

A MATERNAL HIGH FAT DIET REPRESSES THE EXPRESSION OF
ANTIOXIDANT DEFENSE GENES AND INDUCES THE CELLULAR
SENESCENCE PATHWAY IN THE LIVER OF MALE OFFSPRING RATS

BY

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THESIS

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ABSTRACT

Maternal high fat (HF) diet feeding is associated with increased risk of developing metabolism-related diseases in adult offspring, including chronic liver disease. The present study tested the hypothesis that maternal high fat diet leads to a decreased antioxidant defense capacity and causes cellular senescence in liver of adult offspring rats, which might increase risk of developing chronic liver disease. Timed-pregnant Sprague Dawley rats were fed a HF diet (45% of energy from fat) or a control (C) diet (16% of energy from fat) during gestation and lactation. The resulting offspring were fed a control diet after weaning, to generate two offspring groups: C/C and HF/C. At 12 wk of age, male rats were killed and samples were collected for analysis. Maternal HF diet significantly increased plasma triacylglycerol and hepatic thiobarbituric acid reactive substance concentrations and the size of hepatic lipid droplets in offspring rats. The expression of antioxidant defense genes, such as glutathione peroxidase-1 (*Gpx1*), Cu/Zn superoxide dismutase (*Sod1*), paraoxonase enzymes (*Pon1*, *Pon2*, and *Pon3*) were significantly lower in the liver of HF/C pups than in C/C pups. The expression of *p16^{INK4a}*, a marker of cellular senescence, and cyclooxygenase-2 (*Cox2*), a pro-inflammatory marker, was significantly higher in the HF/C offspring group than in the C/C offspring group. Western blot analysis shows that cyclin D1 and phosphorylated retinoblastoma (p-Rb) protein were significantly lower in HF/C offspring than in C/C offspring. The results provide the first evidence that maternal HF diet might alter antioxidant defense capacity and program the *p16^{INK4a}*-dependent cellular senescence in the liver of adult offspring.

Keywords: Aging, Cancer, Oxidative stress, Non-alcoholic fatty liver disease, Lipid

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LIST OF SYMBOLS AND ABBREVIATIONS

| <u>Item</u> | <u>Meaning</u> |
|-------------|---|
| C/C | control diet fed offspring of dams fed with control diet |
| CDK | cyclin dependent kinase |
| cDNA | complementary DNA |
| COX2 | cyclooxygenase-2 |
| d | day (s) |
| °C | degree Celsius |
| DNA | deoxyribonucleic acid |
| g | gram (s) |
| GPx1 | glutathione peroxidase-1 |
| h | hour (s) |
| HCC | hepatocellular carcinoma |
| HF | high-fat diet |
| HF/C | control diet fed offspring of dams fed with high-fat diet |
| kcal | kilocalorie (s) |
| kg | kilogram (s) |
| L | liter (s) |
| LBW | low birth weight |
| LP | low-protein diet |
| µg | microgram (s) |
| µL | microliter (s) |

| | |
|----------------------|---|
| mg | milligram (s) |
| min | minute (s) |
| mL | milliliter (s) |
| mRNA | messenger ribonucleic acid |
| NAFLD | non-alcoholic fatty liver disease |
| NEFA | non-esterified fatty acid |
| ng | nanogram (s) |
| p16 ^{INK4a} | inhibitor of cyclin dependent kinase-4a |
| p21 ^{Cip1} | cyclin dependent kinase inhibitor protein-1 |
| % | percent |
| PON | paraoxonase enzymes |
| qPCR | quantitative polymerase chain reaction |
| Rb | retinoblastoma protein |
| ROS | reactive oxygen species |
| s | second (s) |
| SOD1 | Cu/Zn superoxide dismutase |
| SOD2 | Mn superoxide dismutase |
| TAG | triacylglycerol |
| w | week (s) |

CHAPTER 1

INTRODUCTION

Appropriate maternal nutrition during gestation and lactation plays important role for offspring to perform normal metabolic function, while maternal malnutrition may lead to metabolism related disorders in offspring including obesity, type 2 diabetes, hypertension and hyperlipidemia (Barker et al., 1993). Mitogen-dependent mammalian cell progression is regulated by complexes formed with cyclin dependent kinases (CDKs) and cyclin, which are under tight control of inhibitors of cyclin dependent kinases (CDIs). The expression and activity of CDIs can be affected by inappropriate maternal diet, which will make the offspring more propensity to several diseases in their later life (Tarry-Adkins et al., 2009).

Because of the importance of maternal nutrition on the development of offspring and on the control of cell cycle pathways, knowledge of fetal development of metabolic diseases and cell cycle control are needed for further research. The potential mechanism of how the cell cycle pathways are programmed by maternal nutrition can be understood.

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CHAPTER 2

LITERATURE REVIEW

Maternal Nutrition and Developmental Origins of Diseases

Maternal nutrition during gestation is important for fetus development with regard of the nutrient provided through placenta, while the effect of maternal diet on breast milk plays significant role in postnatal offspring during lactation. Maternal malnutrition is the origin of many metabolic disorders happens to offspring in their later life. Hales and Barker proposed a “thrifty phenotype hypothesis” in 1992, demonstrating the association of malnutrition in fetal and early infant life to the damage of β -cells and the risk of type 2 diabetes in later life (Hales and Barker, 1992). Later, they established the relation of type 2 diabetes, hypertension, and hyperlipidemia to reduced birth weight induced by maternal undernutrition (Barker et al., 1993). Function of different internal organs in offspring can be affected by maternal malnutrition.

Maternal nutrition has tremendous effect on the function of pancreatic β -cells in offspring, demonstrating the association of maternal malnutrition to the development of type 2 diabetes in offspring's later life. Hales et al. studied 468 men born in east Hertfordshire and reported the association between low birth weight (LBW) induced by maternal malnutrition and the impaired glucose tolerance (Hales et al., 1991). Their study demonstrated that the impaired glucose tolerance caused by dysfunction of pancreatic β -cells is strongly linked with the reduced early growth. One study of maternal feed restriction on guinea pigs also showed reduced fetal growth, hyperinsulinemia and insulin resistance in male offspring (Kind et al., 2003). Following studies tried to explain the mechanism of

programmed increased risk of type 2 diabetes caused by maternal protein restriction (Tarry-Adkins et al., 2010; Tarry-Adkins et al., 2009). They first proposed that poor maternal nutrition followed by catch-up growth is associated with increased cellular senescence and decrease antioxidant defense capacity in rat islets. Followed up, they reported increased oxidative stress and fibrosis in 15 month rats offspring from dams fed a LP diet, indicating that maternal malnutrition can lead to type 2 diabetes in offspring's later life.

