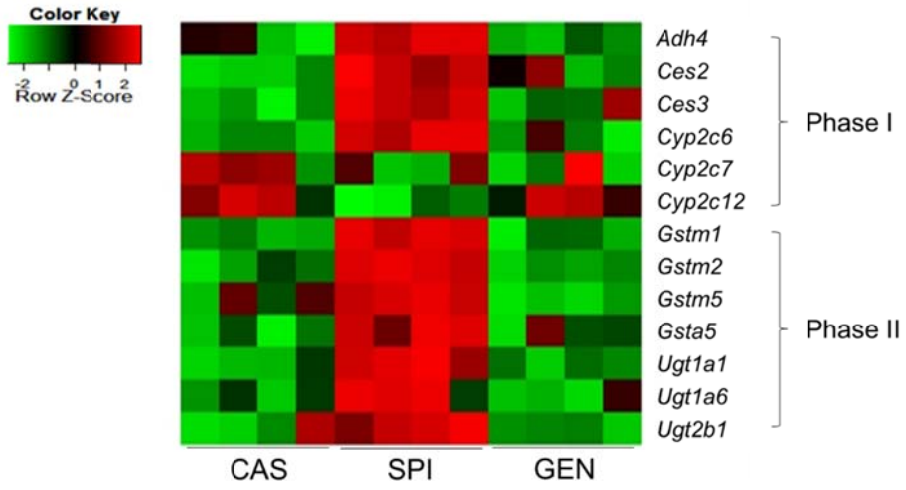


Figure 3.6. Effects of maternal diet on the expressions of genes involved in xenobiotics and drug metabolism in the liver of 3-week-old offspring. The relationship between the samples within a gene was determined by the Z-score normalization with a color indication. Red color represents the up-regulated gene expression compared to the mean, whereas green color represents the down-regulated expression below the mean green. CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

4.4. Effects of maternal diet on the proliferation and apoptosis in the liver of 3-week-old offspring

To address the mechanisms that may have contributed to liver growth and development, changes in cell proliferation and apoptosis-related gene expression were investigated. The SPI group had a significantly higher *Myc* (1.43-fold) and *Gadd45b* (1.49-fold) expression and had a lower *Junb* expression (-1.50-fold) compared to the CAS group (**Appendix 3** and **Figure 3.7A**). In early developmental period, *Myc* was highly expressed, but their expression was dramatically suppressed at postnatal day 21 (Zhang et al., 1988). *Gadd45b*, an anti-apoptotic gene, is also induced by the liver growth and cell proliferation. In addition, relative liver weight was strongly associated with *Gadd45b* expression (r=0.599, p=0.040). Otherwise, Jun-B has been shown to suppress cell proliferation by transcriptional activation of p16 (Passegue and Wagner, 2000). The SPI group also exhibited significantly higher expression (1.32-fold) of *Pik3cb* compared to the CAS group. PI3K p110beta has been shown to be involved in early embryonic development, DNA replication, and S-phase progression (Kumar et al., 2011). However, none of the cell cycle regulator genes (cyclin, cyclin-dependent kinase, and cyclin-dependent kinase inhibitor genes) were included in DEGs. *H19* (3.61-fold) expression was also significantly different among the groups. *H19* transcription is upregulated during the S-phase of growth-stimulated cells (Berteaux et al., 2005).

Previous study found the significant higher level of the PCNA protein in offspring from maternal SPI diet compared to those from maternal CAS and GEN diet,

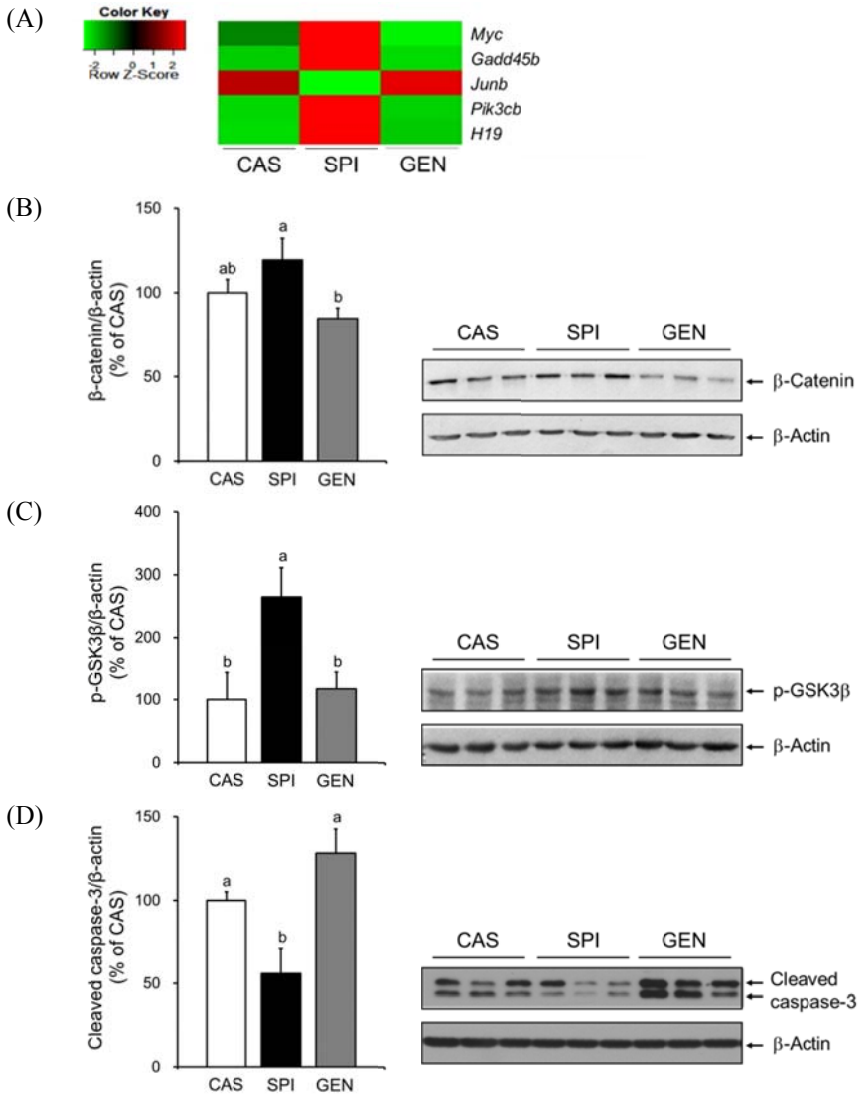


Figure 3.7. Effects of maternal diet on the proliferation and apoptosis in the liver of 3-week-old offspring. (A) Expressions of genes involved in cell proliferation and apoptosis were analyzed by microarray. (B) β-Catenin (n=5), (C) p-GSK3β (Ser9) (n=3), and (D) cleaved caspase-3 (n=3-4) protein levels were determined by immunoblotting. Bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test). CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

implying that a maternal SPI diet is associated with the increased hepatocyte proliferation (Han, 2011). In addition, to further support the microarray observations, β-Catenin and p-GSK3β protein levels were determined. The SPI group exhibited significantly higher β-catenin levels (**Figure 3.7B**), which correlate with cell proliferation during liver growth, regeneration, and development (Scheving and Russell, 2006). β-Catenin mRNA levels were not significantly changed in response to the maternal diet, as determined by microarray analysis, suggesting that changes in protein level may be due to increased stability. Accordingly, the SPI group featured significantly higher p-GSK3β levels compared to the CAS and GEN groups, suggesting that higher β-catenin protein levels could be due to reduced phosphorylation by GSK-3β (**Figure 3.7C**). Furthermore, GSK3-mediated phosphorylation of tuberous sclerosis complex 2 inhibits the mammalian target of rapamycin (mTOR) signaling pathway, resulting in reduced protein synthesis and cellular growth (Julien et al., 2010). In the previous study, the SPI group had significantly higher expressions of phosphorylated mTOR (Han, 2011). Phosphorylation of mTOR on Ser2448 is positively related to its activity (Anand et al., 2002). As the next step, the activity of caspase-3 in the liver was measured by immunoblotting to determine the extent of cell apoptosis and observed a significant difference in the SPI group compared to the CAS and GEN groups (**Figure 3.7D**).

4.5. Effect of maternal diet on the one-carbon metabolism in the liver of 3-week-old offspring

The present study determined whether the maternal diet alters one-carbon metabolism in the offspring livers. First, hepatic SAM and SAH levels were determined because SAM donates a methyl group for CpG methylation (**Table 3.6**). No significant differences in the hepatic SAM/SAH ratio were detected, although the ratio tended to be higher in the SPI group. Homocysteine is metabolically linked to methylation reactions through the methionine cycle. Serum homocysteine levels were significantly higher in the SPI group compared with the CAS and GEN groups (**Figure 3.8A**), and homocysteine levels showed a positive correlation with the relative liver weight (r=0.505, p=0.065). Maternal serum homocysteine levels also tended to be higher in dams fed an SPI diet with no statistical difference (CAS: 6.9 ± 0.4; SPI 7.6 ± 0.5; GEN 6.9 ± 0.4 μmol/L). Hepatic betaine-homocysteine methyltransferase (*Bhmt)*, phosphatidylethanolamine *N*-methyltransferase (*Pemt*), and cystathionine β-synthase (*Cbs*) mRNA levels were tended to lower in the SPI group compared to the CAS and GEN groups (**Figure 3.8B&C**). Significantly reduced glycine *N*-methyltransferase (*Gnmt*) and 5-methyltetrahydrofolate-homocysteine methyltransferase (*Mtr*) mRNA levels were also observed in the SPI group compared with the CAS and GEN groups.

Table 3.6. Effects of maternal diet on the SAM and SAH concentrations and SAM/SAH ratio in the liver of 3-week-old offspring

	Maternal diet		
	CAS	SPI	GEN
SAM (nmol/g liver)	94.4 ± 12.5	121.0 ± 18.1	125.1 ± 5.0
SAH (nmol/g liver)	37.9 ± 6.2	37.1 ± 2.8	47.2 ± 1.8
SAM : SAH	2.6 ± 0.5	3.2 ± 0.3	2.7 ± 0.1

SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine. Data are means ± SEM (n=3). CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

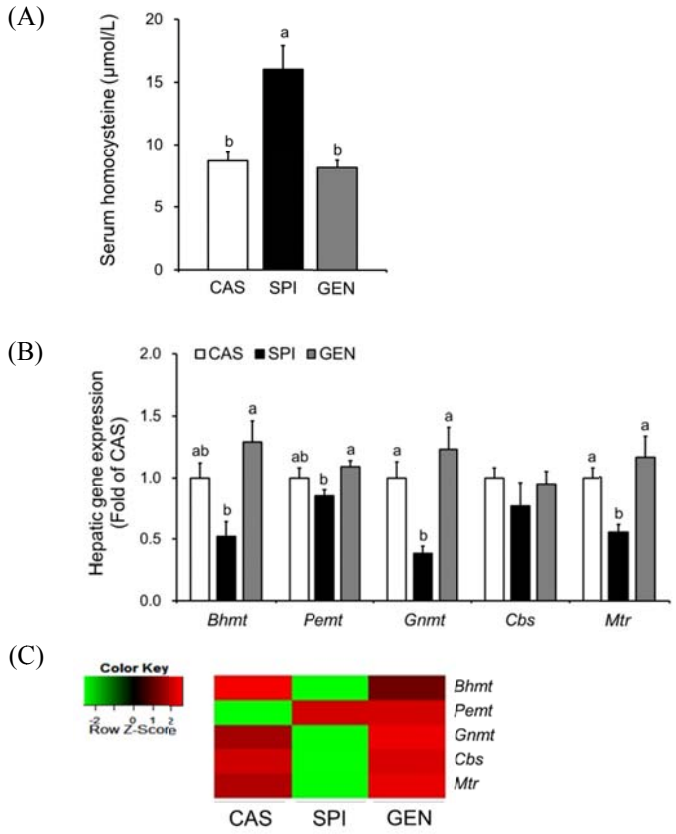


Figure 3.8. Effects of maternal diet on the one-carbon metabolism in the liver of 3-week-old offspring. (A) Serum homocysteine levels were analyzed by HPLC method (n=4-5). (B) Hepatic *Bhmt, Pemt, Gnmt, Cbs, and Mtr* mRNA levels were analyzed by qRT-PCR (n=4). (C) Expressions of genes involved in one-carbon methylation were analyzed by microarray. The mRNA expression level was normalized to β-actin. Each bar represents the mean ± SEM and bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test). CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

4.6. Effects of maternal diet on the epigenetic modification in the liver of 3-week-old offspring

To determine whether increased serum homocysteine levels affect DNA methylation in the SPI group, global DNA methylation was measured by ELISA-based format. Global DNA methylation was significantly lower in the SPI group than in the CAS group (**Figure 3.9A**). But the expression of the DNA methyltransferase genes, *Dnmt1*, *Dnmt3a*, and *Dnmt3b* mRNA levels did not reach significance (**Figure 3.9B&C**).

Changes in DNA methylation and histone modifications are coordinately regulated. Specifically, methylation at H3K9 is positively correlated with DNA methylation (Cheng and Blumenthal, 2010). Therefore, the post-translational histone modification patterns were determined using methylation and acetylation-specific antibodies. Histone methylation determined by immunoblotting with an antibody against H3K9Me3 was significantly lower in the SPI group compared to the CAS and GEN groups (**Figure 3.10A**), whereas H3K9 acetylation was significantly higher in the SPI group (**Figure 3.10B**). In addition, there was a strong negative correlation between the H3K9Me3 and H3k9Ac (**Figure 3.10C**).

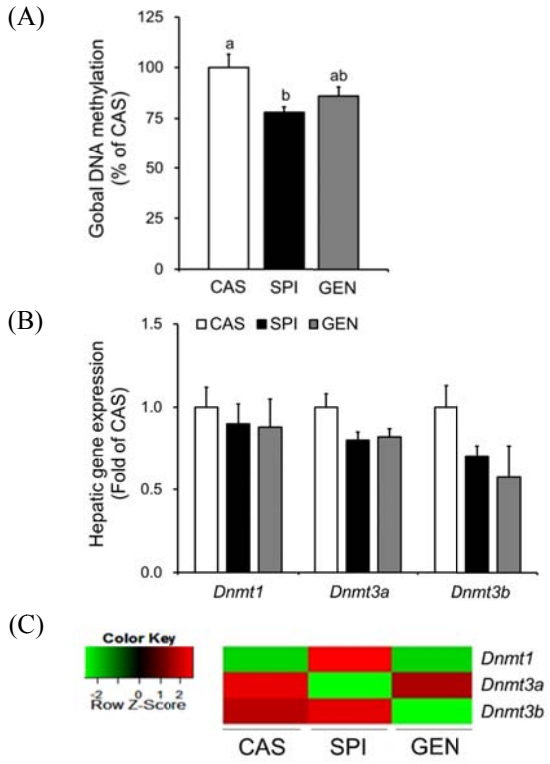


Figure 3.9. Effects of maternal diet on DNA methylation in the liver of 3-week-old offspring. (A) Global DNA methylation was measured by ELISA-based format (n=4). (B) Hepatic *Dnmt* mRNA levels were analyzed by qRT-PCR (n=4). (C) Expressions of genes involved in DNA methylation were analyzed by microarray. The mRNA expression level was normalized to β-actin. Each bar represents the mean ± SEM and bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test). CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

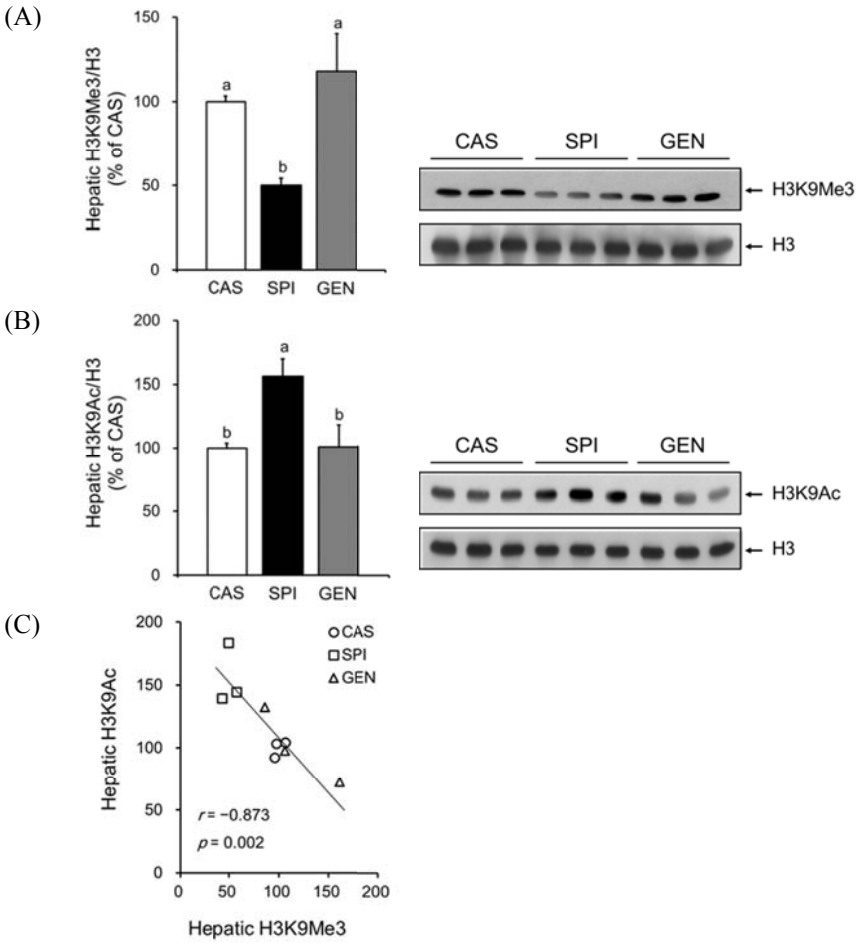


Figure 3.10. Effects of maternal diet on histone modification in the liver of 3-week-old offspring. (A) Trimethylated histone H3-Lysine 9 (H3K9Me3) and (B) acetylated H3K9 (H3K9Ac) protein levels were determined by immunoblotting (n=3). (C) *Pearson*'s correlation between H3K9Me3 and H3K9Ac. Each bar represents the mean ± SEM and bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test). *Pearson* correlation coefficient, *r* and *p*-value are indicated. CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

5. Discussion

The objective of this study was to determine whether maternal consumption of a low-isoflavone soy protein isolate diet and a casein diet supplemented with genistein could alter hepatic gene expression and liver development in 3-week-old rat offspring. Although several studies have reported that maternal diet affects metabolic disease development in adulthood, few studies have performed gene expression profiling in the specific organ of young offspring. Liver differentiation is accelerated after birth, although fetal hepatocytes acquire some metabolic functions during embryonic development (Torre et al., 2011). Therefore, the gene expression profiling is useful for monitoring liver development of 3-week-old offspring. Interestingly, maternal SPI consumption significantly changed hepatic gene expressions related to cell growth and development, suggesting that the dietary protein source may play a pivotal role in the liver development of offspring. Genistein supplementation had less of an effect in regulating hepatic gene expression than the SPI diet, suggesting that the gene expression may be altered by amino acid composition or other components of the SPI, rather than soy isoflavone. The distinct roles of soy protein isolate and genistein in regulating the gene expression were also previously reported. A lifetime dietary intake of soy protein isolate with isoflavone regulated 79 mammary epithelial genes, while a lifetime of genistein intake regulated 96 genes, with minimal overlap in the gene expression patterns in female rat offspring (Su et al., 2007b). To the best of our knowledge, this study is the first to determine the effect of the maternal protein source on epigenetic regulation of gene expression in the liver of 3-week-old offspring.