Maternal malnutrition can lead to the damage of kidney and the related metabolic diseases. When Hales and Barker proposed the “thrifty phenotype hypothesis”, they reported higher risk of hypertension in offspring experienced imbalanced early nutrition without digging into the mechanism related to the dysfunction of kidney. Accelerated senescence and reactive oxygen species have been reported in the kidneys of LBW rats after catch-up growth, suggesting the link between poor early growth induced by maternal malnutrition and the risk of hypertension in later life (Luyckx et al., 2009). Another study reported that maternal LP diet can program significant reduction in nephron number, glomerular volume and renal AT₁ and AT₂ receptor protein expression, indicating the association between elevated blood pressure and morphological change in kidneys of offspring rats induced by maternal protein restriction (Alwasel et al., 2010). It was also reported that offspring from dams fed a LP diet are more sensitive to stress and high-salt diet and may predispose for hypertension in later life (Augustyniak et al., 2010). Furthermore, the hypertension programmed by

maternal malnutrition can highly increase the risk of cardiovascular disease in adult offspring.

Offspring who experienced malnutrition in early life are likely to have higher blood pressure and higher risk of cardiovascular disease in their later life. It was reported that moderate balanced undernutrition in guinea pigs can increase systolic blood pressure in young adult male offspring (Kind et al., 2002). Maternal obesity was reported to induce greater fetal heart connective tissue accumulation, indicating a negative impact of maternal malnutrition in offspring's heart function (Huang et al., 2010). Increased mortality rate induced by maternal malnutrition has been associated to the increased risk of coronary heart disease in those offspring. The association between LBW and increased risk of coronary heart disease has been repeatedly confirmed (Barker, 1997). It was reported that a HF diet during pregnancy can lead to vascular dysfunction in rat weanlings (Koukkou et al., 1998).

Liver, as an important organ in regulating metabolism, can be significantly affected by maternal malnutrition and is greatly associated with chronic disease in later life. It was reported that protein restriction and folic acid supplement during pregnancy can change hepatic gene expression in rat offspring and the difference of DNA methylation on those genes may explain the mechanism (Lillicrop et al., 2005; Lillicrop et al., 2010). Maternal LP diet during gestation and lactation can decrease hepatic lipid content in male offspring, indicating the change of lipid metabolism can be programmed by maternal malnutrition (Qasem et al., 2010; Torres et al., 2010). Programmed hepatic antioxidant capacity and oxidative injury was reported in aging rat offspring from dams fed a LP diet (Langley-Evans and

Sculley, 2005). Hepatic lipid metabolism and gene expression can also be programmed by maternal HF diet during pregnancy and lactation (Bruce et al., 2009; Ghebremeskel et al., 1999; McCurdy et al., 2009; Zhang et al., 2009). Chronic liver diseases, like non-alcoholic fatty liver disease, can be induced in adult offspring from dams of malnutrition during pregnancy (Bruce et al., 2009; Elahi et al., 2009).

Other organs can be affected by maternal malnutrition, like mammary gland, skeleton muscle and adipose tissues. Maternal protein restriction can cause transcriptional repression of p16^{INK4a} in mammary gland through histone modification (Zheng and Pan, 2010). The expression of CCAAT/enhancer-binding protein was programmed through histone modification on the gene in the skeleton muscle of offspring from dams fed a LP diet (Zheng et al., 2011).

To sum up, maternal nutrition during pregnancy and lactation plays pivotal role in providing a healthy early environment for offspring in order to minimize the risk of metabolism disorder in later life. The association between maternal malnutrition and metabolic disease in later life has been established. Maternal HF diet and obesity have been related to hypertension, hyperlipidemia, impaired insulin tolerance, fatty liver disease and other chronic disorders in offspring's later life (Bruce and Hanson, 2010). In this study, I investigated the potential impact of maternal HF diet on the livers of male offspring.

Maternal High-Fat Exposure and Dyslipidemia

Maternal HF exposure during pregnancy can trigger several metabolic disorders in offspring, including cardiovascular disease, hypertension, dyslipidemia

and chronic liver diseases. It was reported that offspring from dams fed a diet high in saturated fat for 10 days before mating, through gestation and lactation have damaged function of vascular system (Koukkou et al., 1998). Plasma triglyceride content has been reported to be increased in those offspring from HF diet fed dams (Chapman et al., 2000; Elahi et al., 2009; Guo and Jen, 1995; Koukkou et al., 1998; Odaka et al., 2010)

Dyslipidemia is defined as abnormal lipid content in blood, hyperlipidemia in most cases. Maternal HF exposure during gestation and lactation can program dyslipidemia in the young or adult offspring, including hypercholesterol and hypertriglyceride in the blood. Offspring rats from dams fed a HF diet had higher plasma triglyceride compared to those from C diet fed dams (Chapman et al., 2000; Guo and Jen, 1995). Blood cholesterol was also significantly higher in offspring rats from long-term HF diet fed dams (Elahi et al., 2009). Hypertriglyceride can be programmed by maternal HF diet during gestation and lactation and can cause damage to cardiovascular system and problem of hepatic lipid metabolism

Liver, as a critical organ for lipid metabolism, can be greatly affected by maternal HF exposure. mRNA level of genes related to hepatic lipid metabolism, including peroxisome proliferator activated receptor-alpha (ppar-alpha) and carnitine palmitoyl transferase-1a (cpt-1a) can be changed by maternal HF diet (Shankar et al., 2010; Zhang et al., 2009). The concentration of docosahexaenoic acid (DHA) was significantly lower in livers of offspring from dam rats fed a HF diet for 10 days before mating, through gestation and lactation, indicating that maternal

HF exposure can lose the cardio-protective and anti-inflammatory function of the body (Ghebremeskel et al., 1999). The expression of hepatic genes can be changed in offspring by chronic maternal HF exposure through epigenetic modification, including the alteration of histone modification and DNA methylation (Aagaard-Tillery et al., 2008). It was also reported that maternal HF diet can trigger lipotoxicity in fetal livers (McCurdy et al., 2009). Several models of maternal HF exposure have reported that maternal HF diet can program chronic liver disease including non-alcoholic fatty liver disease and steatosis in offspring (Bruce et al., 2009; Elahi et al., 2009). Those metabolic disorders in livers of offspring, which were programmed by maternal overnutrition, are highly associated with the dyslipidemia in those offspring.

Some other function of the offspring can be affected by chronic maternal HF exposure. It has been reported that immune function of offspring was altered by maternal HF diet during gestation and lactation (Odaka et al., 2010). Mild maternal overnutrition can cause the increased adiposity and altered the brain appetite regulator in offspring (Rajia et al., 2010).

To sum up, maternal HF exposure has vital impact on the metabolic function in offspring, including the increased blood lipid content, increased hepatic triglyceride concentration, propensity to chronic liver diseases and dysfunction of other organs.

Hepatic Lipid Metabolism and Non-Alcoholic Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD), which is defined as the abnormal accumulation of lipid, primarily in the form of triacylglycerols (TAG) in the livers of

individuals who do not consume significant amounts of alcohol (20 g ethanol per day) and in whom other known causes of steatosis, such as certain drugs and toxins, have been excluded (McCullough, 2004), is the most common chronic liver disease. NAFLD prevalence is 10-24% in normal population, and it increases to 25-75% in obese population (Angulo, 2002). Two “hits” theory has been proposed for the pathophysiology of NAFLD while the exact cause still remains unclear. The first “hit” is insulin resistance, which leads to steatosis. The second “hit” is oxidative stress, which leads to the production of lipid peroxidation and the activation of inflammatory cytokines (Youssef and McCullough, 2002). There are many potential possibilities behind the reason of NAFLD (Angulo, 2002; Day and James, 1998), including medications, metabolic and genetic factors, nutritional status, and other uncommon factors.