Under poor maternal nutrition, liver growth was suppressed to protect the essential organ development (Hyatt et al., 2008). In the present study, the increased liver proliferation was observed in the SPI group, implying that this may be associated with the early growth retardation. In addition, the expression pattern of the specific genes, particularly, xenobiotic-metabolizing enzymes were up-regulated in the SPI group compared to the CAS and GEN groups. Previous study showed the association between liver enlargement and the induction of xenobiotic metabolizing enzymes (Schulte-Hermann, 1979). In the present study, the expression of the specific genes (Cyp2c7 and Cyp2c12) related to cytochrome P450s were lower in the SPI group than in the CAS group, whereas that of Cyp2c6 was higher in the SPI group. Consistent with the present finding, the previous study observed the ontogeny of cytochrome P450s in developing mouse liver (Cui et al., 2012). Expression of Cyp2c66 (homologue of rat Cyp2c6) was appeared and peak at PND 10 and decreased to adult, whereas that of Cyp2c29/38/39 (homologue of rat Cyp2c7) and Cyp2c40/67/68/69 (homologue of rat Cyp2c12) was appeared at PND 10 and then increased to adult. In addition, these xenobiotic genes may be regulated by the nuclear receptor superfamily, such as constitutive androstane receptor (CAR, NR3I1) (Yang and Wang, 2014). Particularly, CAR induced the cytochrome P450 isoforms along with the increase of the liver weight and DNA synthesis (Wei et al., 2000). In spite of more clear explanation of the roles of xenobiotic metabolizing enzymes, they are more likely to be essential for early liver development and long-term health consequences because the liver is a major organ for the detoxification after birth. Furthermore, β-catenin is

involved in altered expressions of both phase I and phase II drug-metabolizing enzymes (Giera et al., 2010). Recently, the influence of β-catenin on the xenobiotic-induced expression of cytochrome p450 isoforms and glutathione *S*-transferase superfamily was reported (Behari, 2010; Braeuning, 2012). Wnt/β-catenin signaling is pivotal role in organogenesis and morphogenesis during peri- and postnatal stages of development (Monga, 2014). During PND 5-20, total β-catenin protein level was increased along with the liver cell proliferation, suggesting that β-catenin is critically involved in hepatocyte proliferation (Apte et al., 2007). In β-catenin null mice, liver weight-body weight ratio was decreased compared to the wild-type mice, showing the role of β-catenin in liver size growth and cell proliferation during the postnatal day. Concomitantly, the peak expression of β-catenin was observed during embryonic day 10-12 in the normal liver development followed by the gradually decrease, indicating that β-catenin is responsible for hepatic growth and morphogenesis (Micsenyi et al., 2004). Glycogen synthase kinase-3β (GSK3β) was negatively regulated the liver cell proliferation (Ji et al., 2015). The effect of GSK-3β inhibition on liver proliferation was mediated by Wnt/β-catenin signaling. β-Catenin localization is also important during liver development. β-Catenin translocation to the nucleus promoted its target genes, such as *c-Myc* (Dang, 1999). In the present study, maternal SPI diet seems to retard the liver growth and development during early stage of development, so β-catenin protein level was relatively higher in offspring of the SPI group than in those of the CAS group. In addition, β-catenin was a positively correlated with p-GSK3β (r=0.706, p=0.034) and its target gene, *Myc* (r=0.799, p=0.003).

The availability of methyl donors may be an important factor in tissue development (Van den Veyver, 2002). SAH hydrolase catalyzes the hydrolysis of SAH to homocysteine, which is remethylated to methionine or is degraded via the transsulfuration pathway. Folate-dependent remethylation by the B_{12}-dependent enzyme methionine synthase requires the donation of a methyl group from 5-methyltetrahydrofolate. Betaine, derived from choline oxidation, is the methyl group donor in folate-independent remethylation by BHMT (Williams and Schalinske, 2007). In addition to increased serum homocysteine levels, the hepatic mRNA levels of Hcy-related enzymes (BHMT, MTR, and CBS) and methylation enzymes (GNMT and PEMT) were reduced in the SPI group compared to the CAS and GEN groups regardless of hepatic SAM and SAH levels. In the present study, although the sulfur-amino acid amount among the maternal diets was not adjusted, maternal serum methionine level was not lower in the SPI group compared to the CAS group, and offspring hepatic SAM level was also not affected. It is likely that the reduced hepatic mRNA levels may contribute the higher serum Hcy level distributing the capacity of Hcy remethylation and transsulfuration. Sustained hepatic SAM may be maintained by the methyl donor pathways rather than removed by the Hcy level. Significantly lower *Mtr* mRNA level was associated with the capacity of Hcy remethylation via folate-dependent enzymes (Christensen et al., 2010). GNMT is suggested to be a tumor suppressor gene because $Gnmt^{-/-}$ mice develop liver hepatocellular carcinomas (Liao et al., 2009). Therefore, altered methylation due to the reduced expression of major

methyltransferases may be involved in regulating gene expression and the accompanying increased proliferation and hepatomegaly in the SPI group.

Epigenetic control of gene expression is largely regulated by DNA methylation and the post-translation modification of histones which play a pivotal role in controlling gene expression during the early development (Aguilera et al., 2010; Dolinoy et al., 2007b). Changes of global DNA methylation and methylation capacity through methyltransferases in response to the diet (the methyl donor and choline) (Cordero et al., 2013; Le et al., 2014) and environment (glucocorticoid) (Crudo et al., 2012) were associated with the organ and disease development. In the present study, the SPI group had a significantly lower global DNA methylation and the low pattern of the *Dnmt3a* and *Dnmt3b* mRNA levels compared to the CAS group. There are major three DNA methyltransferases. *Dnmt1* is responsible for the maintenance of global methylation patterns on DNA, whereas *Dnmt3a* and *Dnmt3b* are the de novo methyltransferases in fetal and postnatal organs and establish methylation patterns at the unmethylated CpGs (Dolinoy et al., 2007b). In particular, Dnmt3 family showed a catalytic function in global DNA methylation during mouse embryonic development (Auclair et al., 2014). Partial global hypomethylation was induced by the decrease in *Dnmt3a* or *Dnmt3b*, and the reduction of one enzyme can be compensated for by the other. However, previous reports did not agree about the association between the availability of methyl groups in the maternal diet and global DNA methylation in offspring, suggesting that DNA methylation may respond to the supply of methyl groups in a complex fashion. When dams were fed a low-protein diet, global DNA

methylation was different in various fetal tissues with DNA hypermethylation in the offspring livers, but not in the hearts or kidneys (Rees et al., 2000). Conversely, no significant differences was observed in hepatic global DNA methylation in the fetuses of dams fed diets containing either 9 or 18% casein, with folate provided at either 1 or 5 mg/kg diet (Engeham et al., 2010). Therefore, this study determined whether histone modification changes contribute to the reduction of global methylation in the SPI group compared to the CAS group. Global DNA hypomethylation in the *Dnmt3a/3b* knockout embryonic stem cells suppressed the cell differentiation associated with apoptosis (Jackson et al., 2004). Global DNA hypomethylation and the reduced DNMTs were involved in the induction of histone hyperacetylation. In addition, hypomethylation of H3K4 and H3K9 and hyperacetylation of H3K9 have been shown to be related with DNA hypomethylation (Delage and Dashwood, 2008), and DNA methylation status under the methyl deficiency induced the loss of H3K9Me3, and this loss was associated with global DNA hypomethylation (Pogribny et al., 2007a). Previous study showed an age-associated with the site specific histone modification in liver (Kawakami et al., 2009). Acetylation of H3K9 was affected by aging related to cell proliferation, so its expression had been decreased following by aging rat liver. In the present study, H3K9Ac level was positively correlated with the relative liver weight of offspring (r=0.817, p=0.007), whereas H3K9Me3 level was negatively correlated (r=–0.598, p=0.089). Moreover, H3K9Me3 has been shown to be necessary for cells to enter the terminal differentiation pathway; the loss of the H3K9Me3

compromises the balance between cell proliferation and differentiation, favoring cell proliferation (Zhou and Pan, 2011).

In the present study, a lower growth rate in the SPI group compared to the CAS and GEN groups was observed. Food intake of dams fed an SPI diet was significantly lower (~ 86%) than that of dams fed either CAS or GEN diet during pregnancy, which was not observed during pre-pregnancy or lactation periods. The maternal body weight at the termination of experiment was not significantly different among the groups. Several studies have shown that the impaired growth and metabolic function due to maternal malnutrition during the fetal and neonatal periods increase the risk of adult chronic disease development including high blood pressure and glucose tolerance (Ross and Desai, 2005).

In summary, hepatic proliferation and development was associated with the changes in global gene expression, one-carbon metabolism genes, global DNA methylation, and histone modification mediated by the maternal diet in male rat offspring on postnatal day 21. Although global DNA methylation and histone modification do not detect region-specific epigenetic changes, the maternal SPI diet had a dramatic effect on gene regulation, suggesting that epigenetic modification of specific genes, especially ones related to cell cycle regulation, would be more obvious in the SPI group. Further studies are required to determine the epigenetic modification of gene-specific expression by the maternal diet.

IV. Study 2

Effects of maternal low-isoflavone soy protein isolate consumption on lipid metabolism of rat offspring

1. Abstract

The consumption of soy protein isolate has been shown to alleviate hyperlipidemia, and amino acid profile and isoflavone have been proposed to contribute to the beneficial effects of soy protein isolate on lipid metabolism. Therefore, the present study investigated whether the component(s), either amino acid profiles of soy protein isolate or genistein, would regulate lipid metabolism in offspring. Hepatic gene expression levels were also determined to investigate the underlying mechanism of lipid metabolism regulation. Female Sprague Dawley rats were fed a casein (CAS) diet, a low-isoflavone soy protein isolate (SPI) diet, or a casein plus genistein (250 mg/kg diet, GEN) diet for two weeks before mating, and during pregnancy and lactation. Serum and hepatic triacylglycerol levels were significantly lowered in dams fed an SPI diet compared to those fed either CAS or GEN diet. Moreover, there was a positive correlation between maternal hepatic triacylglycerol level and the body weight and fat mass of offspring, whereas the relative liver weight of offspring was negatively correlated. The ratio of serum branched chain amino acids to aromatic amino acids was also correlated with the body weight and fat mass of offspring. Otherwise, serum cholesterol levels of dams and offspring were lowered by both SPI and GEN groups. In addition, specific gene expression altered by maternal SPI diet in microarray analysis of the **Study 1** was associated with the activation of peroxisome proliferator-activated receptor alpha signaling, indicating that maternal hypolipidemic effect and serum amino acid levels may contribute to lower body weight gain and fat mass of offspring. The down-regulated expression of genes involved in HDL-cholesterol assembly and

transport may reflect the cholesterol-lowering effect observed in the SPI group. Therefore, these data suggest that the consumption of maternal SPI diet regardless of isoflavone content has more profound effect on lipid metabolism regulation in offspring. Further study is required to assess the sustainability of the effect of maternal SPI diet on liver metabolism in later life.

2. Introduction

It has been shown that the consumption of a soy-protein diet reduces serum triacylglycerol and cholesterol in rats (Ascencio et al., 2004; Tovar et al., 2005). Furthermore, maternal consumption of soy protein during lactation significantly reduced serum cholesterol, triacylglycerol, and phospholipid levels of offspring compared to casein (Lu and Jian, 1997). In particular, genistein, the major isoflavone in soy, has been shown to alleviate metabolic diseases by reducing serum and hepatic lipid levels (Gudbrandsen et al., 2005). Other soy protein isolate-derived dietary components may play important roles in preventing metabolic diseases because dietary soy protein, with low isoflavone, reduces plasma and hepatic cholesterol and/or triacylglycerol in rats (Fukui et al., 2002; Shukla et al., 2007).

It has been shown that the amino acid composition of soy protein is associated with its hypolipidemic effects. A recent study reported differences in body weight and fat pad weight between offspring of dams fed a soy protein diet (with isoflavone) and offspring of dams fed a casein plus genistein diet, although similar levels of serum genistein were observed between two groups of dams (Simmen et al., 2010). Soy protein isolate contains higher amounts of several amino acids including cysteine, arginine, and glycine than casein. Its low proportion of methionine among the total sulfur-containing amino acids has been shown to be involved in the beneficial effect of dietary soy protein on lipid metabolism (Sugiyama et al., 1986). A hypocholesterolemic effect was also observed in animals fed dietary proteins with low ratio of methionine:glycine (Gudbrandsen et al., 2005; Morita et al., 1997). In addition,

glycine supplementation reversed hypertension in offspring induced by a maternal low-protein diet (Brawley et al., 2004; Jackson et al., 2002). These results suggest that genistein as well as other component(s) in maternal soy protein isolate diet may regulate lipid metabolism in offspring of dams. The **Study 1** observed that maternal low-isoflavone soy protein isolate (SPI) consumption altered the expressions of multiple genes, which regulate cell proliferation and apoptosis, drug metabolism, and lipid metabolism in offspring liver. Therefore, the present study compared the effects of the early-life consumption of the SPI diet with those of a casein supplemented with genistein diet on hepatic lipid metabolism of dams and their offspring at 3 weeks of age.

3. Materials and Methods

3.1. Diets and animals

See the method section of the **Study 1.**

3.2. Serum biochemical analysis

After collection, blood was centrifuged at 10,000 × *g* for 15 min and stored at –80°C until analyzed. Serum glucose, triacylglycerol, total cholesterol, and HDL-cholesterol levels were determined using commercial kits (Asan Pharm. Co., Ltd, Korea). Serum free fatty acid levels were measured using a commercially available kit (Shinyang Diagnostics, Korea), and serum insulin levels were measured using an ELISA kit (Millipore, USA). The insulin resistance index was estimated by homeostasis model assessment (HOMA-IR) with the following formula: serum glucose × serum insulin/22.5, with serum glucose in mmol/mL and serum insulin in μU/mL. The activities of serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were measured by using the commercial kit (Asan Pharm. Co., Ltd, Korea).

3.3. Serum free amino acid analysis

Free amino acids in serum were determined using the HPLC method. Serum samples were mixed with 2 mM norvaline as an internal standard and 20% sulphosalicylic acid to precipitate the protein and kept for 60 min in an ice bath. After the centrifugation at 12,000 x g for 5 min at 4°C, the clear supernatant containing free amino acids was

removed and filtered through a 0.2 μm filter. Each amino acid as the calibration standards was obtained from Agilent Technologies (USA). 16 amino acids included were aspartate, glutamate, serine, histidine, glycine, threonine, arginine, alanine, tyrosine, valine, methionine, phenylalanine, isoleucine, leucine, lysine, proline, and cystine, and 3 extended amino acids included were asparagine, glutamine, and tryptophan. Samples and standards were analyzed by the Agilent 1200 HPLC (Agilent Technologies, USA) with the fluorescence detector (FLD) and ultraviolate detector (UV). The column was an Inno C18 column (4.6 mm x 150 mm, 5 μm, Innopia, Korea). The derivatization reagents of amino acids were o-phthalaldehyde (OPA) for the primary amino acids and 9-flurorenylmethyl chloroformate (FMOC) for proline and sarcosine. The separation was performed with the gradient elution, and the mobile phases were prepared by the following conditions: (A) 10 mM Na_2HPO_4 and 10 mM $Na_2B4O_7{\cdot}10H_2O$, pH 8.2 and (B) Acetonitrile:Methanol:Water (45:45:10, v/v/v%). The flow rate was 1.5 ml/min, and the column and sample temperature was maintained at 40°C and 20°C, respectively. For OPA, the FLD was set at 340 nm excitation and 450 nm emission, and for FMOC, 266 nm excitation and 305 nm emission. As not to be detected by the FLD, cystine was observed on UV at the absorbance of 338 nm.

3.4. Hepatic lipid analysis

Total lipids were extracted according to the method of Folch *et al*. (Folch et al., 1957). Briefly, hepatic tissue was homogenized in 20 volumes (w/v) of ice-cold PBS, and the protein content was measured using the commercial kit (Bio-Rad, USA). The

homogenate containing the equal amount of protein (1 mg/ml) was mixed with methanol-chloroform (1:2, v/v). After overnight incubation at 4°C, 240 μL of 0.88% KCl was added for aggregation of non-lipid contents and centrifuged at 1,000 × *g* for 15 min at 4°C. The bottom layer was transferred to the new tube, and hepatic triacylglycerol and cholesterol levels were determined by enzymatic colorimetric methods using commercial kits (Asan Pharm. Co., Ltd, Korea).

3.5. Total RNA extraction and quantitative real-time PCR analysis

See the method section in the **Study 1**.

3.6. Statistical analysis

Statistical analyses were performed using SPSS version 19.0 software (SPSS Inc., USA). For all experiments, one-way analysis of variance (One-way ANOVA) followed by Duncan's multiple range test or an independent *t*-test was used to determine statistical significance among the groups. Data were expressed as means ± SEM and differences were considered statistically significant at $p < 0.05$. Correlation between two variables was analyzed by *Pearson* correlation coefficient.

4. Results

4.1. Effects of maternal diet on the body weight change and biochemical parameters in dams

The food intake of the dams fed an SPI diet was significantly lower than that of dams fed either CAS or GEN diet during pregnancy (~ 86% of other dam groups), which was not observed during pre-pregnancy or lactation periods (**Table 4.1**). There was no significant difference in maternal body weight change during both pregnancy period and whole experimental period among the groups. As shown in **Table 4.1**, serum triacylglycerol, total cholesterol, and HDL-cholesterol levels were significantly lower in dams fed an SPI diet than in those fed a CAS diet. Serum free fatty acid levels tended to be lower in dams fed an SPI diet, but it did not reach a significant difference. There was no significant difference in serum glucose and insulin levels among the groups. Consistent with observations in the sera, hepatic triacylglycerol and cholesterol levels were also significantly lower in dams fed an SPI diet than in those fed either CAS or GEN diet.

Table 4.1. Diet intake and serum and hepatic biochemical parameters of dams

	Diet		
	CAS	SPI	GEN
Diet intake (g/day)			
Pre-pregnancy	18.8 ± 1.5	18.7 ± 0.8	20.2 ± 0.7
Pregnancy	26.2 ± 0.6[a]	22.5 ± 0.6[b]	26.1 ± 0.6[a]
Lactation	41.2 ± 0.3	40.5 ± 1.5	40.1 ± 1.1
Body weight (g)	299.5 ± 12.6	299.9 ± 3.4	304.0 ± 11.2
Liver weight (g)	11.6 ± 0.5	10.5 ± 0.3	12.1 ± 1.1
Serum			
Glucose (mg/dL)	94.5 ± 6.6	99.3 ± 10.1	104.2 ± 13.3
Triacylglycerol (mg/dL)	81.8 ± 10.4[a]	48.7 ± 7.3[b]	80.4 ± 15.5[a]
Total cholesterol (mg/dL)	92.0 ± 5.7[a]	59.4 ± 6.0[b]	77.0 ± 8.2[ab]
HDL-cholesterol (mg/dL)	74.8 ± 4.9[a]	49.0 ± 4.8[b]	56.9 ± 4.1[b]
Free fatty acids (μmol/L)	722.1 ± 88.5	617.1 ± 32.1	694.9 ± 75.4
Insulin (ng/mL)	0.49 ± 0.1	0.32 ± 0.08	0.86 ± 0.37
HOMA-IR	2.86 ± 0.76	1.73 ± 0.48	6.54 ± 3.72
Liver (μg/mg protein)			
Triacylglycerol	81.0 ± 2.7[a]	63.5 ± 3.9[b]	87.3 ± 5.2[a]
Cholesterol	5.8 ± 1.3[a]	2.5 ± 0.5[b]	5.4 ± 0.6[a]

HOMA-IR, homeostasis model of assessment – insulin resistance, CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

Data are means ± SEM (n=5–9). Means in the same row with different superscript are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test).

4.2. Effects of maternal diet on serum free amino acid levels in dams

Serum levels of tryptophan and tyrosine were significantly higher in dams fed an SPI diet than in dams fed the CAS diet, whereas those of leucine ($p < 0.05$, Student *t*-test) and valine were significantly lower in dams fed an SPI diet (**Table 4.2**). The ratio of the branched amino acids (BCAA, leucine, isoleucine, and valine) to aromatic amino acids (AAA, tryptophan, phenylalanine, and tyrosine) was lower in dams fed an SPI diet than in those fed the CAS and GEN diets. Particularly, there was a strong positive correlation between maternal AAA levels and the relative liver weight of offspring (r=0.678, p=0.005), whereas the body weight of offspring was negatively related to the maternal AAA levels (r=–0.798, p<0.0001). Maternal SPI consumption resulted in a lower body weight of their male offspring than CAS and GEN consumption (see the Result section of the **Study 1**). The relative epididymal fat weight was also significantly lower in the SPI group than in the CAS and GEN groups. Conversely, the relative liver weight was significantly higher in the SPI group than in the CAS group.

Table 4.2. Serum free amino acid profiles of dams

Amino acid (μmol/L)	Diet		
	CAS	SPI	GEN
Alanine	489.2 ± 44.7	464.5 ± 33.7	490.1 ± 13.8
Arginine	170.6 ± 5.0	191.3 ± 13.3	141.0 ± 23.3
Asparagine	76.8 ± 3.6	74.7 ± 6.6	72.2 ± 1.5
Aspartate	47.8 ± 3.0	48.8 ± 9.4	40.3 ± 4.3
Cystine	26.6 ± 1.6	24.4 ± 2.8	20.3 ± 1.9
Glutamate	186.9 ± 9.2	198.2 ± 24.0	203.6 ± 24.0
Glutamine	610.5 ± 42.9	550.7 ± 34.1	513.7 ± 30.8
Glycine	234.7 ± 16.0	255.6 ± 25.8	225.1 ± 20.5
Histidine	56.0 ± 1.2	52.1 ± 2.8	50.3 ± 1.3
Isoleucine	95.8 ± 6.9	80.1 ± 3.4	85.3 ± 8.1
Leucine	145.5 ± 9.5	117.6 ± 5.7	129.2 ± 12.6
Lysine	477.4 ± 25.0	477.7 ± 62.0	431.1 ± 47.5
Methionine	52.9 ± 1.2^{a}	52.9 ± 1.6^{a}	40.2 ± 6.3^{b}
Phenylalanine	61.9 ± 1.5^{ab}	63.2 ± 3.4^{a}	54.1 ± 2.6^{b}
Proline	172.4 ± 6.5	148.9 ± 9.8	169.5 ± 11.2
Serine	416.9 ± 18.0	401.4 ± 24.9	373.7 ± 12.7
Threonine	662.0 ± 97.0	426.3 ± 37.3	608.9 ± 100.9
Tryptophan	62.9 ± 1.4^{b}	78.8 ± 6.6^{a}	53.5 ± 3.7^{b}
Tyrosine	47.8 ± 1.9^{b}	64.6 ± 5.8^{a}	46.2 ± 3.2^{b}
Valine	152.4 ± 8.6^{a}	119.4 ± 3.5^{b}	137.7 ± 10.4^{ab}
BCAA/AAA	2.3 ± 0.1^{a}	1.5 ± 0.1^{b}	2.3 ± 0.2^{a}

CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet); BCAA, branched-chain amino acids (valine, isoleucine, leucine); AAA, aromatic amino acids (tyrosine, phenylalanine, tryptophan). Data are means ± SEM (n=5). Means in the same row with different superscript are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test).

4.3. Effects of maternal diet on lipid metabolism of 3-week-old offspring

As shown in **Table 4.3**, serum triacylglycerol levels were significantly lower in the SPI group than in other two groups. Serum free fatty acid levels were significantly lower in the SPI group than in the GEN group. Consistent with observation of dams, serum total cholesterol levels were lower in the SPI and GEN groups. However, unlike with their corresponding dams, offspring did not show any significant difference in the hepatic triacylglycerol or cholesterol levels.

Table 4.3. Effects of maternal diet on serum and hepatic biochemical parameters of 3-week-old offspring

	Maternal diet		
	CAS	SPI	GEN
Serum			
Triacylglycerol (mg/dL)	100.5 ± 8.5[a]	75.8 ± 5.8[b]	116.8 ± 8.0[a]
Total cholesterol (mg/dL)	84.4 ± 5.3[a]	58.8 ± 3.0[b]	54.3 ± 5.5[b]
HDL-cholesterol (mg/dL)	37.4 ± 3.1[a]	26.7 ± 1.7[b]	32.4 ± 2.2[ab]
Free fatty acids (μmol/L)	805.8 ± 67.9[ab]	739.8 ± 51.8[b]	945.6 ± 76.9[a]
Liver (μg/mg protein)			
Triacylglycerol	88.5 ± 3.8	87.4 ± 3.2	85.4 ± 2.9
Cholesterol	5.7 ± 0.8	5.4 ± 0.8	6.7 ± 1.1

CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

Data are means ± SEM (n=9–11). Means in the same row with different superscript are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test).

4.4. Effects of maternal diet on the gene expression related to lipid metabolism in the liver of 3-week-old offspring

The **Study 1** found the distinct expression of the genes involved in PPARα signaling pathway (**Figure 4.1**). Lipid-lowering effect observed in offspring of the SPI group may be related to PPARα regulation. Although the maternal diet did not alter the mRNA levels of *Pparα*, a gene encoding a key transcription factor that regulates many aspects of lipid metabolism such as fatty acid oxidation, fatty acid transport, and fatty acid synthesis, the expression of PPARα-regulated genes was affected by maternal SPI diet. The higher expression of some of its downstream target genes, including *Lpl* and *Apoa2*, was observed in the SPI group (**Figure 4.2A & 4.3**). The expression of the hepatic lipogenic gene, *Fasn,* was significantly lower in the SPI group than in the CAS and GEN groups, whereas the mRNA level of *Me1*, which is activated by the PPARα, was higher in the SPI group (**Figure 4.2A**). The mRNA levels of other lipogenic genes, *Thrsp* and *Scd1*, also tended to be lower in the SPI group compared to the CAS and GEN groups. Significantly higher *Cpt1b* and *Hadh* mRNA levels were observed in the SPI group compared to the CAS group. In addition, the hepatic expression of some selected genes important in the cholesterol metabolism was altered by the dietary changes (**Figure 4.2B & 4.3**). Although the mRNA level of *Lxra*, a gene encoding a major transcription factor involved in cholesterol metabolism, was not affected by the maternal diets, its downstream target genes were significantly changed. The mRNA level of *Cyp3a1,* which plays an important role in bile acid degradation (Xie et al., 2001), was significantly higher in the SPI group compared to the CAS and GEN

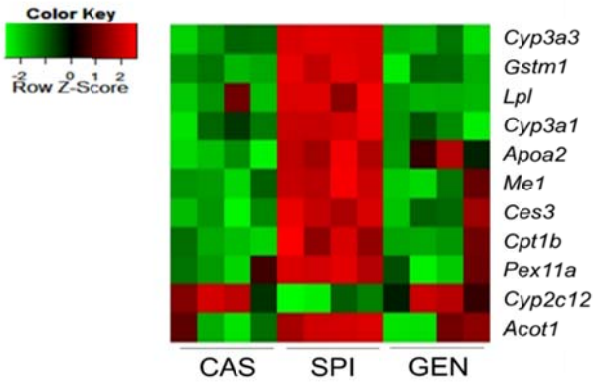


Figure 4.1. Effects of maternal diet on the expressions of PPARα-target genes in the liver of 3-week-old offspring. The relationship between the samples within a gene was determined by the Z-score normalization with a color indication. Red color represents the up-regulated gene expression compared to the mean, whereas green color represents the down-regulated expression below the mean green (see Method section in the **Study 1**). CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

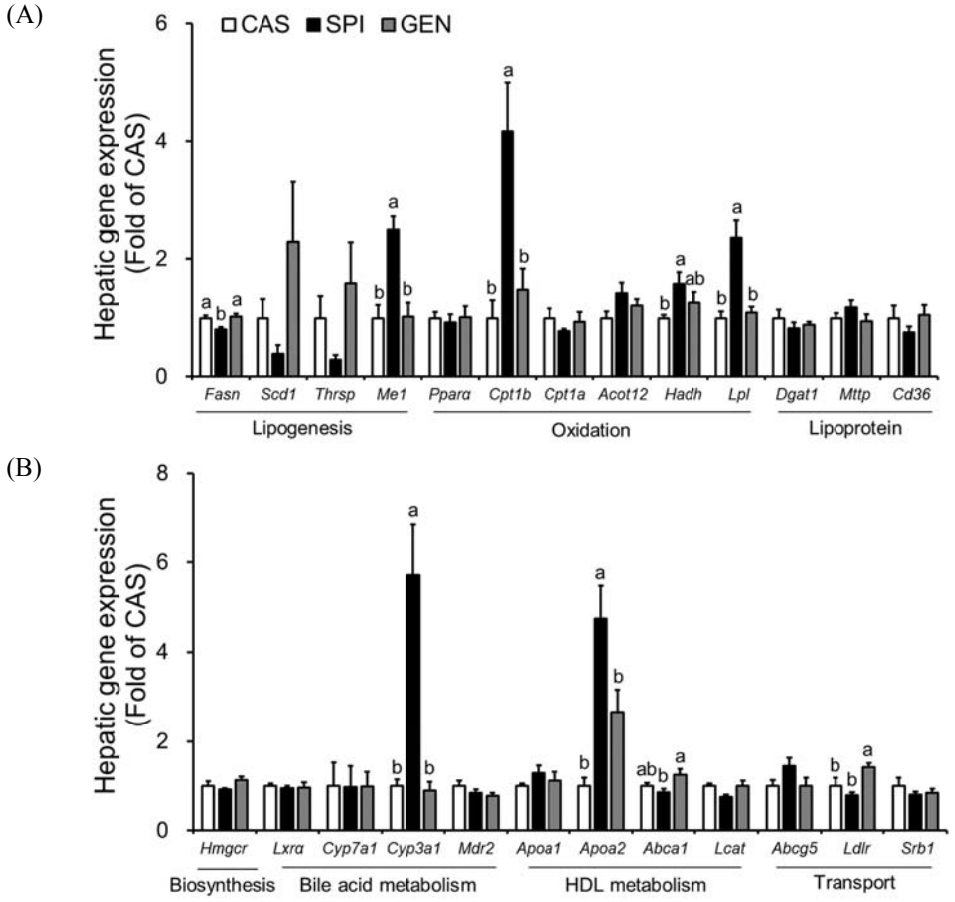
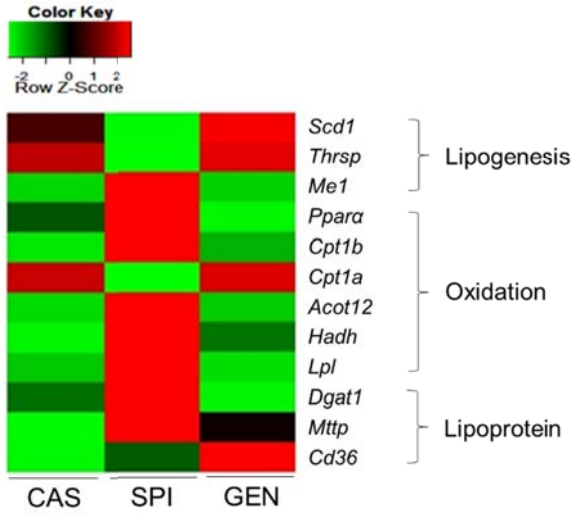


Figure 4.2. Effects of maternal diet on the gene expression related to lipid metabolism in the liver of 3-week-old offspring. Relative mRNA level of each gene involved in (A) triacylglycerol and (B) cholesterol metabolism was determined by qRT-PCR (n=4). The mRNA expression level was normalized to β-actin. Each bar represents the mean ± SEM and bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test). CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet).

(A)



(B)

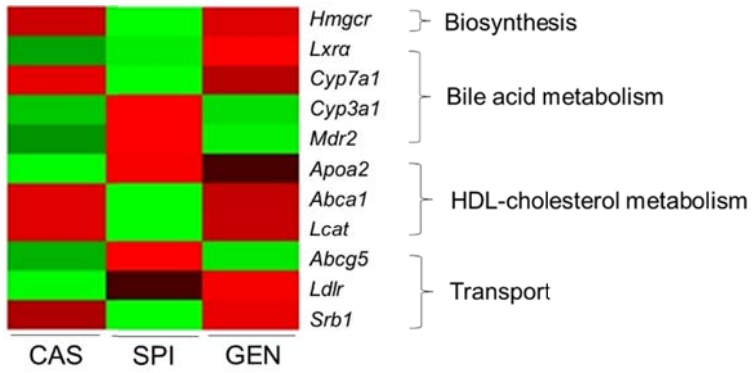


Figure 4.3. Effects of maternal diet on the gene expression related to lipid metabolism observed in microarray in the liver of 3-week-old offspring. The relationship between the samples within a gene was determined by the Z-score normalization with a color indication. Red color represents the up-regulated gene expression compared to the mean, whereas green color represents the down-regulated expression below the mean green (see Method section in the **Study 1**). CAS, casein; SPI, low-isoflavone soy protein isolate; GEN, casein plus genistein (250 mg/kg diet)

groups. The mRNA level of *Ldlr* was significantly higher in the GEN group compared to both the CAS and SPI groups. However, the mRNA levels of *Hmgcr* and *Cyp7a1*, a gene encoding an enzyme catalyzing the rate determining step in sterol and bile acid biosynthesis, were not altered among the maternal diet groups. Interestingly, HDL-cholesterol regulatory genes, *Abca1*, *Lcat*, and *Srb1*, showed a tendency for being lower in the SPI group than in other two groups.

5. Discussion

Several studies have suggested that the consumption of isoflavone with soy protein has different biological effects than the consumption of isoflavone without soy protein (Badger et al., 2002). In the present study, female Sprague-Dawley rats were fed either casein, soy protein with low isoflavone content, or casein supplemented with genistein for two weeks before mating, and during pregnancy and lactation to investigate the effects of maternal dietary protein source and isoflavone content on lipid metabolism in their offspring. The maternal SPI consumption significantly lower serum triacylglycerol and total cholesterol levels in the offspring compared to maternal CAS consumption. In contrast, maternal intake of genistein did not alter serum triacylglycerol levels of offspring, but rather than the lower total cholesterol levels compared to the CAS group. These changes in serum lipid parameters were also observed in dams fed the same diet. However, the SPI diet lowed hepatic triacylglycerol and cholesterol levels only in dams, but not in their offspring. In comparison to epigenetic markers with serum triacylglycerol and total cholesterol levels of offspring, the SPI group had more sensitive response to histone modification compared to the GEN group. H3K9Me3 and H3K9Ac levels were associated with the levels of triacylglycerol (r=0.852, p=0.031 and r=–0.820, p=0.046, respectively) and cholesterol (r=0.777, p=0.069 and r=–0.740, p=0.093, respectively) in the SPI group, whereas these levels were not associated with the levels of triacylglycerol (r=–0.154, p=0.804 and r=–0.409, p=0.494, respectively) and cholesterol (r=–0.041, p=0.948 and r=0.164, p=0.792, respectively) in the GEN group. However, global DNA methylation

was related to serum cholesterol level (r=0.780, p=0.023 for SPI and r=0.818, p=0.024 for GEN) rather than triacylglycerol (r=0.402, p=0.324 for SPI and r=0.080, p=0.865 for GEN) in both SPI and GEN group. In the previous study, H3K9Me3 level was suppressed and correlated with the cell proliferation and PPARα activation (Pogribny et al., 2007b). The **Study 1** observed that PPARα signaling target genes were higher in the SPI group than the CAS group, implying the lipid-lowering effect affected by the maternal diet may be mediated by the epigenetic modification. In the present study, the hypolipidemic effect of the SPI diet is supported by higher hepatic mRNA levels of several genes involved in lipid metabolism, including *Lpl, Me1, Cpt1b, Cyp3a1*, and *Apoa2* activated by PPARα signaling. PPARα activation through the hypolipidemic drugs is involved in liver hyperplasia through the peroxisome proliferation and increases the cytochrome P450 enzyme activity (Walker et al., 1996). PPARα activation is involved in dual effect on lipid metabolism. PPARα stimulates not only lipogenesis mediated by the upregulation of malic enzyme but also fatty acid oxidation through the upregulation of its target genes, such as *Cpt1*. Moreover, PPARα activation is required for the lipoprotein synthesis and assembly related to VLDL and HDL-cholesterol. Amino acid metabolism is also regulated by the PPARα activation. WY14643, which is the agonist of PPARα, decreases plasma triacylglycerol level and increases ketone body (Sheikh et al., 2007). During the rapid growth, liver hyperplasia was generated by the PPARα activation through the reduction in arginine level. In addition, hepatic enlargement induced by WY14643 changed the hepatic gene expression involved in the amino acid metabolism related to the cell proliferation,

suggesting that amino acid mobilization may be involved in the hepatic cell growth and division. It is mediated by the reduction of the rate-limiting enzymes related to the degradation of phenylalanine, tryptophan, methionine, tyrosine, and alanine. Lipid-lowering effect induced by WY14643 may be responsible for the compensation of the increased demand for hepatic protein synthesis. The increased expression of *Lpl* has been reported to be involved in the increased hepatic uptake of circulating triacylglycerol (Grinberg et al., 1985), especially around birth and throughout the suckling period (Panadero et al., 2006). The present study also confirms the increased fatty acid beta-oxidation in the offspring of the SPI group, in which the levels of *Cpt1b* and *Hadh* mRNA were significantly higher. A previous study reported that fetal liver may contain a mixture of adult liver- and muscle-type of CPT1 (Saggerson and Carpenter, 1986), and the protein level showing homology with *Hadh* was higher in adipose tissue of piglets of dams fed the low protein diet (Sarr et al., 2010). Thus, it is possible that the increased fatty acid oxidation may occur to compensate for the increased import of free fatty acids into the liver, thereby resulting in similar hepatic triacylglycerol levels between the CAS and SPI groups. The present study observed a different regulation pattern for lipid metabolism between the SPI and GEN groups. Maternal SPI diet changed both triacylglycerol and cholesterol levels, whereas maternal GEN diet only influenced cholesterol levels in dams and offspring. Higher *Cyp3a1* mRNA levels may be responsible for the hypocholesterolemic effect of the SPI diet by increasing a degradation of bile acid (Xie et al., 2001). The drug-metabolizing P450s such as CYP3a and CYP2 families may be involved in the regulation of the

cholesterol biosynthesis (Rezen et al., 2011). Particularly, *Cyp3a1*, human homologue to *Cyp3a4*, is regulated by farnesoid X receptor (FXR), one of the nuclear receptors expressed in liver, playing a central role in the homeostasis of cholesterol and bile acids. FXR deficient mice induce the increased levels of plasma cholesterol and triacylglycerol regardless of their regulation gene expressions in liver (Lambert et al., 2003). An increased mRNA expression of *Apoa2,* a gene encoding the second most abundant structural apolipoprotein associated with HDL (Birjmohun et al., 2007), may have beneficial effects in the regulation of lipoprotein metabolism in the SPI group. However, other genes involved in HDL metabolism tended to be lower in the SPI group compared to the CAS group, and the ratio of total cholesterol to HDL-cholesterol did not differ between the CAS and SPI groups, suggesting that another mechanism of serum cholesterol lowering effect in the SPI group may be related to the overall cholesterol production rate. Low serum triacylglycerol level in the SPI group compared to the CAS and GEN groups also seems to affect the assembly of HDL, so the reduction of serum total cholesterol level in the SPI group may be consistent with the low HDL-cholesterol level.