Different animal models have been identified to study the pathophysiology of NAFLD. Firstly, effect of different dietary factors in modulating liver steatosis has been repeatedly reported: saturated fat, monounsaturated fatty acids, polyunsaturated fatty acids, *trans* fatty acids, n-3 fatty acids, glycemic index and fiber, sucrose and fructose, and protein (Zivkovic et al., 2007). Several animal models have been reported to establish the association between diet and the genesis of NAFLD: methionine-choline deficiency (MCD) diet, HF diet and sucrose diet with or without high caloric intake (Basaranoglu et al., 2010). A diet high in fat can increase hepatic lipid content and plasma insulin concentration, causing insulin resistance and increased risk of NAFLD in obese populations (Vilar et al., 2008). Being consistent with a typical American cafeteria diet, a HF diet containing

45% kcal from fat can trigger NAFLD in murine (Aagaard-Tillery et al., 2008; Bruce et al., 2009). Interestingly, n-3 polyunsaturated fatty acids have been reported to ameliorate the degree of liver injury in a high-fat, high-caloric diet induced NAFLD model (Svegliati-Baroni et al., 2006). A novel rats model found that the chemical hypoxemia can enhance the HF diet induced NAFLD (Takayama et al., 2009). The widely used MCD diet can develop liver inflammation as early as three days after feeding by eliminating two essential components for very-low-density-lipoprotein (VLDL) production and hepatic β -oxidation (Fan and Qiao, 2009). By using the MCD diet, animal models with severe liver damage can be developed (Larter and Yeh, 2008; Schattenberg and Galle, 2010). Another commonly used diet, atherogenic diet (cholic acid and high cholesterol), has been reported to trigger NAFLD (Larter and Yeh, 2008; Schattenberg and Galle, 2010). Furthermore, female rats fed a diet high in unsaturated fat, not a HF diet, have been reported to be a powerful model to study the pathogenesis of NAFLD (Tipoe et al., 2009). Secondly, genetically modified animal models have been developed to study the pathogenesis of NAFLD, including ob/ob mice (lacking leptin), db/db and fa/fa mice (lacking leptin receptor), *KK-Ay/a* mice, SREBP-1c mice, and other murine models (Schattenberg and Galle, 2010). Third, the animal models using combination of genetic modification and dietary challenge have been reported to induce NAFLD (Fan and Qiao, 2009). Lastly, maternal high-fat diet during gestation and lactation has been reported to program NAFLD in the offspring (Bruce et al., 2009; Oben et al., 2010).

Although the exact mechanism behind the pathophysiology of NAFLD remains elusive, studying the hepatic lipid metabolism can partially help to explain the abnormal accumulation of triglyceride in the liver. The imbalance among four major pathways of lipid metabolism contributes to the development of NAFLD, including the *de novo* lipogenesis, the fatty acid oxidation, the formation and secretion of VLDL, and the hepatic uptake of fatty acids (Angulo, 2002; Fabbrini et al., 2010; Tessari et al., 2009). The rate of *de novo* lipogenesis is regulated by the FA synthase complex, acetyl-CoA carboxylase (ACC), diacylglycerol acyltransferase (DGAT), stearoyl-CoA desaturase 1, and several nuclear transcription factors. *De novo* lipogenesis can be regulated independently by insulin and glucose through the nuclear transcription factors (Fabbrini et al., 2010). Fatty acid oxidation happens in mitochondria (majority), peroxisomes and microsomes (much less). Carnitine palmitoyltransferase-1 (CPT-1) is the rate-limiting and key component of regulation of the mitochondrial fatty acid β -oxidation (Tessari et al., 2009). Intrahepatocellular fatty acids that are not oxidized will be esterified and incorporated in the lipoprotein and be secreted as VLDL. The secretion of VLDL can contribute to the decrease of TAG content in the hepatocyte. The progression of NAFLD also comes from the combined result of adipose tissue lipolysis, hepatic lipolysis of the circulating TAG, and the up take of free fatty acid by other tissues.

The development of NAFLD is commonly accompanied with the changes of gene expression related to lipid metabolism, inflammatory response, and cell cycle control (Kirpich et al., 2011). The degree of nuclear factor-kappa B (NF- κ B)

activation, mRNA expression of tumor necrosis factor alpha (TNF- α), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) increased in the rats with NAFLD induced by a HF diet, while the mRNA expression of glutathione peroxidase (GPx) and catalase (CAT) decreased significantly (Tipoe et al., 2009). An animal model of NAFLD induced by maternal HF diet demonstrated up-regulation of the lipogenesis, oxidative stress, and inflammatory pathways (Bruce et al., 2009). Yan Wang et al. reported an increase of apoptosis in an animal model of NAFLD induced by HF diet, associated with the c-Jun NH2-terminal kinase (JNK) activation (Wang et al., 2008). Interestingly, the accumulation of p21Cip1, an inhibitor of cyclin dependent kinase and a marker of cellular senescence, was observed in NAFLD patients (Nakajima et al., 2010).

To sum up, with the rapidly increasing prevalence of NAFLD, molecular and metabolic basis associated with the pathogenesis of the disease is urgent to be identified. Future work to study the correlation of lipid metabolism, liver's function of regeneration, and the hepatocellular inflammatory response may contribute to understand the mechanism behind the phenomenon.

p16^{INK4a}

Various regulation of cell cycle has been identified in offspring from dams experienced undernutrition through gestation and lactation and in patients at different stages of chronic liver diseases. In order to establish the role of cell proliferation and senescence plays in those conditions, it is crucial to clarify the mechanism behind the complicated regulation of mammalian cell cycle.

Mitogen-dependent progression through the first gap (G1) phase and DNA synthesis (S) phase during the mammalian cell cycle is cooperated by several classes of cyclin dependent kinases (CDKs) whose activities are regulated by particular CDK inhibitors (CKIs). p16^{INK4a} belongs to the family of inhibitors of CDK 4 (INK4) which can only bind to CDK 4 or 6 and modifies the phosphorylation of retinoblastoma (Rb) protein who plays pivotal role in mediating the expression of downstream genes regulated by the complex formed by Rb and E2F protein (Dyson, 1998; Hunter and Pines, 1994; Mitnacht, 1998; Morgan, 1995; Serrano et al., 1993; Sherr, 1994; Sherr and Roberts, 1999).

p16^{INK4a} was firstly identified as a specific inhibitor of cyclin D/CDK4 in 1993 by Serrano et al. (Serrano et al., 1993). They reported that the protein of p16^{INK4a} can bind to CDK4 and inhibit the catalytic activity of CDK4/cyclin D in phosphorylating the downstream target proteins. In this report, they proposed p16^{INK4a} to be both upstream and downstream of retinoblastoma protein (Rb) in a negative feedback loop. Further research on how this inhibitor of CDKs works to regulate cell cycle progression and how p16^{INK4a} is regulated in the tightly control of cell proliferation or programmed death.