It is likely that low serum and hepatic triacylglycerol and cholesterol levels in dams fed an SPI diet potentially change the postnatal growth of their offspring. There was a positive correlation between maternal hepatic triacylglycerol and the body weight (r=0.428, p=0.042) and relative epididymal fat weight (r=0.363, p=0.088) of offspring, but the relative liver weight of offspring showed a negative correlation (r=–0.411, p=0.052). Interestingly, maternal SPI diet rather than maternal GEN diet was

related to offspring changes. Previous study suggested that maternal fat accumulation during pregnancy and lactation may differently influence on lipid metabolism in fetal and postnatal development (Herrera et al., 2006). Maternal low triacylglycerol and cholesterol levels may be associated with the reduction of lipid capacity pool of offspring in the early pregnancy as well as maternal milk quality during lactation consequently alter the postnatal development and lipid metabolism. A formal study observed that circulating maternal lipids, not glucose, was correlated with fetal growth during pregnancy (Schaefer-Graf et al., 2008). Epididymal fat weights in the SPI group were also significantly lower, suggesting that less deposition of fat mass rather than growth retardation was probably be involved in a lower body weight gain of the SPI group. A previous study reported significantly lower body weight and retroperitoneal fat pad weight of the offspring of dams fed a soy protein isolate with isoflavone diet compared to the offspring of dams fed a casein plus genistein (250 mg genistein/kg diet) diet, suggesting that the low body weight and fat weight observed in the offspring of dams fed a soy protein isolate with isoflavone diet may not be attributable to genistein (Simmen et al., 2010). Consistent with this, different levels of phytoestrogen exposure during the perinatal period did not alter the weight gain of either male or female offspring (Becker et al., 2005).

Hepatocyte proliferation and lipid metabolism may be influenced by the maternal serum free amino acid levels. The low circulating levels of the essential amino acids found in maternal protein restriction resulted in the reduced fetal amino acid levels (Rosario et al., 2011). It was related to the decrease in the specific placental

transporters, such as system A and L. Na^+-dependent transporter, system A, transfers the neutral amino acids, such as alanine and serine, whereas Na^+-independent transporter, system L, transfers glutamine and branched-chain amino acids, particularly, leucine. These transporters were regulated by mTOR signaling involved in the protein synthesis and cell growth. Furthermore, maternal serum free amino acid levels may be associated with the biochemical properties and the growth rate of the offspring. Particularly, maternal serum BCAA/AAA ratio was correlated with the body weight (r=0.679, p=0.005), relative epididymal fat (r=0.549, p=0.034), and serum triacylglycerol level (r=0.513, p=0.061) of offspring. The level of the tyrosine in infant plasma, not that of the phenylalanine, may play an important role in the hepatocyte maturity in infants (Clark et al., 1989). The low body weight infants had a poor tyrosine tolerance, so the ratio of BCAA to the aromatic amino acids (AA, phenylalanine and tyrosine) was significantly lowered. The less metabolism of AA in liver led to the higher in AA level in plasma, whereas BCAA was taken up from the plasma as a substitute energy source to generate glucose. In addition, the levels of phenylalanine and tyrosine may be involved in the milk yield (Jansen et al., 1991; Jansen et al., 1986). The levels of the phenylalanine and tyrosine in dam serum were not difference between the control and low-protein diet, but those of the BCAA were significantly lowered in low-protein diet than in the control diet. It is likely that the BCAA may affect the availability of the AAA by competing with each other via leucine preferring system (L system). In addition, tryptophan may be required for protein synthesis in offspring liver. In weaning piglets, dietary tryptophan deficiency decreased

the rate of protein synthesis in muscle and liver (Cortamira et al., 1991). As a different manner with the maternal SPI diet, the maternal GEN diet exhibited the lower level of the maternal serum sulfur amino acid, methionine, compared to that of the maternal CAS diet. Although its mechanism is not clearly explained, at this time, it is likely that there is a different regulation pathway between the SPI and the GEN diet consumption. These results suggest that the reduced methionine level in dams fed with the GEN diet may be related to the betaine-sparing effect. In addition, the maternal consumption of a GEN diet, but not an SPI diet, showed significantly higher mRNA levels of *Ldlr* in offspring, suggesting that increased lipoprotein uptake may be involved in the hypocholesterolemic effect observed in the GEN group. Consistently, a previous study showed a higher expression of *Ldlr* gene in male Zucker rats fed a high-isoflavone diet compared with casein-fed rats, but not in rats fed a protein diet with low ratios of methionine-glycine and lysine-arginine for 6 weeks (Gudbrandsen et al., 2005). Several studies in humans and experimental animals have demonstrated that the in utero environment may have an impact on fetal developmental processes (Ross and Desai, 2005). Programming by early life events can alter overall homeostatic regulatory mechanisms, thereby playing a critical role in influencing the susceptibility of offspring to certain diseases in adulthood (Gluckman et al., 2008). It is proposed that nutrition during the fetal and neonatal periods can affect the risk of chronic diseases in adulthood as well as fetal development (Wu et al., 2004).

Taken together, maternal consumption of the SPI and genistein distinctively regulated serum lipid parameters and hepatic gene expressions involved in lipid

metabolism both in dams and their offspring. Interestingly, more pronounced effects were observed in offspring of the SPI group, suggesting an important role of maternal SPI consumption regardless of isoflavone levels in lipid metabolism regulation. Therefore, further studies would be needed to investigate whether metabolic programming rather than direct effects by maternal nutrition would be involved in the altered lipid metabolism in offspring.

V. Study 3

Effects of maternal consumption of soy protein isolate on the liver damage and cholesterol metabolism of adult rat offspring fed an ethanol diet

1. Abstract

In **Study 1** and **2**, the consumption of a low-isoflavone soy protein isolate (SPI) rather than genistein showed a significant lipid-lowering effect compared to that of casein (CAS) and altered hepatic expression of genes related to methyl group and lipid metabolism of 3-week-old male rat offspring. A follow-up study was performed to compare the effect of maternal dietary protein source between the CAS and SPI diet. Therefore, this study investigated whether the adaptive response of offspring to chronic ethanol consumption would affect the progression of liver damage in later life. Female Sprague-Dawley rats were fed a CAS or an SPI diet for two weeks before mating and during pregnancy and lactation. After feeding the standard chow diet from weaning to 8 weeks of age, pairs of male offspring originated from the same dam were fed either a control or an ethanol liquid diet (Groups: CAS/CON, CAS/EtOH, SPI/CON, and SPI/EtOH). After 6 weeks of pair-feeding, levels of serum transaminases and hepatic tumor necrosis factor-alpha and monocyte chemoattractant protein-1 mRNA were significantly elevated in the SPI/EtOH group compared to the CAS/EtOH group. Concomitant with lower hepatic ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine in the SPI/EtOH group compared to the CAS/EtOH group, hepatic mRNA levels of phosphatidylethanolamine *N*-methyltransferase, glycine *N*-methyltransferase, and 5-methyltetrahydrofolate-homocysteine methyl-transferase were significantly lowered. Moreover, endoplasmic reticulum stress response associated with the reduced methylation capacity was accelerated by the SPI group. Unexpected severe liver damage observed in the SPI/EtOH group was followed by

lower levels of serum total and HDL-cholesterol. The significantly lower hepatic mRNA levels of scavenger receptor class B type 1, lecithin:cholesterol acyltransferase, and ATP-binding cassette transporter A1 as the key regulator genes responsible for HDL-cholesterol production and assembly were observed in the SPI/EtOH group compared to the CAS/EtOH group. In conclusion, relatively severe liver damage was observed in the offspring of the SPI group compared to those of the CAS group, suggesting for the first-time that maternal SPI diet as a potential mediator may contribute to the offspring's susceptibility to the development of liver damage in adulthood through regulating one-carbon metabolism and cholesterol metabolism.

2. Introduction

Liver is the largest organ and responsible for the various adaptation of metabolic homeostasis and detoxification (Hyatt et al., 2008). These functions in fetal and prenatal liver growth and development are closely associated with placental and/or maternal nutrition in specific gestation stage. Liver growth is induced by the combination of hepatocyte hypertrophy and proliferation. The growth of multiple organs of offspring showed a different adaptive pattern (Desai et al., 1996; Latini et al., 2004; Morrison et al., 2010). Among the organogenesis, liver size and growth are more sensitively affected by utero-perturbation during critical period of development to protect and compensate for other organs' function (Hyatt et al., 2008). Under poor maternal nutrition status, liver growth was greatly decreased compared to other organs like brain and heart, which are hardly influenced. This compromised response may contribute to induce a rapid hepatic cellular proliferation in late gestation and early postnatal life and unfavorable effect on glucose and lipid metabolism (Morrison et al., 2010). Therefore, fetal liver growth programming is a pivotal role to improve offspring survival and long-lasting effect on maintaining metabolic homeostasis in later health.

Alcohol is mainly metabolized in liver and a major contributor to liver-related injury in both early and later life (Keegan et al., 1995; Meyers et al., 2002). The metabolic consequences of hepatic ethanol exposure may develop the progression of alcoholic liver injury including fatty liver, hepatocyte necrosis, inflammation, fibrosis, and cirrhosis (Lieber, 2000). Break of the balance of liver homeostasis in chronic ethanol exposure causes an increase in homocysteine level associated with the

disturbance of methylation capacity (Ji, 2012; Sozio and Crabb, 2008). The perturbation of the endoplasmic reticulum (ER) homeostasis stimulated by homocysteine is also a key factor in the pathogenesis of ethanol related liver damage (Godfrey and Barker, 2001; Ji, 2012). Homocysteine and ER stress response up-regulated by ethanol feeding contributes to dysregulate lipid metabolism (Chen et al., 2012a). Although there is a controversial issue related to HDL-cholesterol level in ethanol intake, chronic ethanol exposure may exacerbate HDL-cholesterol metabolism through the down-regulation of scavenger receptor class B type 1, ATP-binding cassette transporter A1, and lecithin:cholesterol acyltransferase. The previous study suggests the connection between HDL-cholesterol mechanism and the impaired liver function.

To date, most studies related to fetal programming have been conducted with inadequate nutritional status (Chen and Nyomba, 2004; Hales and Ozanne, 2003; Yao and Nyomba, 2008). Their offspring developed the adverse effects such as insulin resistance and glucose intolerance. Furthermore, most researches using soy protein were not designed to show the influence of the later life consequences of early life dietary exposure. There are few studies of soy protein related to maternal diet effect on metabolic changes in the adult offspring. As well, the adaptive responses of offspring to metabolic challenges in later life have not been well understood. Previous study showed the interaction between soy protein isolate in the diet and formation of aberrant crypt foci (ACF) in the growing offspring (Linz et al., 2004). They also confirmed the inhibitory effect of SPI on occurrence of ACF in offspring from maternal exposure to

ethanol during pregnancy, but soy protein isolate effect-related to ethanol was limited on the developmental stage like other reports.

The **Study 1** and **2** observed the lipid-lowering effect and the significant changes of hepatic gene expression in three-week-old male offspring from dams fed an SPI diet rather than genistein compared to dams fed CAS. Regardless of the limited amino acids of SPI *per s*e, maternal dietary composition based on AIN-93G was not modified except for soybean oil, but dams were received an adequate nutrition during the pre-pregnancy, pregnancy, and lactation. Adult offspring used in the present study were originated from the **Study 1** and **2**, but the SPI diet was not provided after the postnatal d21 and during ethanol consumption. Therefore, in this study, the first aim was to investigate whether the adaptive response of offspring to maternal diet affects the development of chronic ethanol-induced liver damage in later life. The second was to identify the correlation between the degree of liver damage and cholesterol homeostasis.

3. Materials and Methods

3.1. Diets and animals

Seven-week-old virgin females Sprague-Dawley rats (Oriental Bio Inc., Korea) were obtained and maintained under the controlled room temperature of 22 ± 3°C and the humidity of 50 ± 10% with a 12 h light-dark cycles (8 am ~ 8 pm). The study scheme was presented in **Figure 5.1**. Briefly, after one week acclimation period, rats were randomly split into two sole protein sources and fed either a CAS or an SPI diet in powdered form made according to the AIN-93G guidelines (Reeves et al., 1993) except that soy oil was replaced with corn oil. All dam diets were provided for 2 weeks prior to pregnancy. At 10 weeks of age, female rats were caged with proven male breeders overnight. Mating was confirmed the next morning by the presence of a virginal plug. After 5 days mating period, dams were housed individually and continued on the same diet during pregnancy and lactation. Within the first day of birth (postnatal day 0, PND 0), litter size was adjusted to 8 pups per litter (2 females and 6 males) to normalize the growth. At weaning on PND 21, male offspring were removed from their dams and weaned to standard chow diet until 8 weeks of age. Then, male offspring from the same dam either a CAS or an SPI diet were divided into two groups and fed either an ethanol or a pair-fed control diet, respectively (Groups: CAS/CON, CAS/EtOH, SPI/CON, and SPI/EtOH). Ethanol groups were fed the Lieber-Decarli liquid diet (#710260; Dytes Inc., USA) that consisted of 36% ethanol-derived calories (Lieber and DeCarli, 1989). Pair-fed control groups were received an ethanol-free isocaloric diet containing maltose dextrin (#710027) instead of ethanol. Ethanol was introduced into the liquid

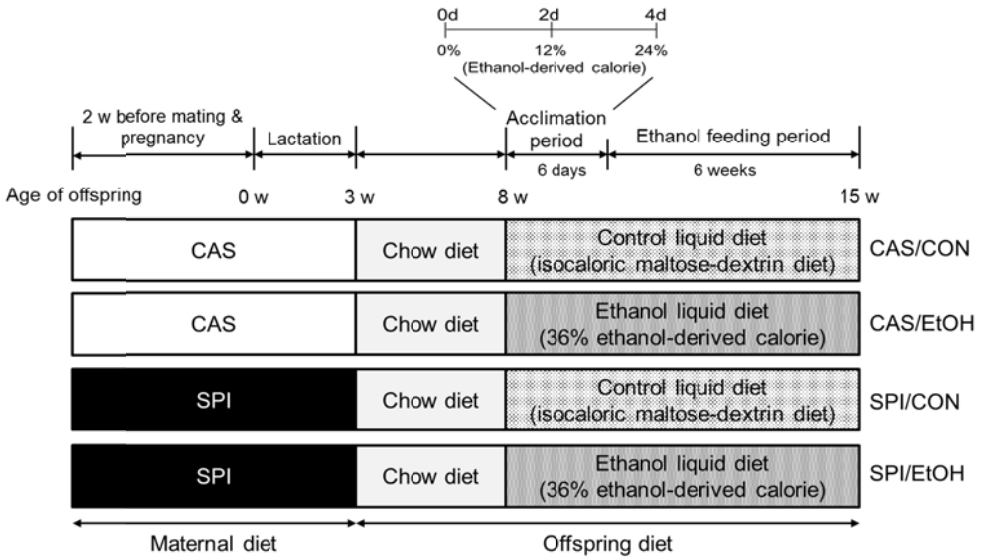


Figure 5.1. Overview of the study design. CAS, casein; SPI, low-isoflavone soy protein isolate; CAS/CON, casein control; CAS/EtOH, casein ethanol; SPI/CON, SPI control; SPI/EtOH, SPI ethanol.

diet gradually starting from 0% of energy to 24% over the acclimation period, and then rats were maintained on the 36% ethanol of calories for another 6 weeks. Ethanol-fed rats were allowed liquid diet *ad libitum*, and their daily intake was monitored. The pair-fed rats were given the same amount of ethanol group during the following 24 h feeding time. Weight gain was measured once a week. At the end of the experiment period, to make a similar fasting condition, liquid diet was provided twice within 24 h before 16 h fasting. Blood samples were rapidly taken from heart puncture, and serum was obtained by the centrifugation at 3,000 rpm for 20 min at 4°C. Liver and epidydimal adipose tissues were removed, snap-frozen immediately in liquid nitrogen, and stored at -80°C until use. All treatment protocols for this study were approved by Seoul National University Institutional Animal Care and Use Committee (SNUIACUC).

3.2. Serum and hepatic biochemical analysis

Serum triacylglycerol, total cholesterol, and HDL-cholesterol concentrations were measured using the commercial kits (Asan Pharm. Co., Ltd, Korea). The activities of serum glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were measured by using the commercial kit (Asan Pharm. Co., Ltd, Korea). Serum monocyte chemoattractant protein-1 (MCP-1) was determined by using the commercial kit (R&D systems, USA). Total lipids were extracted according to the method used by Folch et al. (Folch et al., 1957), and hepatic triacylglycerol and cholesterol levels were determined by the same commercial kit used in serum (see the

method section in the **Study 2**).

3.3. Total RNA isolation and quantitative real-time PCR analysis

See the method section in the **Study 1.**

3.4. Semi-quantitative RT-PCR analysis

After extracting total RNA of liver using RNAiso-plus reagent (Takara, Japan), it was reverse-transcribed using the Superscript®II first-strand synthesis system II (Invitrogen, USA) for RT-PCR. According to the previous report (Toda et al., 2006), the synthesized cDNA was amplified using X-box binding protein 1 primers (*Xbp-1*, forward: 5'-GAA CCA GGA GTT AAG GAC ACG C-3' and reverse: 5'-GGG GAT CTC TAA GAC TAC AGG CT-3'). *Xbp-1* primers generate cDNA products of the unspliced and spliced *Xbp-1* mRNA. The unspliced fragment was further digested by *Pst 1* (Promega, USA), and cDNA products from the unspliced mRNA yielded two short fragments after digestion. PCR reaction was performed under the following condition: initiation at 94°C for 4 min and PCR cycling for 25 cycles (denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min).

3.5. Determination of serum homocysteine and hepatic SAM and SAH

See the method section in the **Study 1.**

3.6. Statistical analysis

All experiments were initially compared using Two-way ANOVA (maternal diet, ethanol diet, and maternal x ethanol interaction) in CAS/CON, CAS/EtOH, SPI/CON, and SPI/EtOH groups. One-way ANOVA followed by Duncan's multiple range test were used after Two-way ANOVA. Also, Student's *t*-test was carried out. All significant differences were assessed using SPSS software (Version 19.0, USA). The significant level was set at an alpha = 0.05, and the results were expressed as means ± SEM (the standard error of the mean). Correlation between two variables was analyzed by *Pearson* correlation coefficient.

4. Results

4.1. Effects of maternal diet on the liver damage in adult offspring of chronic ethanol consumption

A present study determined whether adult male offspring from maternal with a CAS or an SPI diet could alter the response of the liver to chronic ethanol consumption leading to liver damage. As expected, offspring fed an ethanol diet had significantly higher relative liver weight and tended to have lower relative epididymal fat weight than those fed a control diet without the body weight differences (**Table 5.1**). As primary hepatic damage indicators, serum GOT and GPT levels were evaluated (**Figure 5.2A**). In the present study, serum GOT and GPT levels were affected by ethanol feeding, and interestingly, the SPI/EtOH group underwent detectable severe liver damage compared to the CAS/EtOH group, and there was a significant maternal x ethanol interaction. Even though offspring was received the same amount of ethanol dose and there was no difference in dietary intake between the CAS and SPI groups (97.2 ± 3.3 g/d and 100.5 ± 3.8 g/d, respectively), they showed the different degree of liver damage.

Table 5.1. Effects of maternal diet on the body weight change and organ weights in adult offspring of chronic ethanol consumption

Maternal diet	CAS		SPI	
Offspring diet	CON	EtOH	CON	EtOH
Body weight (g)				
3w	63.3 ± 0.9		54.7 ± 1.3*	
8w	400.1 ± 3.7		384.4 ± 6.9	
15w (Final)	533.4 ± 12.3	497.9 ± 10.8	531.8 ± 22.8	511.6 ± 20.0
Organ weight (g)				
Liver	15.0 ± 0.4	16.9 ± 0.8	15.3 ± 1.3	17.0 ± 1.3
Epididymal fat	14.0 ± 1.3	10.7 ± 0.7	12.7 ± 1.1	10.4 ± 1.6
Relative liver weight (g/100g body weight)				
Liver	2.8 ± 0.1^{b}	3.4 ± 0.1^{a}	2.9 ± 0.1^{b}	3.3 ± 0.1^{a}
Epididymal fat	2.6 ± 0.2^{a}	2.1 ± 0.1^{ab}	2.4 ± 0.1^{ab}	2.0 ± 0.2^{b}

CAS, casein; SPI, low-isoflavone soy protein isolate; CON, control; EtOH, Ethanol. Data are means ± SEM (n=8). Effects of maternal diet and offspring diet were analyzed by Two-way ANOVA. Means in the same row with different superscript are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test). * CAS vs. SPI at $p < 0.05$ (Student's *t*-test).