In the absence of CKIs, mitogen-dependent cell progression through G1 phase to S phase is positively regulated by the CDKs, whose activities rely mostly on the binding of cyclins. D-type cyclins are an important group of molecules that can bind to two major types of CDKs, CDK 4 and 6 (Hunter and Pines, 1994). D-type cyclins have a very short turnover time, about 30 min, if they are not binding to the specific CDKs. The complex formed by cyclin D and CDKs is fundamental to

cell cycle regulation of Rb protein. Rb protein, whose phosphorylation is mainly catalyzed by CDK4/6/cyclin D, is not phosphorylated through G1 phase. Hypophosphorylation of Rb protein arrests cells in G1 phase and phosphorylation can relieve this inhibition.

With functional inhibitors of cyclin D-dependent kinases, p16^{INK4a}, the enzyme activity of CDK4/6/cyclin D to phosphorylate the downstream protein will be inhibited. p16^{INK4a} can bind to CDK4 in competition with cyclin D and inhibit the phosphorylation of Rb protein (Hannon and Beach, 1994). p16^{INK4a} can inhibit CDK6/cyclin D assemble in the similar way. It was reviewed that p16^{INK4a} accumulates progressively as cells age and may be induced by cellular senescence (Sherr and Roberts, 1999).

Phosphorylation of Rb protein plays the central role in the p16^{INK4a} regulated cell cycle pathway. Cyclin D activated CDK4/6 and cyclin E activated CDK 2 are responsible for the phosphorylation of Rb protein at different sites (Mittnacht, 1998). Different kind of CDIs charge for different CDK regulated phosphorylation of Rb protein and p16^{INK4a}, as discussed above, can compete with cyclin D for the binding site of CDK4/6. The downstream target of Rb protein, E2F family, is an important regulator for several E2F-dependent transcriptions that are necessary for DNA synthesis. There are many members in the E2F family and they play vital roles in regulating the cell progression through G1 phase to S phase (Dyson, 1998)

The abnormal expression of p16^{INK4a} happens in various abnormal conditions. It has been reported that the gene of p16^{INK4a} is deleted in many cases

of human cancers (Nobori et al., 1994). The gene of p16^{INK4a} is located on the chromosome 9p21, which appears to be deleted in multiple types of cancers. In this aspect, the deletion of p16^{INK4a} can be used in negatively diagnosing specific kinds of cancer. The level of p16^{INK4a} plays critical role in balancing development and tumorigenesis (Elledge et al., 1996). p16^{INK4a} transcription can be regulated through DNA methylation and aberrant methylation was observed in some diseases (Hinshelwood et al., 2009; Nishida et al., 2008). The loss of p16^{INK4a} in mice showed increased rate of tumor development, while the overexpression of the gene arrests the cells in G1 phase.

In conclusion, p16^{INK4a} is an important inhibitor of CDK4/6 in competing with cyclin D for the binding site. The balanced expression and function of p16^{INK4a} and CDKs are needed to maintain normal cell cycle progression.

p21^{Cip1}

p21^{Cip1} belongs to the family of CDKs inhibitor protein and is a universal inhibitor of CDKs which include cyclin D-, E- and A-dependent kinases (Sherr and Roberts, 1999; Xiong et al., 1993). p21^{Cip1} binds to and inhibits a wide range of cyclin/CDKs complexes including cyclin D-dependent CDK4, cyclin A dependent CDK2 and cyclin E dependent CDK2 (Hunter and Pines, 1994). In some cases, the inhibition of forming the cyclin-CDK complex requires more than one p21^{Cip1} molecules.

The expression of p21^{Cip1} is regulated by the p53 tumor suppressor protein. mRNA expression of p21^{Cip1} is induced by DNA damage in a p53 positive cell line, while the expression of p21^{Cip1} remains the same under the same treatment in p53

negative cells (Xiong et al., 1993). On the promoter of p21^{Cip1} gene, there exists a binding site for p53 protein and the binding of p53 acts as a transcription factor for p21^{Cip1}. In normal cells, p21^{Cip1} binds with CDK, cyclin and proliferating cell nuclear antigen to form a complex (Xiong et al., 1992). The inhibiting function of p21^{Cip1} on cell cycle progression after DNA damage relies on the induction of p53 and the resulting induction of p21^{Cip1}.

The expression of p21^{Cip1} was abnormal in several conditions, including cancer, adipocyte differentiation and maternal protein restriction. Loss of p21^{Cip1} expression was observed in 79% of colon cancer patients in a study of host-tumor interactions (Ogino et al., 2009). The interaction of p21^{Cip1} with cigarette smoking can affect the susceptibility to esophageal squamous cell carcinoma (Taghavi et al., 2010). In a p21^{Cip1} knockout model, vigorous apoptosis was observed as well as the activated p53 (Inoue et al., 2008). It has been reported that mRNA expression of p21^{Cip1} can be induced in the kidney and pancreatic islet cells of offspring from dams fed a LP diet (Luyckx et al., 2009; Tarry-Adkins et al., 2010; Tarry-Adkins et al., 2009). The increased expression of p21^{Cip1} and the associated hepatocellular senescence were observed in non-alcoholic fatty liver disease patients (Nakajima et al., 2010).

In conclusion, mitogen-dependent cell cycle progression is under tightly control of the elaboration of cyclin, CDKs and the CDIs. The abnormal expression of p16^{INK4a} and p21^{Cip1} was highly involved in several conditions including tumorigenesis and cellular senescence. The loss of expression of the inhibitors

can lead to the uncontrolled cell proliferation, while the increased expression may cause aging and senescence.

Aging and Cellular Senescence

Cellular senescence was primarily described as the limited lifespan of cells in culture (Hayflick, 1965). According to the different causes of senescence, it can be divided into four classes: telomere-dependent senescence, DNA-damage-initiated senescence, oncogene-induced senescence and oxidation- and other-inducer-induced senescence (Campisi and dda di, 2007). As discussed in previous paragraphs, p53-p21^{Cip1} and p16^{INK4a}-Rb pathways are involved in cellular senescence. Generally speaking, DNA damage acts as a senescence signal and causes the activation of p53 to induce the inhibiting function of p21^{Cip1} in the cell cycle progression, while other senescence signals induce the expression of p16^{INK4a} and cause the cell cycle arrest.

Although both p53-p21^{Cip1} and p16^{INK4a}-Rb pathways are involved in a senescence response, the roles played by these two CKIs are not equivalent. Cells undergo senescence induced solely by DNA damage and p53 activation can resume after the inactivation of p53 (Campisi and dda di, 2007). However, cells senesce completely because of p16^{INK4a}-Rb activation cannot resume even after the inactivation of p16^{INK4a} or Rb. The expression of p16^{INK4a} was used as a biomarker of aging and senescence (Krishnamurthy et al., 2004). In this aspect, studying the p16^{INK4a}-Rb senescence pathway is critical to understand how to maintain normal cell function.