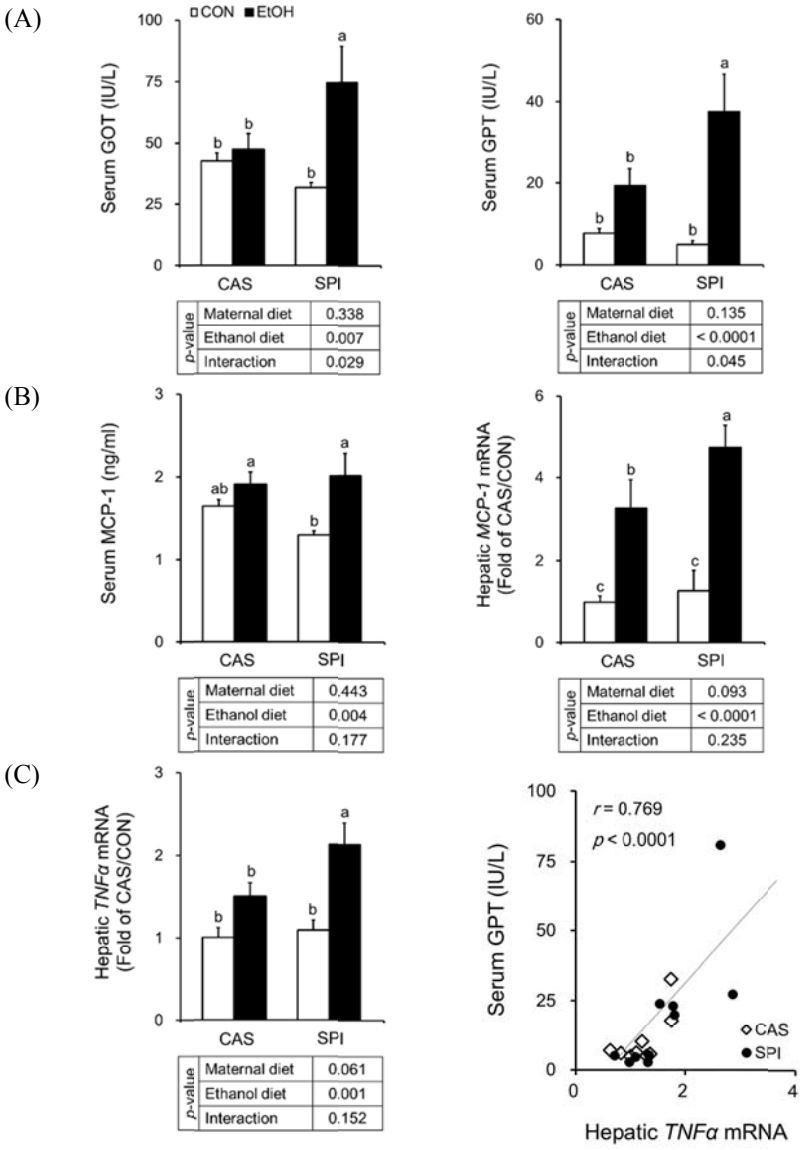


Figure 5.2. Effects of maternal diet on the liver damage markers in adult offspring fed an ethanol diet. (A) Serum levels of GOT and GPT (n=8), (B) serum levels and hepatic mRNA levels of MCP-1 (n=5-8), (C) hepatic *TNFa* mRNA expression (n=4-5) and *Pearson*'s correlation between serum GPT levels and hepatic *TNFa* mRNA expression. The mRNA expression level was normalized to β-actin. Data are expressed as means ± SEM. Effects of maternal diet and offspring diet were analyzed by Two-way ANOVA. Bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test). *Pearson* correlation coefficient, *r* and *p*-value are indicated.

4.2. Effects of maternal diet on the pro-inflammatory response in adult offspring of chronic ethanol consumption

More severe liver damage was seen in the SPI/EtOH group, so pro-inflammatory mediators, TNFα and MCP-1, were associated with alcoholic liver injury. Previous study reported that TNFα and MCP-1 may be considered to be harmful in context of liver injury (Mandrekar et al., 2011). Concomitant with other damage markers observed in this study, levels of serum MCP-1 and hepatic *MCP-1* mRNA were markedly higher in the SPI/EtOH group than the SPI/CON group (**Figure 5.2B**). Hepatic mRNA level of *TNFα* was also enhanced in the SPI/EtOH group, and the pattern of hepatic *TNFα* mRNA level was positively correlated with serum GPT levels (**Figure 5.2C**).

4.3. Effects of maternal diet on the one-carbon metabolism in adult offspring of chronic ethanol consumption

To figure out this different response pattern in the degree of liver injury between the CAS and SPI groups, firstly, the present study focused on homocysteine metabolism and methylation status because alcohol-induced homocysteine increase may be associated with the methylation capacity, ER-stress, and dyslipidemia, leading to the progression of liver damage. In **Study 1**, offspring from maternal SPI diet had significantly higher in serum homocysteine level and lower in methylation enzymes without the change of SAM/SAH ratio. Here, this study observed whether these methylation responses would be sustained in SPI group, and there would be interrelation between homocysteine and one-carbon mechanism and the severity of alcohol-induced liver injury. Like the damage markers, GOT and GPT levels, serum homocysteine level was significantly higher in the SPI/EtOH group compared to the SPI/CON group, but not between the CAS groups (**Figure 5.3A**). Chronic ethanol consumption also significantly reduced hepatic SAM/SAH ratio in the SPI/EtOH group compared to the CAS/EtOH group, and there was a significant maternal x ethanol interaction. These results may be related to hepatic SAM and SAH levels, so hepatic SAM/SAH ratio was correlated with hepatic SAM level (r=0.670, p<0.0001) and hepatic SAH level (r=−0.553, p=0.002). Moreover, many methylation enzymes can be responsible for homocysteine level and methylation potential. Hepatic mRNA levels of *Bhmt*, *Mat1a*, and *Cbs* were not different between the CAS/EtOH and SPI/EtOH groups (**Figure 5.3B**), suggesting that chronic ethanol consumption may similarly

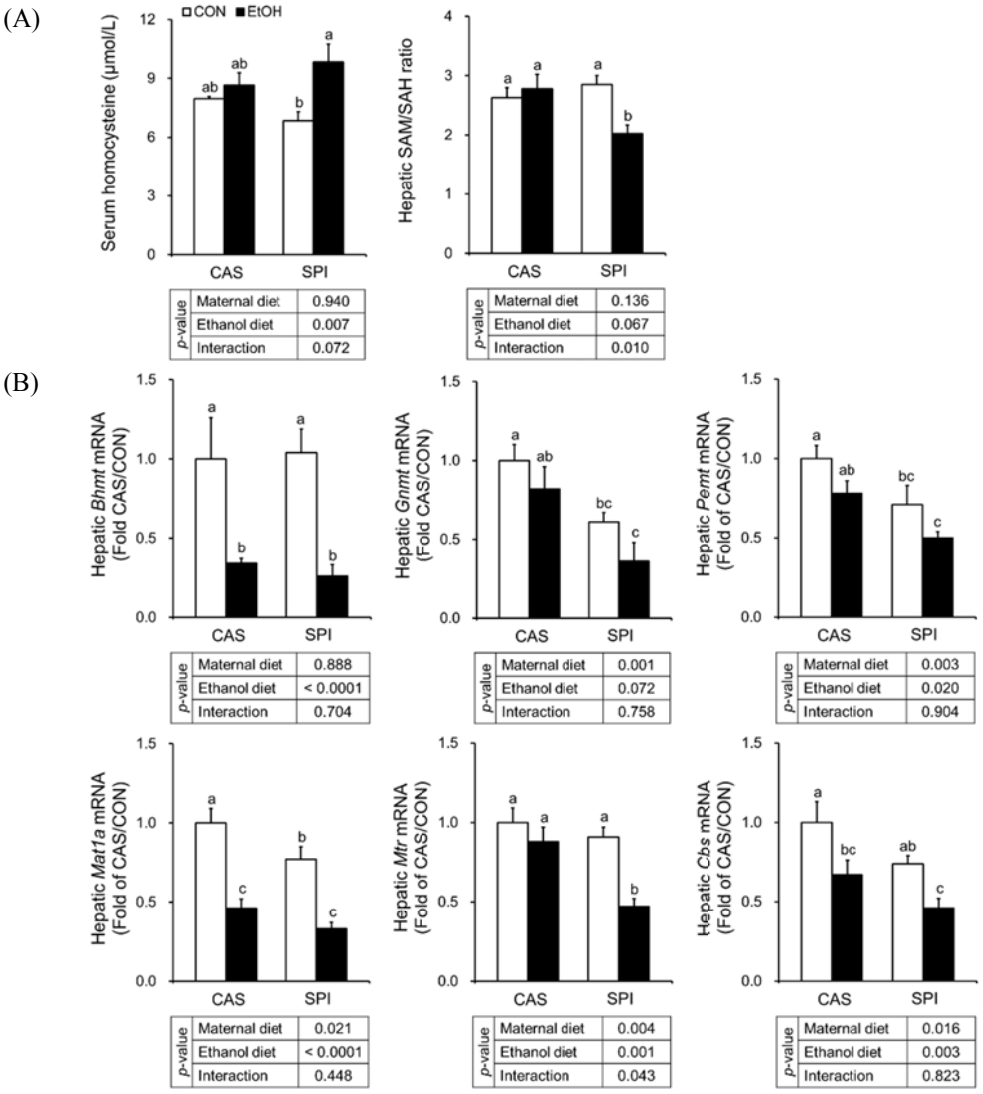


Figure 5.3. Effects of maternal diet on the one-carbon metabolism in adult offspring fed an ethanol diet. (A) Serum levels of homocysteine (n=5) and hepatic SAM/SAH ratio (n=7-8) and (B) hepatic mRNA levels of *Bhmt*, *Gnmt*, *Pemt*, *Mat1a*, *Mtr*, and *Cbs* (n=4-5). The mRNA expression level was normalized to β-actin. Data are expressed as means ± SEM. Effects of maternal diet and offspring diet were analyzed by Two-way ANOVA. Bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test).

affect the production of methionine, SAM, and cysteine. However, hepatic mRNA levels of *Mtr*, *Pemt*, and *Gnmt* were significantly lower in the SPI/EtOH group compared to the CAS/EtOH group (**Figure 5.3B**). The mRNA levels of *Cbs*, *Mtr*, *Mat1a*, *Pemt*, and *Gnmt* had a significant maternal effect. SAM acted as the universal methyl donor for all methylation reactions, whereas these reactions were inhibited by SAH (Halsted and Medici, 2011). Previous studies showed that alcohol intake suppressed PEMT activity equally with the decreased SAM/SAH ratio (Kharbanda, 2007). Taken together, in non-stress condition, methylation capacity seemed to compensate for the inadequate enzyme levels in the SPI/CON group compared to the CAS/CON group, whereas these sustained lower levels of methylation enzymes may contribute to stimulate the response to alcoholic liver injury and make liver damage worse.

4.4. Effects of maternal diet on the ER stress in adult offspring of chronic ethanol consumption

Alcohol and/or homocysteine promoted liver damage through ER stress response (Barve et al., 2006). The reduced methylation capacity was also associated with ER stress response. As shown in **Figure 5.4A and B**, the mRNA levels of the spliced *Xbp-1* and *Chop*, as the ER stress indicators, were significantly higher in the SPI/EtOH group compared to the SPI/CON group. *Chop* mRNA level also had a significant maternal x ethanol interaction, indicating that *Chop* mRNA level in response to ER stress may be responsible for apoptosis in the SPI/EtOH group. In particular, the mRNA levels of the spliced *Xbp-1* and *Chop* had a positive correlation with those of hepatic *TNFα* mRNA (r=0.507, p=0.027 and r=0.518, p=0.023, respectively) and serum MCP-1 (r=0.637, p=0.003 and r=0.585, p=0.008, respectively).

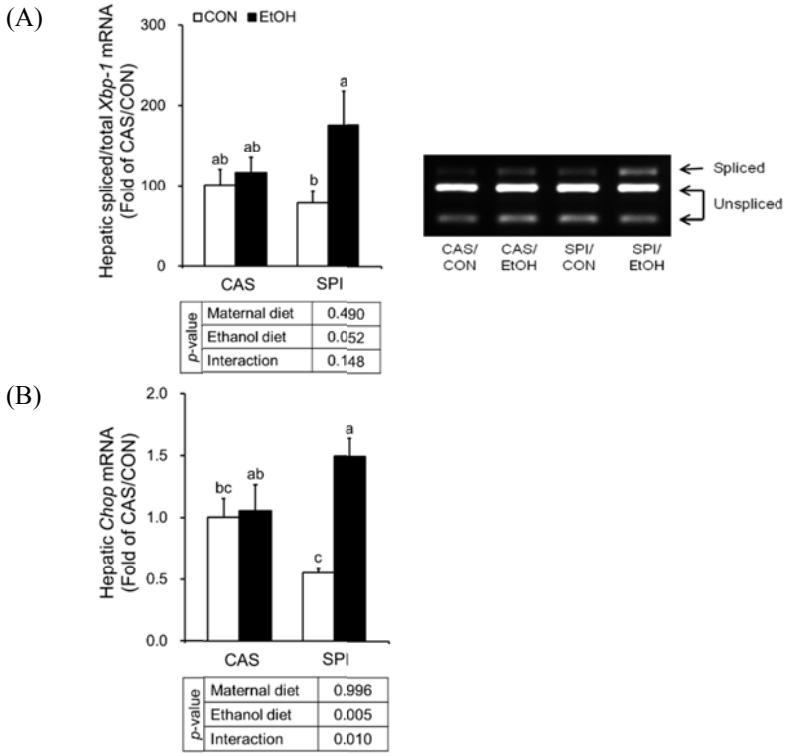


Figure 5.4. Effects of maternal diet on the ER stress response in adult offspring fed an ethanol diet. Hepatic (A) spliced *Xbp-1* (n=5) and (B) *Chop* mRNA expression (n=4-5) was analyzed by the semi-quantitative RT-PCT and qRT-PCR, respectively. The mRNA expression level was normalized to β-actin. Data are expressed as means ± SEM. Effects of maternal diet and offspring diet were analyzed by Two-way ANOVA. Bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test).

4.5. Effects of chronic ethanol-induced liver damage on cholesterol metabolism of adult offspring

As the liver function was disturbed, lower levels of cholesterol were observed (Ghadir et al., 2010). The progression of liver damage may contribute to impair the key genes related to cholesterol synthesis and cholesterol clearance, specially, HDL-production and assembly. Chronic ethanol consumption caused more severe liver damage in the SPI/EtOH group compared to the CAS/EtOH group. Therefore, the association between the dysregulation of cholesterol metabolism and the degree of liver damage was confirmed. Ethanol feeding significantly increased serum and hepatic cholesterol levels in the CAS/EtOH group compared to the CAS/CON group, but not in between the SPI groups (**Figure 5.5A and B**). Serum cholesterol levels had also significant maternal effect and maternal x ethanol interaction. This cholesterol-lowering effect in the SPI/EtOH group may reflect metabolic disturbance as a result of severe liver damage induced by chronic ethanol consumption. Previous study proposed that plasma homocysteine level may be associated with a negative effect on HDL cholesterol level by suppressing the mRNA levels of *Apoa1*, *Lcat*, and *Abca1* (Velez-Carrasco et al., 2008). In addition, the lower ratio of SAM/SAH can lead to decrease HDL-cholesterol levels (Obeid and Herrmann, 2009). Without changing *Hmgcr* mRNA level, *Ldl*r mRNA level was significantly reduced by ethanol feeding and showed a maternal effect (**Figure 5.6**). As anticipated, the mRNA levels of *Abca1* and *Lcat* were significantly suppressed in the SPI/EtOH group compared to other groups and showed significant maternal effect and maternal x ethanol interaction (**Figure 5.7**).

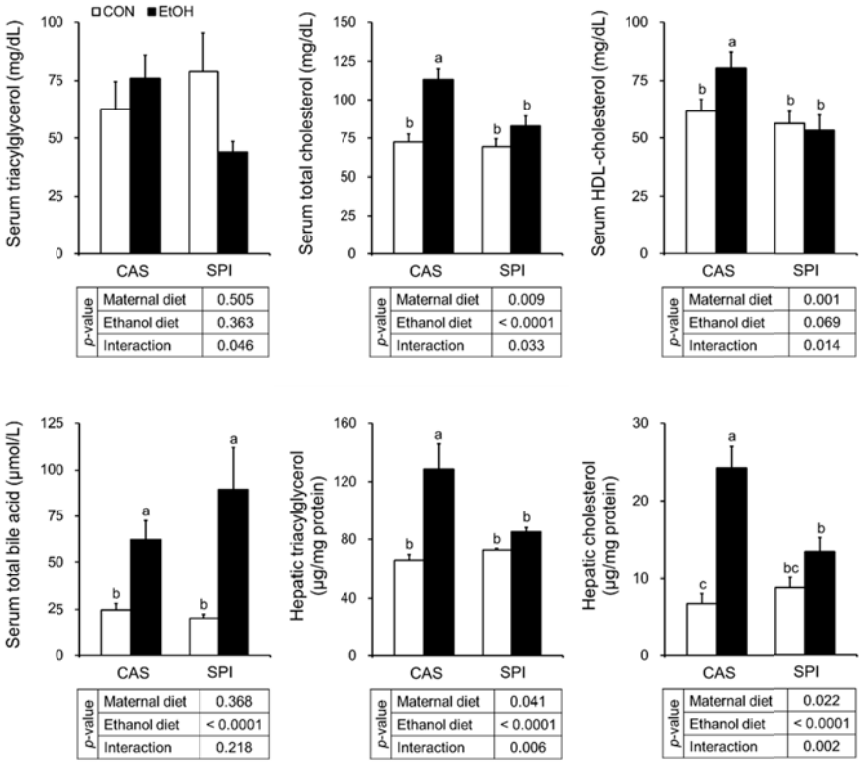


Figure 5.5. Effects of maternal diet on serum and hepatic biochemical parameters in adult offspring fed an ethanol diet. (A) Serum triacylglycerol, total cholesterol, HDL-cholesterol, and total bile acid levels (n=8) and (B) hepatic triacylglycerol and cholesterol levels (n=7-8). Data are expressed as means ± SEM. Effects of maternal diet and offspring diet were analyzed by Two-way ANOVA. Bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test).

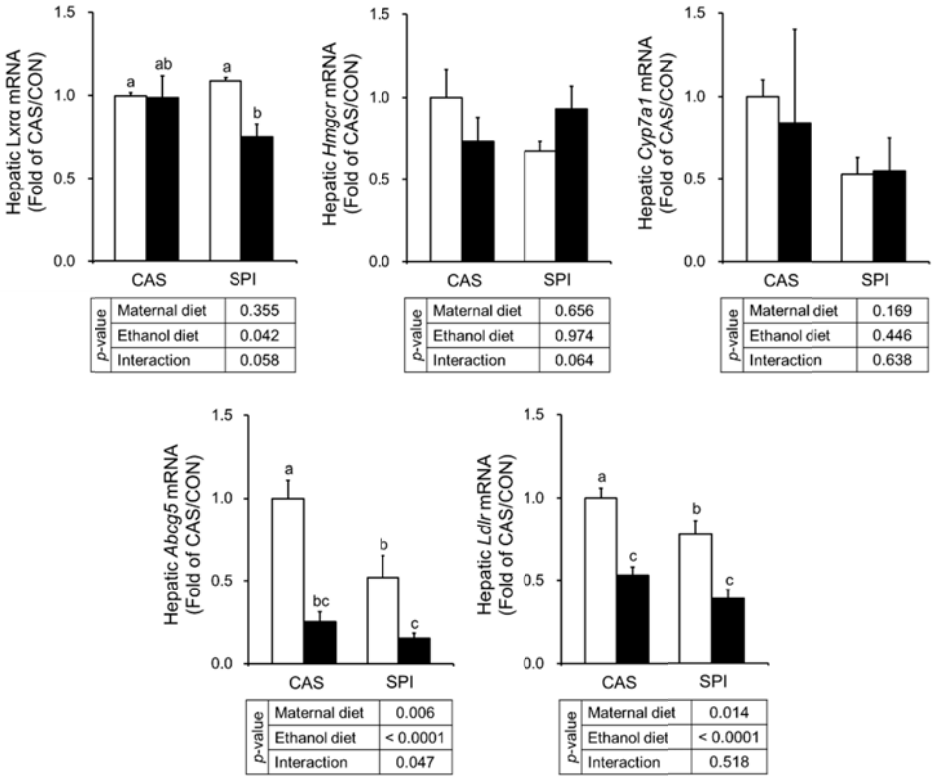


Figure 5.6. Effects of maternal diet on the gene expression related to cholesterol and bile acid metabolism in the liver of adult offspring fed an ethanol diet (n=4-5). The mRNA expression level was normalized to β-actin. Data are expressed as means ± SEM. Effects of maternal diet and offspring diet were analyzed by Two-way ANOVA. Bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test).

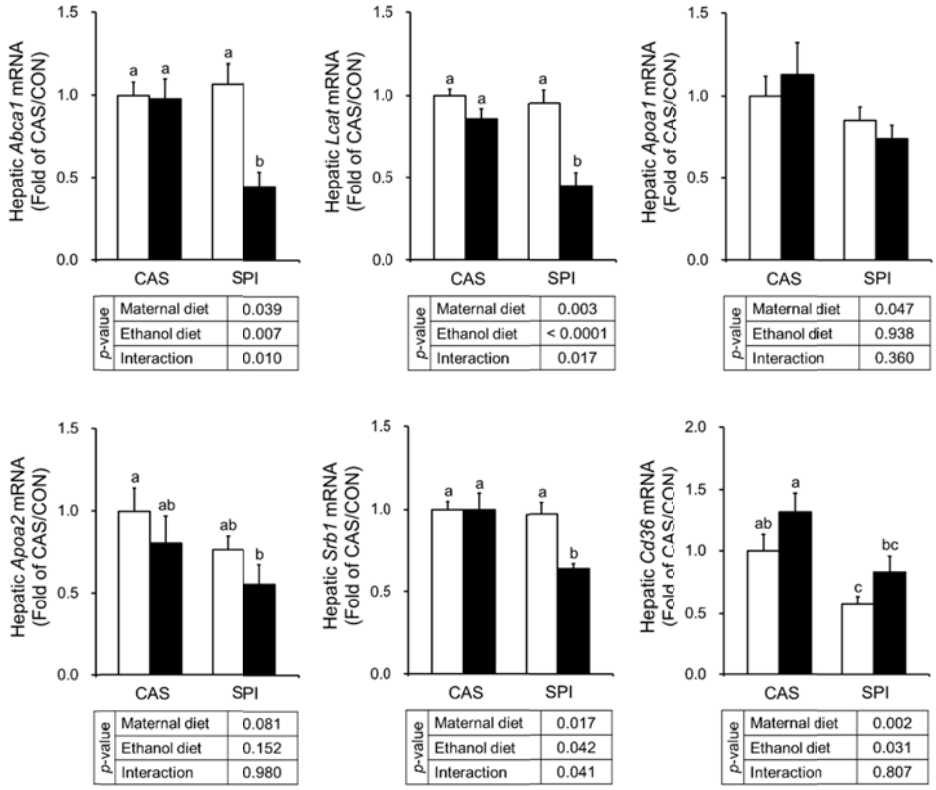


Figure 5.7. Effects of maternal diet on the gene expression related to HDL-cholesterol production and assembly in the liver of adult offspring fed an ethanol diet (n=4-5). The mRNA expression level was normalized to β-actin. Data are expressed as means ± SEM. Effects of maternal diet and offspring diet were analyzed by Two-way ANOVA. Bars with different superscripts are significantly different at $p < 0.05$ (One-way ANOVA with Duncan's multiple comparison test).

In addition, the mRNA level of *Srb1*, HDL-cholesterol specific transport, was significantly lowered in the SPI/EtOH group (**Figure 5.7**) and had maternal effect and maternal x ethanol interaction. Hepatic *Cd36* mRNA level was also lower in the SPI/EtOH group than in the CAS/EtOH group. *Cyp7a1* mRNA level was not changed by ethanol feeding, whereas *Abcg5* mRNA level in both ethanol groups was significantly inhibited and showed maternal effect and maternal x ethanol interaction (**Figure 5.6**). Chronic ethanol consumption disturbed the mRNA levels of *Mttp* (CAS: 0.98 ± 0.12; SPI: 0.63 ± 0.08, p=0.059) and *Dgat1* (CAS: 0.81 ± 0.0.6; SPI 0.58 ± 0.07, p=0.032) in the SPI/EtOH group. *Mttp* mRNA level also had a maternal x effect interaction (p=0.025), and *Dgat1* mRNA level was significantly affected by maternal effect (p=0.014). Similarly to cholesterol, serum and hepatic triacylglycerol levels showed a significant maternal x ethanol interaction, and hepatic triacylglycerol level had a significant difference between the ethanol groups (**Figure 5.5A and B**).

In *Pearson's* correlation analysis, there was a correlation between liver damage marker, GPT, and total cholesterol level (**Table 5.2**). Interestingly, serum GPT level had a powerful correlation with the key genes involved in HDL-cholesterol metabolism (**Table 5.3**).

Table 5.2. Correlation between serum GPT level and serum and hepatic parameters of adult offspring

Parameters	Correlation coefficient (*r*)	*p*-value
Serum		
Triacylglycerol	–0.212	0.243
Free fatty acid	–0.268	0.139
Total cholesterol	0.345	0.053
HDL-cholesterol	–0.035	0.847
HDL/total cholesterol	0.784	0.000
Total bile acid	0.850	0.000
Liver		
Triacylglycerol	0.316	0.089
Free fatty acid	0.055	0.771
Cholesterol	0.264	0.159

Correlation between two variables was analyzed by *Pearson* correlation coefficient.

Table 5.3. Correlation between serum GPT level and hepatic gene expression related to cholesterol metabolism

Hepatic gene expression (mRNA)	Correlation coefficient (*r*)	*p*-value
Abcg5	–0.521	0.022
Abca1	–0.453	0.045
Apoa2	–0.453	0.045
Lcat	–0.715	0.000
Ldlr	–0.682	0.001
Lxrα	–0.593	0.006
Mttp	–0.592	0.008
Srb1	–0.513	0.030

Correlation between two variables was analyzed by *Pearson* correlation coefficient.

5. Discussion

The present study investigated the effects of maternal diet originated from the different sole protein sources, casein or soy protein isolate, on ethanol-induced liver injury in adult offspring. As mentioned earlier, the previous studies have shown the beneficial effects of soy protein isolate on lipid and glucose metabolism compared to a casein (Ikeda et al., 2009; Rajasree et al., 2009). These effects may be associated with the phytochemicals, such as isoflavones, and the different amino acid profiles. The **Study 1** and **2** observed that after the birth, liver proliferation was dramatically increased in offspring from maternal SPI diet compared to that from maternal CAS diet. So, the present study was interested in whether the impact of the different amino acid composition between CAS and SPI can be sustained on liver function in adult offspring.

Offspring from maternal exposure to the SPI diet significantly showed much higher GOT and GPT levels in chronic ethanol consumption, whereas that from maternal CAS diet induced slightly increased GOT and GPT levels. This different pattern may influence the severity of liver damage. To determine the degree of liver injury in the ethanol feeding groups, firstly, the alteration of homocysteine and one-carbon methyl-group mechanism was observed. In **Study 1**, one-carbon metabolism was influenced by maternal diet, so the connection between maternal diet and ethanol-induced liver damage in offspring later life was determined. Serum homocysteine level was significantly elevated in the SPI/EtOH group compared to the SPI/CON group, and there was a strong correlation between serum GOT and GPT levels and homocysteine level (r=0.699, p=0.001 for GOT and r=0.681, p=0.001 for GPT). In

addition, SAM/SAH ratio as the methylation capacity was significantly reduced by ethanol exposure only in the SPI/EtOH group. These homocysteine and methylation response changed by the ethanol feeding have been well explained (Lieber, 2002; Obeid and Herrmann, 2009; Sozio and Crabb, 2008). Classically, ethanol exposure disturbed methionine metabolism and induced the impaired methylation capacity. Particularly, the possible interaction between homocysteine level and SAM/SAH ratio and liver damage was well reviewed (Obeid and Herrmann, 2009). Similarly, the change of serum homocysteine level and hepatic SAM/SAH ratio was more sensitively stimulated by the SPI/EtOH group compared to the CAS/EtOH group. So, methylation enzymes at the transcriptional level were determined. The mRNA levels of *Cbs*, *Mat1a* and *Bhmt* were not different between the CAS/EtOH and SPI/EtOH group, suggesting that cysteine, methionine, and SAM production may be similarly affected by the ethanol intake, whereas those of *Mtr*, *Pemt* and *Gnmt* were significantly affected by maternal diet. In particular, *Gnmt* mRNA level in the SPI groups was positively correlated with the SAM/SAH ratio like the previous reviews (Kharbanda, 2007), suggesting that *Gnmt* mRNA level may be an important factor for the development of liver damage. Consistently, *Gnmt* deficient mice induced the suppression of *Srb1* and *Abca1* level in liver and aorta, contributing to the altered cholesterol metabolism and increased inflammation response (Chen et al., 2012a). Interestingly, there was a negative correlation between liver damage indicator, GPT, and one-carbon metabolism genes, *Bhmt* (r=–0.526, p=0.017), *Mat1a* (r=–0.573, p=0.008), *Cbs* (r=–0.623, p=0.003), *Mtr* (r=–0.661, p=0.002), *Gnmt* (r=–0.424, p=0.063), and *Pemt* (r=–0.530,

p=0.016). In addition, compared to the **Study 1**, the result patterns of *Mtr*, *Pemt*, and *Gnmt* mRNA levels were sustained regardless of ethanol feeding. This suggests that the reduction of one-carbon methyl-group mechanism induced relatively severe liver damage in the SPI/EtOH group compared to the CAS/EtOH group. Even though its mechanism has remained obscure, it is likely that maternal SPI diet serves as the secondary endogenous inducer for liver damage. Therefore, this study suggests that these sustained lower levels in the SPI group may be a contributor to accelerate liver damage caused by chronic ethanol exposure. A balance between hepatocyte death and survival is the important factor for liver homeostasis (Wullaert et al., 2007). This homeostatic control mechanism becomes unstable because ethanol intake increases homocysteine level stimulating the progression of liver damage through the ER stress response (Barve et al., 2006). This study mentioned that the reason why the ER stress mechanism is up-regulated by the ethanol feeding has not been fully understood, but the higher level of homocystiene would be considered as the ER stress inducer. The previous studies mentioned that hyperhomocysteinemia had lower PEMT activity in ethanol exposure and led to the higher phosphatidylethanolamine/phosphatidylcholine ratio, resulting in a fatty liver (Ji, 2012; Obeid and Herrmann, 2009). So the ER stress response likely linked to the development of liver damage. For the spliced *Xbp-1* and *Chop* data, ER stress response tended to be induced only in the SPI/EtOH group compared to the CAS/EtOH group. Moreover, levels of serum MCP-1 and hepatic *MCP-1* and *TNFa* mRNA were significantly higher in the SPI/EtOH group compared to the SPI/CON group. These pro-inflammatory factors were strongly correlated with

other damage indicators observed in the present study. There was also a significant correlation between MCP-1 and homocysteine levels (r=0.785, p<0.0001) like the previous study (Wang et al., 2002). Even though further study is necessary to define the association between maternal diet and liver damage, ER stress and inflammation response were relatively stimulated by maternal SPI diet compared to maternal CAS diet. Therefore, as proposed, this study confirmed that maternal SPI diet likely serves as a risk cofactor of ethanol-induced liver damage similar to endotoxin.

The present study observed hypocholesterolemic effect on more severe degree of liver damage in the SPI/EtOH group compared to the CAS/EtOH group. In clinical study, an elevated plasma HDL-cholesterol level was observed among alcoholics without liver disease, but not in alcoholics with liver disease (Athukorala et al., 1988). Interestingly, offspring from dam fed the SPI diet showed more sensitive response to ethanol exposure, implying that the degree of liver damage may contribute to the dysregulated HDL-cholesterol metabolism. These results suggest that homocysteine or TNFα-induced ER stress may contribute to the decreased HDL production via the down-regulation of its key genes. However, the present study observed no significant change of serum and hepatic triacylglycerol in the SPI groups compared to the CAS groups. Likewise, the mRNA levels of genes involved in fatty acid synthesis and fatty acid oxidation were not significant difference between ethanol feeding groups. These responses may reflect that the degree of ethanol-induced liver damage in the SPI/EtOH group seems not to affect triacylglycerol metabolism despite the increased homocysteine and ER stress indicating a fatty liver. In addition, even though the

cellular mechanism has not been fully elucidated, there was a stronger correlation between maternal SPI diet and the regulation of cholesterol rather than that of triacylglycerol. Accordingly, alcoholic liver damage-involved maternal SPI diet is likely to be a major cause of the disturbed cholesterol mechanism.

In conclusion, offspring from dams fed the SPI diet developed more severe ethanol-induced liver damage compared to those from dams fed the CAS diet. Under no damage condition, the SPI/CON group likely works through the suitable buffer system, so there may be no significant difference in damage indicators and serum and hepatic biochemical parameters between the CAS/CON group and the SPI/CON group. However, under ethanol exposure, maternal SPI diet is likely to act as a secondary insult causing severe damage to liver, so lower levels of methylation enzymes responsible for homocysteine and methylation potential seem to be a key risk factor in liver dysfunction. As well, aminotransferases and proinflammatory levels showed a powerful correlation with key genes, particularly *Lcat*, responsible for HDL production and assembly. Therefore, these results suggest that the maternal SPI diet may be regarded as a potential mediator of liver damage in response to ethanol stress later in life.

VI. Conclusion

Pre- and early postnatal life is an important period to develop the organs, so the manipulation of the availability of the dietary components during pregnancy and lactation may determine the organ plasticity. Particularly, liver is an important organ, which is sensitively influenced by the utero-environment, so poor or inadequate maternal nutritional status may control the process of liver development and growth. Because liver is responsible for the various adaptation of metabolic homeostasis, early liver programming affected by the maternal diets is a critical role in long-term health consequence. Recently, the preference for the plant protein source has gradually increased. Soy protein contains various bioactive components such as isoflavones, saponins, and polyphenols. These components may contribute to the lipid-lowering effect. Moreover, the different amino acid profiles of soy protein from the animal protein source may act as a potential beneficial effect on lipid-lowering response. From now on, very few studies have reported the role of the maternal soy protein isolate in association with the early liver development and later risk of disease in offspring. Therefore, the study was conducted to investigate the effect of maternal SPI diet for two weeks before mating and throughout pregnancy and lactation on liver development and its function in earlier and later life. This study was mainly focused on two parts, whether the effect of maternal SPI diet would alter hepatic growth and function (Short-term study), and whether its effect would be sustained in liver damage status later in life (Long-term study).

In **Study 1**, the role of maternal SPI diet in the liver development of male rat offspring at 3-week of age and then hepatic gene expression and epigenetic regulation

was investigated. When the SPI diet was provided as a major protein source of dams, the SPI group had a significantly lower body weight and fat mass than the CAS and GEN groups, whereas the relative liver weight was higher in the SPI group than in the CAS and GEN groups. Hepatocyte hypertrophy and proliferation may be related to the rate and capacity of liver growth. The previous study suggests the patterns of gene expression during liver development show an age-dependent response, so gene expression profiles may be a useful tool to monitor the liver development and function. In the present study, the significantly altered gene expression related to cell growth and development was observed in the SPI group compared to the CAS and GEN groups, suggesting that maternal dietary protein source may play an important role in the liver development of offspring. Through the further analysis to figure out the relationship between maternal SPI diet and offspring liver growth rate, the distinctly different expression patterns of genes involved in xenobiotic and drug metabolism were found, and these gene expression showed a strong correlation with the relative liver weight. However, genistein supplementation had a less of an effect in regulating hepatic gene expression than the SPI diet, suggesting that the gene expression may be altered by amino acid components rather than isoflavone. It is likely that these different expression patterns may influence the functional capacity in liver disease in later life. Interestingly, the SPI group affected serum homocysteine and its corresponding gene expression. Moreover, hypomethylation and hyperacetylation of H3K9 related to DNA hypomethylation were observed in the SPI group, and there was a positive association with the relative liver weight. From these results, **Study 1** suggests that maternal SPI

diet seems to act as a potential inhibitor. Therefore, the consumption of the SPI diet contributes to the relatively slow development of liver in offspring and consequent changes in the hepatic gene expression profiles and epigenetic modification at 3-week of age.

In **Study 2**, the role of maternal diet in lipid metabolism was investigated based on the unexpected results observed in the **Study 1**. Here, lipid-lowering effect of maternal SPI diet was associated with the body weight gain and fat mass compared to maternal CAS and GEN diets. Particularly, maternal hepatic triacylglycerol level showed a positive correlation with the offspring body weight and fat mass and a negative correlation with the offspring relative liver weight. The ratio of serum BCAA to AAA in dams was also correlated with offspring body weight and fat mass. In **Study 1**, the gene expression related to the activation of PPARα signaling was higher in the SPI group than in the CAS and GEN groups, suggesting that these changes may be responsible for the regulation of energy homeostasis compensating for hepatic proliferation. In the present study, though lipid-lowering effect of the SPI diet on dams and their offspring was not directly explained, this study implies that maternal SPI diet could contribute to the different effect on liver function of offspring.

In **Study 3**, the sustainable effect of maternal dietary protein source on the development of liver damage in adulthood was investigated. Firstly, this study was designed to figure out the role of maternal dietary protein source in early liver development of offspring, and in **Study 1** and **2**, the consumption of the SPI diet rather than genistein supplementation showed a different response pattern in the liver

development and its function of offspring. Therefore, a follow-up study was performed to determine the adaptive response of offspring to chronic ethanol-induced liver damage in later life. There was a different response in offspring of the maternal SPI diet compared to those of the CAS diet. Unexpectedly, relatively severe liver damage was observed in the SPI/EtOH group compared to the CAS/EtOH group. According to the induction of the liver damage, the SPI/EtOH group developed an impaired lipid metabolism, particularly, HDL-cholesterol metabolism. Although a direct hypolipidemic effect of maternal SPI diet was disappeared, adult offspring exposure to ethanol feeding had a significantly lower levels of cholesterol and HDL-cholesterol in the SPI group than in the CAS group. Although the mechanism is unknown, it is proposed that offspring exposure to maternal SPI diet, which acts as a potential endogenous factor, may sensitively respond to the induction of the liver damage. **Figure 6.1** shows the summary results of these studies.

In conclusion, this study compared for the first time the effects of maternal dietary protein source, casein and soy protein isolate with a low-isoflavone, on liver development and function of male offspring at early and later time points. These results present the maternal dietary protein source may be responsible for the liver development and growth in early life, which may influence the offspring's susceptibility to the development of liver disease on later life.