The expression of p16^{INK4a} is regulated by the transcriptional activator and inhibitor during cellular senescence. Ets family has many members of transcription factors, including Ets1, Ets2, PEA3, SAP1 and ELK1, which can be activated by Ras-Raf-MEK signaling through phosphorylation (Ohtani et al., 2001). In young diploid fibroblast cells, the expression of p16^{INK4a} can be induced by the transcription activator Ets2 and be inhibited by the direct interaction with a helix-loop-helix protein Id1. In senescence cells, it was found that the induction of p16^{INK4a} is associated with the increase of the transcription activator Ets1 and the decrease of the inhibitor Id1. Another member of the Ets family of transcription factors, ESE3, was detected to be induced in cellular senescence, which agreed with the increased expression of p16^{INK4a} (Fujikawa et al., 2007). Members of another family of transcription factors, Id proteins, have basic-helix-loop-helix structure and are repressed in senescent cells (Hara et al., 1994; Zebedee and Hara, 2001). The expression of p16^{INK4a} can be regulated by other transcription factors through a manner of histone modification (Feng et al., 2009; Jung et al., 2010). The network of interaction and collaboration of the transcription activators and inhibitors decides the expression of p16^{INK4a} in senescence pathway.

Cellular senescence can prevent the cells against cancer development. Senescent bile ductular cells increase in chronic liver diseases (Sasaki et al., 2010) and the proliferative activity of hepatocytes was decreased in human liver cirrhosis (Delhaye et al., 1999). Hepatocellular telomere shortening and senescence are considered as markers of liver cirrhosis (Wiemann et al., 2002). However, the complex of senescent hepatocytes and ductular cells was lost before the

development of early hepatocarcinogenesis (Ikeda et al., 2009). Study on the replicate senescence in different stages of chronic liver diseases confirmed that the accumulation of senescent cells happens more often in the cirrhosis tissue surrounding the hepatocellular carcinoma (Paradis et al., 2001). A significant number of cells in premalignant tumors undergo oncogene-induced senescence and cells bypass the senescence are easier to develop tumor (Braig and Schmitt, 2006; Ozturk et al., 2009). Experiencing cellular senescence provides protection for the premalignant cells against cancer development.

The production of reactive oxygen species (ROS) accumulates as normal by-product of metabolism, which can increase to a significant amount that is harmful to cells and may lead to ROS-induced senescence when cells are experiencing stress. Substantial amount of evidence have shown that the induction of cellular senescence can be carried out through p53-p21^{Cip1} and p16^{INK4a}-Rb pathways. Although the mechanism of how ROS contributes and induces senescence remains illusive, studies have demonstrated that ROS, oxidative stress and DNA damage collaborate to trigger senescence in a manner of DNA damage response (Ozturk et al., 2009). Oxidative stress can induce cellular senescence in cultured mouse biliary epithelial cells, suggesting the reason why progressive bile duct was lost in biliary cirrhosis (Sasaki et al., 2005). It was also reported that excessive ROS production can trigger the p53 activated senescence, which contributed to inflammatory disorders and associated aging (Kim et al., 2009). Mild hyperoxia can induce senescence via DNA-damage response (Chen et al., 1995; von et al., 1995). Decreased level of antioxidant

defense capacity and increased level of oxidative stress and ROS contribute to the induction of cellular senescence and body's propensity to some chronic diseases (Burhans and Heintz, 2009).

In conclusion, cellular senescence induced by excessive oxidative stress and ROS via p21^{Cip1}, p16^{INK4a}-Rb pathway is highly associated with the development of chronic diseases.

Antioxidant Defense Genes

Oxidative stress and the abnormal accumulation of ROS are well-known causes of cellular senescence. In cultured human retinal pigment epithelial (RPE) cells, H₂O₂ treatment triggered cellular senescence via p21^{Cip1}-Rb pathway (Yu et al., 2009). p16^{INK4a} and p21^{Cip1} accumulate in response to oxidative stress and induce cellular senescence via specific pathways, while antioxidant defense capacity of the body reduces the excessive ROS and protect the cells from senescence.

Oxidative stress results from the accumulation of ROS (O₂^{•-}, H₂O₂, [•]OH), which are the normal by-product of metabolism but can excessively accumulate when organism losses the ability to eliminate them. Superoxide dismutase (Sod) is the enzyme converts O₂^{•-} to H₂O₂ (Beyer et al., 1991) and glutathione peroxidase (Gpx) catalyzes the reaction converting H₂O₂ to H₂O (Ursini et al., 1995). Both of them act very important role in antioxidant defense reactions. Three members exist in the family of Sod enzymes: Cu/ZnSOD (Sod1), MnSOD (Sod2) and ECSOD (Sod3) (Miao and St Clair, 2009). It was reported that loss of catalytic activity of Sod1 is associated with aging and aging-associated disease. In a Sod1

knock-out mice model, high rate of death from liver tumor was observed (Miao and St Clair, 2009). Alteration of Sod2 expression and activity has been reported to be related to the incidence of ROS-mediated cancer development. Loss or decreased activity of Gpx can lead to an oxidative-stress-mediated growth retardation and impairment of physical situation (Esposito et al., 2000; Shin et al., 2008). Studies on the interaction between maternal diet and antioxidant capacity in offspring show that selenium supplement in maternal diet is important to establish an effective antioxidant protection system in fetus (Pappas et al., 2008).

There are three members in the paraoxonase (PON) enzyme family: PON1, PON2 and PON3. The sufficient expression and activity of these three enzymes can provide efficient reduction in low density lipoprotein (LDL) oxidation (Aviram and Rosenblat, 2004). More importantly, the decreased expression and activity of PON1 has been associated with chronic liver disease (Camps et al., 2009). It is important for the body to maintain a reasonable level of these enzymes in order to establish the effective antioxidant defense capacity.

Cyclooxygenase 2 (Cox2) is the enzyme that catalyzes the conversion of arachidonic acid to some pro-inflammatory factors including prostanoids and thromboxanes (Funk, 2001). The expression of Cox2 presents extremely low in hepatocytes under normal condition but it will be induced if there exist lipid peroxides and oxidative stress. Expression of Cox2 is related to chronic liver disease and stress and Cox2-inhibitors suppress proliferation of hepatocellular carcinoma (HCC) cells (Hu, 2003). The induction of Cox2 is associated with pro-inflammatory mediators and fatty liver disease (Hsieh et al., 2009; Kniss, 1999; Yu

et al., 2006). It was also reported that, by using Cox2-inhibitors, we can improve immune system function and decrease liver damage in septic rats (Li et al., 2009).

In conclusion, increased oxidative stress and decreased antioxidant defense capacity can act as mediator for stress-induced senescence and chronic diseases. Organism's ability to eliminate the harmful substrates produced during metabolism is important to maintain a normal function of operating.

Conclusion

Appropriate maternal diet is an important predetermining requirement for the offspring to maintain a healthy condition in their life. Maternal malnutrition during pregnancy and lactation can program metabolic disorders in offspring, including obesity, type 2 diabetes, hypertension and fatty liver disease. Among the several organs that can be affected by inappropriate maternal nutrition, liver is an important one to be targeted because of the central role it plays in metabolism. Previous studies have shown the significant impact of maternal malnutrition on metabolic function of livers of offspring. It still remains elusive that how maternal HF diet during gestation and lactation can affect the livers of offspring.

Tightly control of cell cycle is performed in normal proliferating mammalian cells through maintaining consistent amount of cyclin, CDKs and CKIs. It was reported that cancer and other disorders could be induced when there exists abnormal expression of CKIs. However, the impact of maternal HF diet on cell cycle control has been hardly addressed.

The regulation of antioxidant defense genes and cell cycle control genes has been well defined while studying the mechanism of cellular senescence in

several *in vivo* and *in vitro* studies. Loss of antioxidant defense genes and increased oxidative stress can induce the accumulation of CKIs, which are responsible for the arrest of cell cycle. It needs to be further identified the impact of maternal HF diet on the expression of antioxidant defense genes and cell cycle control genes.

To further our understanding of this topic, we planed to study the impact of maternal HF diet through gestation and lactation on the livers of offspring rats by evaluating the expression of antioxidant defense genes and cellular senescence genes. Oxidative stress and down stream factors in the pathway will be evaluated in the livers of offspring to illustrate the mechanism and regulation of any programmed difference between offspring from HF diet fed dams and those from C diet fed dams.

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CHAPTER 3

A MATERNAL HIGH FAT DIET REPRESSES THE EXPRESSION OF ANTIOXIDANT DEFENSE GENES AND INDUCES THE CELLULAR SENESENCE PATHWAY IN THE LIVER OF MALE OFFSPRING RATS

Abstract

Maternal high fat (HF) diet feeding is associated with increased risk of developing metabolism-related diseases in adult offspring, including chronic liver disease. The present study tested the hypothesis that maternal high fat diet leads to a decreased antioxidant defense capacity and causes cellular senescence in liver of adult offspring rats, which might increase risk of developing chronic liver disease. Timed-pregnant Sprague Dawley rats were fed a HF diet (45% of energy from fat) or a control (C) diet (16% of energy from fat) during gestation and lactation. The resulting offspring were fed a control diet after weaning, to generate two offspring groups: C/C and HF/C. At 12 wk of age, male rats were killed and samples were collected for analysis. Maternal HF diet significantly increased plasma triacylglycerol and hepatic thiobarbituric acid reactive substance concentrations and the size of hepatic lipid droplets in offspring rats. The expression of antioxidant defense genes, such as glutathione peroxidase-1 (*Gpx1*), Cu/Zn superoxide dismutase (*Sod1*), paraoxonase enzymes (*Pon1*, *Pon2*, and *Pon3*) were significantly lower in the liver of HF/C pups than in C/C pups. The expression of *p16^{INK4a}*, a marker of cellular senescence, and cyclooxygenase-2 (*Cox2*), a pro-inflammatory marker, was significantly higher in the HF/C offspring group than in the C/C offspring group. Western blot analysis shows that cyclin D1 and phosphorylated retinoblastoma (p-Rb) protein were significantly lower in HF/C

offspring than in C/C offspring. The results provide the first evidence that maternal HF diet might alter antioxidant defense capacity and program the p16^{INK4a}-dependent cellular senescence in the liver of adult offspring.

Keywords: Aging, Cancer, Oxidative stress, Non-alcoholic fatty liver disease, Lipid

Introduction

Epidemiological studies have shown that maternal HF diet and the associated overweight affect insulin signaling (Shankar et al., 2010), brain appetite regulation (Rajia et al., 2010), immune function (Odaka et al., 2010), hepatic gene expression (Zhang et al., 2009), blood pressure (Elahi et al., 2009), plasma lipid (Elahi et al., 2009), and liver lipid metabolism (Chapman et al., 2000; Ghebremeskel et al., 1999; Higham et al., 1984; Innis, 1988; McCurdy et al., 2009). Long term HF diet consumption is associated with the increased plasma lipid level in offspring of mice (Elahi et al., 2009). Additionally, maternal HF diet has been shown to be highly associated with chronic liver damage in offspring adults and non-alcoholic fatty liver disease (NAFLD) was reported in offspring of dams fed with HF diet (Bruce et al., 2009; McCurdy et al., 2009).

p16^{INK4a} (Inhibitor of cyclin dependent Kinase 4a) is a vital cell cycle control gene that binds to cyclin-dependent kinase4 (CDK4) and inhibits the formation of the active CDK4/cyclin D complex (Serrano et al., 1993). p21^{Cip1} (cell cycle inhibitory protein 1) belongs to the Cip/Kip family, whose actions affect the activities of cyclin D, E and A dependent kinases (Sherr, 1994). p16^{INK4a} and p21^{Cip1} inhibit the active formation of cyclin/CDK, thereby decreasing the phosphorylation of Rb (Mitnacht, 1998). Hypophosphorylation of Rb protein increases its binding to E2F, which correlates with a decrease of E2F-regulated transcription (Dyson, 1998). E2F plays an important role as a transcription factor in either activating or suppressing crucial genes. The phosphorylated Rb protein

will release E2F to activate the transcription of downstream DNA synthesis related genes which will increase the cells progression from G1 to S phase (Sherr, 1994).

The p16^{INK4a}-Rb senescence pathway is known to be induced by various stressful stimuli, including oxidative stress (Zhang, 2007). It has been reported in previous studies that decreased expression and activity of GPx1 in response to oxidative stress decreased antioxidant defense capacity of the organ (Esposito et al., 2000; Shin et al., 2008). Expression of COX2 is more related to chronic liver disease and stress and COX2-inhibitors suppress proliferation of hepatocellular carcinoma (HCC) cells (Hu, 2003) and to decrease liver damage (Li et al., 2009). The induction of COX2 is associated with pro-inflammatory mediators and fatty liver disease (Hsieh et al., 2009; Kniss, 1999; Yu et al., 2006). Another indicator of antioxidant defense response, SOD1, has been reported to convert harmful superoxide radicals to hydrogen peroxide and therefore neutralize the reactive oxygen species (de Haan et al., 1995). The three members of the paraoxonase (PON) family of enzymes, PON1, PON2, and PON3 are efficient in reducing low density lipoprotein (LDL) oxidation (Aviram and Rosenblat, 2004). The decreased expression and activity of PON1 has been associated with chronic liver disease (Camps et al., 2009).

The relationship between diet and cellular senescence has been observed in other tissues. Poor maternal nutrition can lead to low-birth-weight offspring that have accelerated cellular senescence after catch-up growth (Luyckx et al., 2009; Tarry-Adkins et al., 2009). p16^{INK4a} was induced in pancreatic islet cells of low-birth-weight offspring rats (Tarry-Adkins et al., 2009). Cellular senescence in

kidney was also triggered by the accelerated postnatal growth following maternal under-nutrition through the p16^{INK4a}-pRb pathway (Luyckx et al., 2009). Additionally, during the development of emphysema in chronic obstructive pulmonary disease, p16^{INK4a} was detected to link environment or nutritional stress and chronic disease (Karrasch et al., 2008).