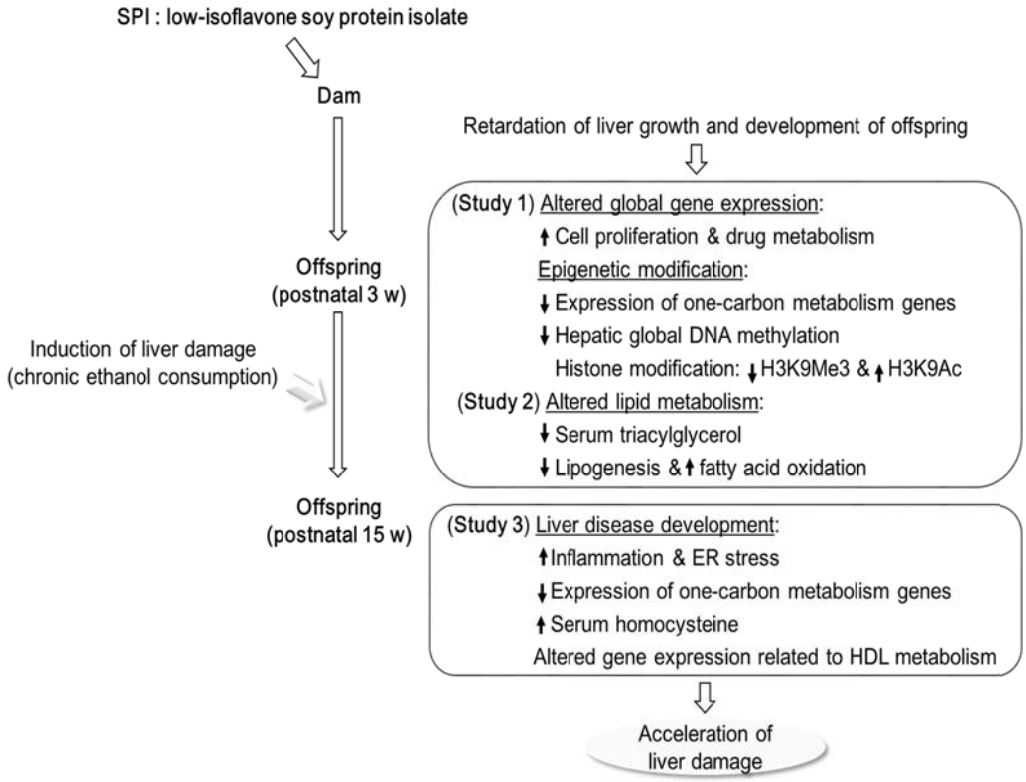


Figure 6.1. Programming of the liver development and disease by maternal SPI diet in early and later life of offspring.

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국 문 초 록

어미 쥐가 섭취한 단백질 종류가 자손 쥐의 간질환 발생에 미치는 영향에 대한 연구

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연구배경: 모체 자궁 내 영양상태와 환경인자는 성인기 질환발생과 관련된 태아 발달과정에 영향을 미친다. 간은 다양한 신진대사 항상성의 조절에 관여하기 때문에 어미식이로부터 영향을 받은 태아의 간발달은 성인기 건강유지를 위해 중요하다. 대두단백질은 중성지방과 콜레스테롤 감소를 통해 대사성 질환을 완화시킨다고 보고되었다. 특히, 분리대두단백질의 아미노산 조성과 이소플라본이 지방대사에 기여한다고 보고되었다. 그러나, 어미 쥐가 분리대두단백질(soy protein isolate)을 섭취한 자손의 초기 발달과 성인기 질병위험성과의 연관성에 관한 연구는 거의 보고되지 않았다.

연구목적: 본 연구는 모체가 섭취한 단백질급원에 따른 자손의 간발달과 성인기 간질환 발생에 미치는 단기적 및 장기적 영향에 대한 효과를 보고자 하였다. 분리대두단백질 자체의 영향을 비교하기 위하여 카제인단백질(CAS)군과 카제인단백질에 제니스테인을 첨가한(GEN)군의 식이모델을 사용하였다. 단기적 영향을 보기 위한 **연구 1**과 **2**에서는 이소플라본 함량이 적은 분리대두단백질식이 또는 카제인단백질에 제니스테인을 첨가한 식이를 섭취한 어미 쥐의 3주 자손 쥐의 간에서 간발달에

미치는 영향과 관련된 유전자 발현 및 후생유전학적 조절을 규명하고자 하였다. 장기적 영향을 보기 위한 **연구 3**에서는 성인기 알코올 섭취에 의한 간손상 및 콜레스테롤 대사에 미치는 어미식이의 효과를 알아보고자 하였다.

연구방법: Sprague Dawley 암컷쥐는 임신 2주전, 임신기 및 수유기 동안 카제인단백질(200 g/kg diet), 이소플라본 함량이 적은 분리대두단백질(SPI, 200 g/kg diet), 또는 카제인단백질에 제니스테인(250 mg/kg diet)을 첨가한 식이를 제공하였다. 각 식이를 섭취한 어미 쥐의 수컷 자손 쥐는 출생 후 3주(**연구 1**과 **2**의 실험군: CAS, SPI, GEN)와 15주 (**연구 3**의 실험군: CAS/CON, CAS/EtOH, SPI/CON, SPI/EtOH)에서 분석되었다. 직접적인 단백질급원의 효과와 어미식이의 지속적인 효과를 보기 위해서 CAS와 SPI 식이를 섭취한 어미 쥐의 자손 쥐에게 수유기 후부터 생후 8주까지 일반식이를 제공한 후 동일 어미 쥐의 자손 쥐를 두 그룹으로 나누어 에탄올이나 대조군 액상식이를 pair feeding 방식으로 6주간 제공하였다.

연구결과: **연구 1**에서 체중증가와 지방무게가 생후 3주의 SPI군 자손 쥐에서 CAS군 자손 쥐에 비해 유의적으로 감소되었으나, 상대적 간무게는 증가되었다. 마이크로어레이분석에서 SPI군 자손 쥐는 CAS군과 GEN군의 자손에 비해 세포증식과 분열 및 약물대사 관련 유전자들의 유의적인 발현변화가 관찰되었다. 또한, SPI군 자손 쥐에서 CAS군과 GEN군에 비해 혈청 호모시스테인 증가, 간 단일탄소대사 유전자 감소 및 global DNA 저메틸화가 관찰되었다. SPI군 자손 쥐에서 CAS군과 GEN군에 비해 히스톤 아세틸화(H3K9Ac)는 유의적으로 증가된 반면 히스톤 메틸화(H3K9Me3)는 감소되었다. **연구 2**에서 SPI 식이를 섭취한 어미 쥐의 혈청 및 간의 중성지방과 콜레스테롤 농도가 CAS 식이를 섭취한 어미 쥐보다 감소되었고,

SPI군 자손 쥐에서도 혈청 중성지방과 콜레스테롤이 CAS군 자손 쥐보다 감소되었다. 이는 peroxisome proliferator-activated receptor alpha signaling 관련 유전자 발현변화와 연관성을 보였고 SPI군의 hypolipidemia에 기여하였고, 유전자 발현변화는 상대적 간무게와 연관성을 보였다. **연구 3**에서는 SPI/EtOH군 자손 쥐가 CAS/EtOH군 자손 쥐에 비해서 상대적으로 간손상에 민감하게 반응하였다. Aminotransferases 효소활성은 SPI/EtOH군 자손 쥐에서 CAS/EtOH군 자손 쥐에 비해 유의적으로 증가되었다. 또한, 소포체 스트레스와 염증 반응도 SPI/EtOH군 자손 쥐에서 유의적으로 증가되었고 단일탄소대사 유전자도 SPI/EtOH군 자손 쥐에서 민감하게 영향을 받았다. 혈청 콜레스테롤 농도도 CAS/EtOH군 자손 쥐에 비해 SPI/EtOH군 자손 쥐에서 유의적으로 감소되었고, 간손상지표와 HDL-콜레스테롤 대사조절에 관여하는 유전자 발현간에 유의적인 상관관계를 보였다. 하지만, 3주에서 관찰된 lipid-lowering 효과가 성인기의 대조군간에는 차이를 보이지 않았다.

결론: 어미 쥐의 SPI 식이 섭취는 수컷 자손 쥐의 간 성장과 발달 변화에 영향을 주었고, 어미식이의 잠재적 효과로 인해 자손의 성인기에 섭취한 알코올은 CAS군 자손 쥐보다 SPI군 자손 쥐에서 상대적으로 높은 수준의 간손상을 야기하였다. 따라서, 어미 쥐가 섭취한 단백질 종류가 자손 쥐의 초기 간발달 지연에 기여하였고 성인기 간질병 발생에 대한 자손 쥐의 민감성에 영향을 줄 수 있을 것으로 사료된다.

주요어: 간발달, 간손상, 분리대두단백질, 만성알코올섭취, 모체식이, 자손 쥐

학번: 2008-30456

Appendices

Appendix 1. Primer sequences for qRT-PCR (Taqman)

Gene	Product number	Gene	Product number
Abca1	Rn00710172_m1	*Hadh*	Rn00589352_m1
Abcg5	Rn00587092_m1	*Hmgcr*	Rn00565598_m1
Acot12	Rn00590651_m1	*Lcat*	Rn00500505_m1
Apoa2	Rn00565403_m1	*Ldlr*	Rn00598442_m1
Bhmt	Rn00578255_m1	*Lpl*	Rn00561482_m1
Cd36	Rn00580728_m1	*Mat1a*	Rn00563454_m1
Chop	Rn01458526_m1	*Mdr2*	Rn01529224_m1
Cpt1a	Rn00580702_m1	*Me1*	Rn00561502_m1
Cpt1b	Rn00566242_m1	*Mttp*	Rn01522970_m1
Cyp3a1	Rn03062228_m1	*Pemt*	Rn00564517_m1
Cyp7a1	Rn00564065_m1	*Ppara*	Rn00566193_m1
Dgat1	Rn00584870_m1	*Scd1*	Rn00594894_g1
Fasn	Rn00569117_m1	*Srb1*	Rn00580588_m1
Gnmt	Rn00567215_m1	*Thrsp*	Rn01511034_m1
Actb	Rn00667869_m1		

Abca1, ATP-binding cassette, subfamily A, member 1; *Abcg5*, ATP-binding cassette, subfamily G (WHITE), member 5; *Acot12*, acyl-CoA thioesterase 12; *Apoa2*, Apo-lipoprotein A2; *Bhmt*, betaine-homocysteine methyltransferase; *Cd36*, Cluster of differentiation 36; *Chop*, DNA-damage inducible transcript 3; *Cpt1a*, Carnitine palmitoyltransferase 1α; *Cpt1b*, Carnitine palmitoyltransferase 1b; *Cyp3a1*, Cytochrome P450, family 3, subfamily a, polypeptide 1; *Cyp7a1*, Cholesterol 7α hydroxylase; *Dgat1*, Diacylglycerol O-acyltransferase homolog 1; *Fasn*, Fatty acid synthase; *Gnmt*, glycine *N*-methyltransferase; *Hadh*, hydroxyacyl-Coenzyme A dehydrogenase; *Hmgcr*, 3-Hydroxy-3-methylglutaryl-Coenzyme A reductase; *Lcat*, Lecithin cholesterol acyltransferase; *Ldlr*, Low density lipoprotein receptor; *Lpl*, Lipoprotein lipase; *Mat1a*, Methionine adenosyltransferase 1 alpha; *Mdr2*, multidrug resistance 2; *Me1*, Malic enzyme 1; *Mttp*, Microsomal triacylglycerol transfer protein; *Pemt*, phosphatidylethanolamine *N*-methyltransferase; *Ppara,* peroxisome proliferator activated receptor alpha; *Scd1*, Stearoyl-CoA desaturase 1; *Srb1*, Scavenger receptor class B, member 1; *Thrsp*, Thyroid hormone responsive protein; *Actb*, actin, beta

Appendix 2. Primer sequences for qRT-PCR (SYBR green)

Gene	Forward (5'- 3')	Reverse (5'- 3')
Apoa1	GGCAGAGACTATGTGTCCCAGTTT	TTGAACCCAGAGTGTCCCAGTT
Cbs	TGCATTATCGTGATGCCTGAGAA	GGGAATCGAATCTGGCGTTG
Dnmt1	GTGTGGTGTCTGTGAGGTCTGT	GTTTCTTCTTCTTCCCTTGGTG
Dnmt3a	CTGAAATGGAAAGGGTGTTTGGC	CCATGTCCCTTACACACAGC
Dnmt3b	AGGAAGGATGGGTGGAGTGG	ATTGGGGTCAGGGAGAGGGA
Gadd45b	GTCACCTCCGTCTTCTTGGA	GAGGCGGTGGGACTTACTTT
Lxra	GAACAGATCCGCTTGAAGAA	ATGGCCAGCTCAGTAAAGTG
MCP-1	TAGCATCCACGTGCTGTCTC	CCGACTCATTGGGATCATCT
Mtr	ACTTGCGCAAACTCCGCTATG	TGCCAAGGATTCTGTCAACCTG
TNFa	CCCATTACTCTGACCCCTTT	TGAGCATCGTAGTTGTTGGA
Actb	CACACTGTGCCCATCTATGA	CCGATAGTGATGACCTGACC

Apoa1, Apo-lipoprotein A1; *Cbs*, cystathionine beta-synthase; *Dnmt1*, DNA (cytosine-5-)-methyltransferase 1; *Dnmt3a*, DNA methyltransferase 3A; *Dnmt3b*, DNA methyltransferase 3B; *Gadd45b*, growth arrest and DNA-damage-inducible 45 beta; *Lxra*, Liver X receptor alpha; *MCP-1*, Monocyte chemoattractant protein-1; *Mtr*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *TNFa*, Tumor necrosis factor-alpha; *Actb*, actin, beta

Appendix 3. List of 119 differentially expressed genes (DEGs) by maternal diet in the liver of 3-week-old offspring [1)]

Gene identifier	Gene symbol	Gene description	Fold change[2)]			*p*-value[3)]
			SPI/ CAS	GEN/ CAS	SPI/ GEN	
Amino acid metabolism						
NM_012600.1	*Me1*	Malic enzyme 1	2.27*	1.02	2.23*	0.003
NM_013200.1	*Cpt1b*	Carnitine palmitoyltransferase 1b, muscle	1.83*	1.12	1.63*	0.009
NM_030850.1	*Bhmt*	Betaine-homocysteine methyltransferase	-1.71*	-1.23	-1.39	0.015
NM_017215.2	*Slc1a2*	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	1.51*	1.02	1.48*	0.027
NM_017016.1	*Hdc*	Histidine decarboxylase	1.90*	-1.10	2.10*	0.034
XM_341089.3	*Sdsl*_predicted (*Sdsl*)	Serine dehydratase-like	1.69*	-1.06	1.78	0.040
Apoptosis						
NM_012977.1	*Lgals9*	Lectin, galactose binding, soluble 9	1.64	1.14	1.44	0.035
NM_012603.2	*Myc*	Myelocytomatosis oncogene	1.43*	-1.21	1.73	0.035
NM_001008321.1	*Gadd45b*	Growth arrest and DNA-damage-inducible 45 beta	1.49	-1.02	1.52	0.043
Blood circulation and gas exchange						
NM_031557.2	*Ptgis*	Prostaglandin I2 (prostacyclin) synthase	-1.57	-1.15	-1.36*	0.040
Carbohydrate metabolism						
NM_012683.2	*Ugt1a1*	UDP glycosyltransferase 1 family, polypeptide A1	2.60*	1.11	2.35*	0.001
NM_130407.1	*Ugt1a7*	UDP glycosyltransferase 1 family, polypeptide A7	1.98	1.05*	1.88*	0.001
NM_012600.1	*Me1*	Malic enzyme 1	2.27*	1.02	2.23*	0.003
NM_017270.1	*Adh4*	Alcohol dehydrogenase 4 (class II), pi polypeptide	1.60*	1.02	1.56*	0.004
NM_001014161.1	*Idh2*	Isocitrate dehydrogenase 2 ($NADP^+$), mitochondrial	1.53*	-1.00	1.53	0.010
NM_001039691.1	*Ugt1a6*	UDP glycosyltransferase 1	1.85*	-1.12	2.08	0.013

		family, polypeptide A6				
XM_001074691.1	RGD1559459_predicted	Similar to Expressed sequence AI788959	1.47	-1.07	1.58*	0.021
NM_173295.1	*Ugt2b1*	UDP glucuronosyltransferase 2 family, polypeptide B1	1.63	-1.02*	1.65*	0.022
XM_001074267.1	*Ugt2a3*_predicted	UDP glucuronosyltransferase 2 family, polypeptide A3	2.09*	-1.07	2.24	0.024
XM_341089.3	*Sdsl*_predicted (*Sdsl*)	Serine dehydratase-like	1.69*	-1.06	1.78	0.040
Cell adhesion						
NM_001001505.1	*Habp2*	Hyaluronic acid binding protein 2	-1.71*	1.01	-1.73*	0.002
XM_001067936.1	RGD1561090_predicted	Similar to protein tyrosine phosphatase, receptor type, D	-1.56*	-1.13	-1.39*	0.006
NM_053329.2	*Igfals*	Insulin-like growth factor binding protein, acid labile subunit	-2.04*	1.01	-2.05*	0.009
NM_012977.1	*Lgals9*	Lectin, galactose binding, soluble 9	1.64	1.14	1.44	0.035
Cell cycle						
NM_021836.2	*Junb*	Jun-B oncogene	-1.50*	1.08	-1.62	0.023
NM_012603.2	*Myc*	Myelocytomatosis oncogene	1.43*	-1.21	1.73	0.035
NM_001008321.1	*Gadd45b*	Growth arrest and DNA-damage-inducible 45 beta	1.49	-1.02	1.52	0.043
Cell proliferation and differentiation						
NM_001037217.1	*Mmd2*_predicted (*Mmd2*)	Monocyte to macrophage differentiation-associated 2	-1.97*	-1.23	-1.60*	0.015
NM_021836.2	*Junb*	Jun-B oncogene	-1.50*	1.08	-1.62	0.023
NM_001008321.1	*Gadd45b*	Growth arrest and DNA-damage-inducible 45 beta	1.49	-1.02	1.52	0.043
Cell structure and motility						
XM_217296.4	*Vill*_predicted (*Vill*)	Villin-like	-1.40*	1.08	-1.51*	0.002
XM_001070569.1	*Farp2*_predicted (*Farp2*)	FERM, RhoGEF and pleckstrin domain protein 2	1.59*	1.09	1.45*	0.004
XM_001067936.1	RGD1561090_predicted	Similar to protein tyrosine phosphatase, receptor type, D	-1.56*	-1.13	-1.39*	0.006
NM_001008753.1	*Krt23*	Keratin 23	2.13*	1.20	1.77	0.017
NM_057132.2	*Rhoa*	Ras homolog gene	1.25	-1.29	1.62*	0.035