Liver is an important organ for studying the growth and cell cycle regulation because of its regenerative and metabolic capacity (Fausto et al., 2006). Many studies focus on the role that cell cycle control genes play in hepatocellular carcinoma (HCC), and how nutrition during pregnancy affects chronic liver diseases development are not well understood. The present study was designed to determine the effect of a maternal HF diet on antioxidant defense and p16^{INK4a}-pRb senescence pathway in the liver of offspring rats. We hypothesize that maternal HF diet during gestation and lactation has the potential to program the expression of cellular senescence and antioxidant defense genes by triggering hepatic oxidative stress in rat offspring.

Experimental Methods

Animals and treatment

Timed-pregnant Sprague Dawley rats (Charles River Laboratories) were fed with either a HF or a C diet (Research Diets, Inc., New Brunswick, NJ, table 3-1) during gestation and lactation. Litter sizes were standardized to 10 pups. All offspring were weaned at 3 wk of age and male rats (n=10 of C/C pups and n=9 of HF/C pups from different litter) of the resulting offspring were fed a control diet after weaning, to generate two offspring groups: HF/C, C/C. Rats were kept

individually in standard polycarbonate cages in a humidity- and temperature-controlled room on a 12-h light-dark cycle and had free access to food and water. At 12 wk of age, male rats were killed by CO₂ asphyxiation and the left lobe of the whole liver was collected, rapidly frozen in liquid nitrogen, and stored in -70°C for future use. We certify that all applicable institutional and governmental regulations regarding the ethical use of animals were followed during this research (University of Illinois Institutional Animal Care and Use Committee approval #09112).

Biochemical assays in pups

One hundred milligrams of frozen liver samples were ground using a mortar and pestle with liquid nitrogen and mixed with 0.3 mL saline (0.9% w/v NaCl). Homogenized samples were quickly frozen in liquid nitrogen and kept in -70°C until analysis. The samples were quickly thawed in 37°C and diluted 5 times to 1.5 mL. Twenty microliters of the diluted samples were incubated with 20 µL 1% deoxycholate in 37°C for 5 min, and ten microliters of the samples were analyzed via the Thermo Infinity Triglycerides Liquid Stable Reagent (Thermo Fisher Scientific, Rockford, IL) following company protocol and using a commercially available standard reference kit (Verichem Laboratories, Providence, RI). Lowry assay was performed to determine the protein concentration, which was used to normalize the TAG concentration in liver. To determine plasma TAG levels, plasma samples were thawed on ice and analyzed accordingly following company protocol as for liver samples. NEFA concentration in liver was determined using a commercially kit (HR-2 Series, Wako Diagnostics, Richmond, VA). To determine lipid peroxidation and oxidative stress, a commercial thiobarbituric acid reactive

substances (TBARS) Assay Kit (Cayman Chemical Company, Ann Arbor, MI) was used to measure plasma and hepatic TBARS level accordingly following company protocol.

Liver histology

Frozen liver samples from offspring were embedded in O.C.T. compound (Gentaur, Kampenhout, Belgium) prior to sectioning. All sections were then stained with hematoxylin and eosin (H&E) or oil red O (ORO) kit (Newcomer Supply, Middleton, WI) and evaluated for steatosis and inflammation.

RNA Isolation and cDNA synthesis

To measure the relative mRNA level of target genes, qPCR was performed in a 96-well plate using a 7300 real time PCR System (Applied Biosystems) as previously described (Strakovsky et al., 2010). High efficiency of the machine and presence of a unique product were ensured by checking that the slope of the standard was in the range of -3.3 ± 0.3 and the R^2 to be larger than 0.98. Kinetic analysis was conducted to detect the exponential phase of amplification in each well with 25 ng template cDNA. mRNA level of ribosomal protein *L7a* was utilized as the internal control. In order to determine the gene transcription rate, primers were designed to amplify a region including both exon and intron of that gene. This procedure was adapted from a method described by Lipson and Baserga (Lipson and Baserga, 1989), except that pre-mRNA amounts were quantified by real time PCR. The relative expression level of this region can indicate the pre-mRNA rate of the gene and further present the transcription rate. Primers used for qPCR were

designed using Vector NTI software (InforMax Inc., Frederick, MD) and all the primers used for real-time PCR are listed in Appendix A.

Protein Isolation and Western blotting

Fifty micrograms of frozen liver was ground in liquid nitrogen and put into 500 μ L protein sample buffer (0.125 mol/L Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 1% SDS, 20% glycerol, 0.4% bromophenol blue, protease inhibitor, phosphatase inhibitor). Protein samples were sonicated (Fisher Scientific model 100 Sonic Dismembrator) on ice with 25 pulses and standard long Lowry assay was performed to determine sample protein concentrations. Samples containing 45 μ g of protein were resolved by 10% SDS-PAGE. Western analysis was performed as previously described (Strakovsky et al., 2010) using 1:1000 rabbit monoclonal antibody against Phospho-Rb (Ser807/811) (#9308, Cell Signaling Technology, Beverly, MA), 1:200 Cyclin D1 (sc-20044, Santa Cruz Biotechnology, Santa Cruz, CA) antibody to detect Cyclin D1 protein, 1:1000 COX2 (#4842, Cell Signaling Technology, Beverly, MA) antibody to detect COX2 protein, 1:10000 Actin (sc-1616-R, Santa Cruz Biotechnology, Santa Cruz, CA) antibody to detect Actin protein, peroxidase-conjugated secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and HRP-linked anti-biotin secondary antibody (#7075, Cell signaling). The secondary antibody was detected by applying Super Signal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL) and western blot images were captured and analyzed by Chemi Doc system (Bio-Rad) and Quantity One software (Bio-Rad). The result of p-Rb, Cyclin D1, and

COX2 protein expression level was normalized to the expression of actin, whose expression did not between groups.

Flow cytometry

Fifty milligrams of frozen liver was ground using a mortar and pestle in liquid nitrogen and was homogenized in phosphate buffered saline (PBS) with 5% fetal bovine serum (FBS). The homogenized samples were centrifuged twice at 1.12 g for 2 min to collect the supernatant and the resulting cell suspension was washed twice with PBS with 5% FBS. The cells were counted using a hemocytometer and the concentration was adjusted to approximately 1×10^9 cells/L. The cells were pelleted by centrifugation and were resuspended in PBS. The cell suspension was fixed and stored in -20°C until analysis. Propidium iodide (PI) was used to stain DNA and cell cycle analysis was performed using a BD Biosciences LSR II flow cytometer at the Flow Cytometry Facility (The W.M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign).

Statistical analysis

Results are expressed as means \pm SEM (animal numbers of specific experiments are described in the figure legends). Comparison of mRNA expression, protein level and physiological outcomes between HF/C and C/C groups were performed by Student's *t*-test in SAS v. 9.1 (SAS Institute, Cary, NC). Body weight and food intake data were tested using repeated-measures 1-way ANOVA (SAS software). The presented SEM was calculated based on the SD and

the specific sample size if there exists unequal sample size. Differences between HF/C and C/C pups were considered to be statistically significant for $P < 0.05$.