		family, member A				
Developmental processes						
XM_001075775.1	*Mpv17l*_predicted (*Mpv17l*)	Mpv17 transgene, kidney disease mutant-like	-1.60*	1.09	-1.75*	<0.001
XM_001070569.1	*Farp2*_predicted (*Farp2*)	FERM, RhoGEF and pleckstrin domain protein 2	1.59*	1.09	1.45*	0.004
XM_001067936.1	RGD1561090_predicted	Similar to protein tyrosine phosphatase, receptor type, D	-1.56*	-1.13	-1.39*	0.006
NM_012630.1	*Prlr*	Prolactin receptor	-1.69	-1.25	-1.35	0.039
NM_001013975.1	RGD1307119 (*Notum*)	Hypothetical LOC303743	-1.48*	1.17	-1.73	0.049
Electron transport						
NM_134369.1	*Cyp2t1*	Cytochrome P450, subfamily t, polypeptide 1	-2.12*	-1.19	-1.78*	<0.001
NM_013105.1	*Cyp3a3*	Cytochrome P450, subfamily 3A, polypeptide 3	4.47*	-1.20	5.37*	<0.001
XM_001070818.1	*Cyp2b15*	Cytochrome P450, family 2, subfamily b, polypeptide 15, transcript variant 2	2.77*	1.29	2.15*	<0.001
NM_173144.1	*Cyp3a1*	Cytochrome P450, family 3, subfamily a, polypeptide 1	2.29*	-1.09	2.49*	0.003
NM_053906.1	*Gsr*	Glutathione reductase	1.86*	-1.01*	1.88	0.004
XM_001063361.1	*Cyp2c37*	Cytochrome P450, 2c37	1.76*	1.14	1.54*	0.009
NM_017158.1	*Cyp2c7*	Cytochrome P450, family 2, subfamily c, polypeptide 7	-1.51	-1.31	-1.15*	0.018
NM_031572.1	*Cyp2c12*	Cytochrome P450, family 2, subfamily c, polypeptide 12	-3.05	-1.08	-2.83*	0.031
NM_031557.2	*Ptgis*	Prostaglandin I2 (prostacyclin) synthase	-1.57	-1.15	-1.36*	0.040
Homeostasis						
NM_053329.2	*Igfals*	Insulin-like growth factor binding protein, acid labile subunit	-2.04*	1.01	-2.05*	0.009
NM_021663.2	*Nucb2*	Nucleobindin 2	1.48	-1.08	1.60*	0.026
Immunity and defense						
NM_001024304.1	MGC108896 (*Gstm4*)	Similar to glutathione transferase GSTM7-7	1.54*	1.03	1.50*	<0.001
NM_172038.1	*Gstm5*	Glutathione S-transferase, mu 5	1.31*	-1.19	1.55*	<0.001
NM_017014.1	*Gstm1*	Glutathione S-transferase, mu 1	1.92*	-1.04	2.00*	<0.001
NM_177426.1	*Gstm2*	Glutathione S-	3.14*	-1.03	3.23*	<0.001

		transferase, mu 2				
XM_001075775.1	*Mpv17l*_predicted (*Mpv17l*)	Mpv17 transgene, kidney disease mutant-like	-1.60*	1.09	-1.75*	<0.001
NM_001009920.1	*Yc2*	Glutathione S-transferase Yc2 subunit	2.14*	-1.13	2.41*	0.001
NM_133586.1	*Ces2*	Carboxylesterase 2 (intestine, liver)	2.64*	1.42	1.86*	0.003
XM_345342.3	*C5*	Complement component 5	-1.44*	1.14	-1.64*	0.003
NM_133295.2	*Ces3*	Carboxylesterase 3	3.56*	1.55	2.29*	0.005
NM_053288.1	*Orm1*	Orosomucoid 1	2.01*	1.18	1.71	0.014
NM_001037217.1	*Mmd2*_predicted (*Mmd2*)	Monocyte to macrophage differentiation-associated 2	-1.97*	-1.23	-1.60*	0.015
NM_080581.1	*Abcc3*	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	1.78	1.05	1.70*	0.017
NM_001010921.1	LOC494499 (*Gsta5*)	LOC494499 protein	3.30*	1.35	2.44*	0.019
NM_012723.1	*Pigr*	Polymeric immunoglobulin receptor	-2.29*	1.49	-3.42	0.032
NM_012977.1	*Lgals9*	Lectin, galactose binding, soluble 9	1.64	1.14	1.44	0.035
XM_212849.3	RGD1565045_predicted	Similar to carboxylesterase isoenzyme gene	1.70*	1.17	1.45	0.038
NM_001008321.1	*Gadd45b*	Growth arrest and DNA-damage-inducible 45 beta	1.49	-1.02	1.52	0.043
Intracellular protein traffic						
NM_001013085.1	*Snx10*	Sorting nexin 10	2.72*	-1.09	2.97*	<0.001
NM_198786.2	*Mal2*	Mal, T-cell differentiation protein 2	1.49	-1.06	1.57*	0.009
XM_240417.4	*Mtmr7*_predicted (*Mtmr7*)	Myotubularin related protein 7	-1.82*	1.04	-1.89*	0.011
NM_053487.1	*Pex11a*	Peroxisomal biogenesis factor 11A	1.54*	-1.05	1.61	0.012
NM_012723.1	*Pigr*	Polymeric immunoglobulin receptor	-2.29*	1.49	-3.42	0.032
Lipid, fatty acid and steroid metabolism						
NM_134369.1	*Cyp2t1*	Cytochrome P450, subfamily t, polypeptide 1	-2.12*	-1.19	-1.78*	<0.001
NM_013105.1	*Cyp3a3*	Cytochrome P450, subfamily 3A, polypeptide 3	4.47*	-1.20	5.37*	<0.001

XM_001070818.1	*Cyp2b15*	Cytochrome P450, family 2, subfamily b, polypeptide 15, transcript variant 2	2.77*	1.29	2.15*	<0.001
NM_012683.2	*Ugt1a1*	UDP glycosyltransferase 1 family, polypeptide A1	2.60*	1.11	2.35*	0.001
NM_012598.1	*Lpl*	Lipoprotein lipase	1.78*	-1.06	1.89*	0.001
NM_130407.1	*Ugt1a7*	UDP glycosyltransferase 1 family, polypeptide A7	1.98	1.05*	1.88*	0.001
NM_173144.1	*Cyp3a1*	Cytochrome P450, family 3, subfamily a, polypeptide 1	2.29*	-1.09	2.49*	0.003
NM_013112.1	*Apoa2*	Apolipoprotein A-II	3.49*	1.98	1.76*	0.003
NM_012661.1	*Sts*	Steroid sulfatase	-1.74*	-1.09	-1.60*	0.003
NM_031732.1	*Sult1c1*	Sulfotransferase family, cytosolic, 1C, member 1	2.06*	-1.54	3.16*	0.009
XM_001063361.1	*Cyp2c37*	Cytochrome P450 2C37	1.76*	1.14	1.54*	0.009
NM_013200.1	*Cpt1b*	Carnitine palmitoyltransferase 1b, muscle	1.83*	1.12	1.63*	0.009
XM_240417.4	*Mtmr7*_predicted (*Mtmr7*)	Myotubularin related protein 7	-1.82*	1.04	-1.89*	0.011
NM_001039691.1	*Ugt1a6*	UDP glycosyltransferase 1 family, polypeptide A6	1.85*	-1.12	2.08	0.013
NM_001037217.1	*Mmd2*_predicted (*Mmd2*)	Monocyte to macrophage differentiation-associated 2	-1.97*	-1.23	-1.60*	0.015
NM_017158.1	*Cyp2c7*	Cytochrome P450, family 2, subfamily c, polypeptide 7	-1.51	-1.31	-1.15*	0.018
XM_001074691.1	RGD1559459_predicted	Similar to Expressed sequence AI788959	1.47	-1.07	1.58*	0.021
NM_173295.1	*Ugt2b1*	UDP glucuronosyltransferase 2 family, polypeptide B1	1.63	-1.02*	1.65*	0.022
XM_001074267.1	*Ugt2a3*_predicted	UDP glucuronosyltransferase 2 family, polypeptide A3	2.09*	-1.07	2.24	0.024
NM_031572.1	*Cyp2c12*	Cytochrome P450, family 2, subfamily c, polypeptide 12	-3.05	-1.08	-2.83*	0.031
NM_031557.2	*Ptgis*	Prostaglandin I2 synthase	-1.57	-1.15	-1.36*	0.040
NM_012703.2	*Thrsp*	Thyroid hormone	-3.02	1.19	-3.59	0.045

		responsive protein				
Miscellaneous						
NM_138847.1	*Nip7*	Nuclear import 7 homolog (S. cerevisiae)	-1.22*	-1.72	1.42*	0.006
Neuronal activities						
NM_017215.2	*Slc1a2*	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	1.51*	1.02	1.48*	0.027
XM_001060226.1	RGD1566093_predicted	Similar to Fusion (involved in t(12;16) in malignant liposarcoma)	-1.56*	-1.12	-1.39	0.034
Nucleoside, nucleotide and nucleic acid metabolism						
NM_001009474.1	*Pir*	Pirin	2.10*	1.07	1.97*	0.003
NM_021836.2	*Junb*	Jun-B oncogene	-1.50*	1.08	-1.62	0.023
XM_001080983.1	RGD1564876_predicted (*Slc35e3*)	Solute carrier family 35, member E3	1.43*	-1.10	1.57*	0.029
XM_001071608.1	*Nfix*	Nuclear factor I/X	-1.63*	-1.18	-1.38*	0.030
XM_001059113.1	*Slc35d2*_predicted (*Slc35d2*)	Solute carrier family 35, member D2	1.62*	1.12	1.45	0.034
NM_012603.2	*Myc*	Myelocytomatosis oncogene	1.43*	-1.21	1.73	0.045
NM_012703.2	*Thrsp*	Thyroid hormone responsive protein	-3.02	1.19	-3.59	0.045
NM_057211.1	*Klf9*	Kruppel-like factor 9	-1.51*	-1.17	-1.29	0.048
Oncogenesis						
NM_021836.2	*Junb*	Jun-B oncogene	-1.50*	1.08	-1.62	0.023
XM_001060226.1	RGD1566093_predicted	Similar to Fusion (involved in t(12;16) in malignant liposarcoma)	-1.56*	-1.12	-1.39	0.034
NM_012603.2	*Myc*	Myelocytomatosis oncogene	1.43*	-1.21	1.73	0.035
Other metabolism						
NM_019170.2	*Cbr1*	Carbonyl reductase 1	1.48*	-1.11	1.65*	0.001
NM_012600.1	*Me1*	Malic enzyme 1	2.27*	1.02	2.23*	0.003
NM_053906.1	*Gsr*	Glutathione reductase	1.86*	-1.01*	1.88	0.004
NM_017270.1	*Adh4*	Alcohol dehydrogenase 4 (class II), pi polypeptide	1.60*	1.02	1.56*	0.004
NM_053781.1	*Akr1b7*	Aldo-keto reductase family 1, member B7	-3.28	-1.98	-1.66	0.037
NM_013215.1	*Akr7a3*	Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	1.88	-1.02	1.93	0.041
NM_012703.2	*Thrsp*	Thyroid hormone responsive protein	-3.02	1.19	-3.59	0.045
NM_145084.1	*Retsat*	Retinol saturase (all trans retinol 13,14 reductase)	-2.41	-1.46	-1.64*	0.045

Phosphate metabolism						
XM_001076428.1	*Nudt7*_predicted	Nudix (nucleoside diphosphate linked moiety X)-type motif 7	1.62	1.19	1.37	0.046
Protein metabolism and modification						
NM_152936.1	*Spink3*	Serine peptidase inhibitor, Kazal type 3	-5.20	1.45*	-7.53*	0.001
NM_212524.1	*Abhd5*	Abhydrolase domain containing 5	1.43*	-1.15	1.64*	0.001
NM_017200.1	*Tfpi*	Tissue factor pathway inhibitor	1.39*	-1.10	1.52*	0.002
NM_001001505.1	*Habp2*	Hyaluronic acid binding protein 2	-1.71*	1.01	-1.73*	0.002
NM_017118.1	*Capns1*	Calpain, small subunit 1	1.50*	1.16	1.30*	0.003
NM_017305.2	*Gclm*	Glutamate cysteine ligase, modifier subunit	1.72	-1.09*	1.87*	0.005
XM_001067936.1	RGD1561090_predicted	Similar to protein tyrosine phosphatase, receptor type, D	-1.56*	-1.13	-1.39*	0.006
XM_001072434.1	*Hebp2*_predicted (*Hebp2*)	Heme binding protein 2	1.47	-1.07*	1.58	0.009
XM_001062085.1	RGD1562373_predicted	Similar to 3-ketoacyl-CoA thiolase B, peroxisomal precursor	2.97*	1.46	2.04*	0.010
NM_001024238.1	*Eprs*	Glutamyl-prolyl-tRNA synthetase	-1.63*	-1.63	1.00*	0.013
NM_031056.1	*Mmp14*	Matrix metallopeptidase 14	-1.57	-1.10	-1.42*	0.018
NM_001013975.1	RGD1307119 (*Notum*)	Hypothetical LOC303743	-1.48*	1.17	-1.73	0.049
Signal transduction						
NM_017118.1	*Capns1*	Calpain, small subunit 1	1.50*	1.16	1.30*	0.003
XM_001067936.1	RGD1561090_predicted	Similar to protein tyrosine phosphatase, receptor type, D	-1.56*	-1.13	-1.39*	0.006
NM_031034.1	*Gna12*	Guanine nucleotide binding protein, alpha 12	1.74*	1.05	1.65	0.006
XM_001056150.1	LOC362068	Similar to very large G-protein coupled receptor 1	-1.57*	-1.16	-1.35*	0.008
NM_001037217.1	*Mmd2*_predicted (*Mmd2*)	Monocyte to macrophage differentiation-associated 2	-1.97*	-1.23	-1.60*	0.015
NM_021836.2	*Junb*	Jun-B oncogene	-1.50*	1.08	-1.62	0.023
NM_017215.2	*Slc1a2*	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	1.51*	1.02	1.48*	0.027
NM_057132.2	*Rhoa*	Ras homolog gene	1.25	-1.29	1.62*	0.035

		family, member A				
NM_012630.1	*Prlr*	Prolactin receptor	-1.69	-1.25	-1.35	0.039
NM_001008321.1	*Gadd45b*	Growth arrest and DNA-damage-inducible 45 beta	1.49	-1.02	1.52	0.043
Sulfur metabolism						
NM_012661.1	*Sts*	Steroid sulfatase	-1.74*	-1.09	-1.60*	0.003
NM_017305.2	*Gclm*	Glutamate cysteine ligase, modifier subunit	1.72	-1.09*	1.87*	0.005
NM_012815.2	*Gclc*	Glutamate-cysteine ligase, catalytic subunit	1.76	-1.05*	1.85	0.005
NM_031732.1	*Sult1c1*	Sulfotransferase family, cytosolic, 1C, member 1	2.06*	-1.54	3.16*	0.009
Transport						
NM_012598.1	*Lpl*	Lipoprotein lipase	1.78*	-1.06	1.89*	0.001
NM_198760.1	*Slc16a6*	Solute carrier family 16 (monocarboxylic acid transporters), member 6	1.52*	-1.11	1.69*	0.001
NM_013112.1	*Apoa2*	Apolipoprotein A-II	3.49*	1.98	1.76*	0.003
XM_001079593.1	*Slc16a11*_predicted (*Slc16a11*)	Solute carrier family 16 (monocarboxylic acid transporters), member 11	-1.54*	1.04	-1.61*	0.005
XM_001056150.1	LOC362068	Similar to very large G-protein coupled receptor 1	-1.57*	-1.16	-1.35*	0.008
NM_080581.1	*Abcc3*	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	1.78	1.05	1.70*	0.017
NM_017215.2	*Slc1a2*	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	1.51*	1.02	1.48*	0.027
XM_001080983.1	RGD1564876_predicted	Solute carrier family 35, member E3	1.43*	-1.10	1.57*	0.029
XM_001059113.1	*Slc35d2*_predicted (*Slc35d2*)	Solute carrier family 35, member D2	1.62*	1.12	1.45	0.034
NM_031332.1	*Slc22a8*	Solute carrier family 22 (organic anion transporter), member 8	1.45*	-1.07	1.55	0.045
Biological process unclassified						
XM_001072142.1	*Blvrb*_predicted (*Blvrb*)	Biliverdin reductase B (flavin reductase (NADPH))	1.74*	-1.03	1.80*	<0.001
XM_341530.2	*Hist1h2bm*_predicted (*Hist1h2bm*)	Histone 1, H2bm	-1.78*	-1.07	-1.66*	<0.001
XM_001078182.1	*Snrp1c*_predicte	U1 small nuclear	1.54*	-1.15	1.77*	0.001

	d (*Snrpc*)	ribonucleoprotein 1C				
NM_172033.1	*Plekhb1*	Pleckstrin homology domain containing, family B (evectins) member 1	1.72*	1.40	1.22*	0.001
XM_341193.3	LOC360919	Similar to alpha-fetoprotein	-1.60*	-1.05	-1.52*	0.001
XM_342835.2	*Mrpl50*_predicted (*Mrpl50*)	Mitochondrial ribosomal protein L50	1.81*	1.60	1.13*	0.001
XM_238022.1	RGD1562699_predicted	RGD1562699	-1.61*	1.19	-1.92*	0.001
XM_574666.1	*Cyp2c6*	Cytochrome P450, subfamily IIC6	2.01*	1.09	1.96	0.001
NM_001014206.1	RGD1309534	Similar to RIKEN cDNA 4931406C07	1.73*	1.17*	1.48	0.003
NM_057104.2	*Enpp2*	Ectonucleotide pyrophosphatase/phosphodiesterase 2	-1.79*	-1.03*	-1.74	0.003
XM_001080298.1	*Htatip2*_predicted (*Htatip2*)	HIV-1 tat interactive protein 2, homolog (human)	1.58*	-1.00	1.58*	0.004
XM_573165.2	*Ap1gbp1*	AP1 gamma subunit binding protein 1	-1.61*	1.05	-1.70*	0.008
NM_001004096.1	*Reg4*	Regenerating islet-derived family, member 4	-1.57*	1.13	-1.78*	0.009
XM_001059942.1	*Txnl1*	Thioredoxin-like 1	1.60*	1.03	1.56*	0.011
XM_213540.3	*Actg*_predicted	Actin, gamma, cytoplasmic	1.26*	-1.21	1.53*	0.011
XM_240367.3	RGD1563825_predicted	Similar to ENSANGP00000020885	1.72*	1.09	1.58	0.016
XM_345284.2	LOC365924 (*Tmem56*)	Similar to RIKEN cDNA 4930577M16	1.74*	1.06	1.65*	0.018
XR_009492.1	RGD1309310_predicted	Similar to mKIAA0195 protein	-1.34	1.14	-1.53*	0.020
XR_007416.1	LOC361841	Similar to glyceraldehyde-3-phosphate dehydrogenase	1.51	1.03	1.47*	0.021
XR_007919.1	LOC501282	Similar to lymphocyte antigen 6 complex, locus E ligand	1.47	-1.06	1.56*	0.027
XM_579556.1	LOC497689	Hypothetical gene supported by NM_053433	-1.52	-1.22*	-1.24	0.027
XM_001055592.1	RGD1564089_predicted (*Acot3*)	Similar to peroxisomal long chain acyl-CoA thioesterase Ia	1.38	-1.17	1.62	0.028
XM_343793.3	RGD1565289_predicted (*Apoo*)	Similar to RIKEN cDNA 0610008C08	1.39*	-1.09	1.51	0.031

NM_013027.1	*Sepw1*	Selenoprotein W, muscle 1	-2.16*	-1.39	-1.55*	0.035
XR_009220.1	LOC310902	Similar to Alcohol dehydrogenase 1A (Alcohol dehydrogenase alpha subunit)	-3.50	-1.19	-2.95*	0.037
XR_008905.1	RGD1563867_predicted	Similar to ribosomal protein L36	-1.61	-1.15	-1.40	0.040
XR_000314.1	*H19*	H19 fetal liver mRNA	3.61	1.10	3.28	0.041
XM_220112.4	RGD1306962_predicted	Similar to dJ55C23.6 gene product	1.41*	-1.07	1.51	0.041
XM_217293.4	*Ctdspl*_predicted (*Ctdspl*)	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like	-1.53*	-1.22	-1.25	0.044
NM_031315.1	*Acot1*	Acyl-CoA thioesterase 1	5.25*	1.07	4.92	0.049
XM_343501.3	RGD1305283_predicted (*Cmc1*)	Similar to RIKEN cDNA 2010110K16	-1.18	-1.59	1.34	0.049

[1)] Among 965 DEGs, 119 genes with the fold change ≥ 1.5 between two groups are listed according to the gene ontology. Information of DEGs was based on Illumina array data submission to Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/info/geo_illu.html).

[2)] Mean fold change between two groups (SPI *vs*. CAS, GEN *vs*. CAS, or SPI *vs*. GEN) was analyzed by using an unpaired *t*-test. Values of $p < 0.05$ were considered statistically significant and were marked with an asterisk (*). The + or – sign indicated up or down regulation, respectively.

[3)] *p*-value among the three different experimental groups (CAS, SPI, and GEN) was determined by One-way ANOVA.