Results

Physiological Observations

Food intake and body weight

The food intake of pups fed a maternal high fat diet (HF/C) did not differ from that of pups fed a maternal control diet (C/C). At 12 wk after weaning, there was no significant difference in body weight between the two offspring groups. (Figure 3-1)

Lipid Profiles

Plasma TAG levels of offspring in HF/C group were significantly higher ($P < 0.05$) when compared to that of the C/C group. Hepatic TAG and NEFA concentration did not differ between HF/C and C/C pups. (Table 3-2)

An index of lipid peroxidation and oxidative stress

Plasma TBARS levels did not differ between HF/C and C/C offspring. Hepatic TBARS levels of pups in HF/C group were significantly higher ($P < 0.05$) when compared to that of the C/C group. (Table 3-2)

Liver histology

Liver structure tested by H&E staining did not differ between HF/C and C/C pups (Figure 3-2A). However, differences between lipid droplets were observed in Oil Red O staining in the livers of HF/C and C/C pups. Although both HF/C and C/C pups had red staining (Figure 3-2B), the red droplets staining in HF/C pups were larger than those observed in C/C pups in all samples examined.

Cell cycle analysis on the frozen liver samples did not show difference between the two offspring groups (data not shown).

Genes related to antioxidant defense capacity

The levels of mRNA from antioxidant genes and markers of oxidation were affected in the liver of maternal HF pups. *Gpx1* mRNA expression was significantly lower in the HF/C group ($P<0.05$) when compared to C/C (Table 3-3). *Sod1* mRNA expression was significantly lower ($P<0.01$) in HF/C group than in C/C group (Table 3-3). However, *Sod2* mRNA expression did not differ between the two groups (Table 3-3). The antioxidant enzymes, *Pon1* ($P<0.05$), *Pon2* ($P<0.05$), and *Pon3* ($P<0.01$) were significantly lower in HF/C pups than in C/C pups (Table 3-3).

Cellular senescence pathway

The mRNA level of *Cox2* (Table 3-3) as well as the protein level of Cox2 (Figure 3-3A) was increased 4 to 5-fold ($P<0.01$) in HF/C pups, indicating the inflammatory state of the liver is higher in HF/C pups. The mRNA amount of *p16^{INK4a}* was increased 4.7-fold ($P<0.01$) in the HF/C pups (Table 3-3). *p21^{Cip1}* mRNA level did not differ between HF/C and C/C pups (Table 3-3). *p16^{INK4a}* transcription activity was significantly higher ($P<0.01$) in HF/C pups when compared to C/C pups (Figure 3-3B). Protein level of ETS1 (Figure 3-3C), a transcription factor activating *p16^{INK4a}* transcription, was also increased 3 fold in the liver of HF/C pups, whereas protein content of ID1 (Figure 3-3D), a transcription repressor was unchanged.

To demonstrate that p16^{INK4a}-dependent cellular senescence pathway was affected, cyclin D1 and phosphorylated Rb protein levels were detected by western blotting. Cyclin D1 protein was decreased 80% ($P<0.01$) in the HF/C pups compared with C/C pups (Figure 3-3E). Phosphorylated-Rb protein was more than 60% lower ($P<0.05$) in HF/C rats compared with C/C pups, which consistent with the increase of p16^{INK4a} mRNA (Figure 3-3F).

Discussion

We report in this study a novel finding that a maternal high fat diet during gestation and lactation leads to a decreased expression of genes responsible for the antioxidant defense capacity and an increase in markers of cellular senescence in the liver of offspring rats in spite of feeding a standard AIN 93 diet after weaning.

The effects of maternal HF diet on offspring physiological outcomes include increased body fat, liver weight, liver lipid content, blood glucose and TAG in offspring rats (Guo and Jen, 1995; Koukkou et al., 1998). Long term HF diet was shown to raise plasma lipid content in the offspring (Elahi et al., 2009) and an increase in blood lipids potentially increased the risk of vascular diseases in adulthood (Barker, 1997), which is consistent with our results showing a significantly increase of plasma TAG in offspring fed a control diet whose mothers consumed a HF diet throughout gestation and lactation. Although in the current study no significant difference in liver TAG concentration was observed between C/C and HF/C groups, we noted that the lipid droplets in HF/C pups were much larger than those observed in C/C pups. A study with hepatic steatosis in obese

rats showed that a decrease in the size of the lipid droplets may parallel an improvement of the disease (Stringer et al., 2010). Further research will be required to fully understand how lipid droplet size and locations affects lipid droplet metabolism and NAFLD development.

Cellular senescence has been reported to be programmed by maternal diets. Maternal under-nutrition with postnatal catch-up growth accelerated cellular senescence in pancreatic islet cells (Tarry-Adkins et al., 2009) and kidney (Luyckx et al., 2009) in offspring rats. In the present study, we did not observe any postnatal growth difference in our rat offspring. However, both cyclin D1 and phosphorylated Rb protein were decreased significantly in HF/C offspring showing the activation of the p16^{INK4a} cellular senescence pathway in liver of offspring from mothers fed a HF diet during gestation and lactation. The ability of hepatocellular proliferation in response to chronic liver injury significantly decreases when cirrhosis occurs (Delhaye et al., 1999) and the expressions of cell cycle inhibitors like p16^{INK4a} and p21^{Cip1} increase (Paradis et al., 2001). Activation of the cellular senescence pathways prevents liver regeneration and the continuous liver damage further triggers the fibrotic scarring and cirrhosis (Wiemann et al., 2002). Our study suggests that early activation of the p16^{INK4a}-Rb senescence programmed by maternal HF diet might accelerate chronic liver injury.

Oxidative stress may lead to cellular senescence by causing DNA damage and alteration of telomere length (Chen et al., 1995; von et al., 1995). Oxidation results in the production of reactive oxygen species (ROS), which damage cell components and organ functions. Levels of ROS are controlled by the activity and

amount of antioxidant enzymes. Normally, COX2 expression in hepatocytes is low, however, COX2 expression levels increase rapidly in the presence of oxidative stress (Yu et al., 2006). In the present study, *Cox2* mRNA and COX2 protein levels were significantly higher in HF/C pups, indicating increased oxidative stress in the liver of maternal HF offspring. Low expression, low activity, or deficiency of SOD1 may lead to various diseases, including causes ApoB degradation and induces hepatic lipid accumulation (Uchiyama et al., 2006). Low expression of GPx1 cause reduced body weight than normal animals and increased levels of lipid peroxides in the liver (Esposito et al., 2000). Aberrant expression of COX2 is associated with liver injury (Li et al., 2009), insulin resistance, fatty liver (Hsieh et al., 2009), steatohepatitis (Yu et al., 2006), and HCC (Hu, 2003). The expression and enzymatic activity of PONs have been associated with the cell ability to reduce oxidative stress (Camps et al., 2009). A measurement of serum PON1 activity can be used as an indicator of liver function and chronic liver impairment (Camps et al., 2009). Our study demonstrated decreased mRNA expression of *Sod1*, *Gpx1*, *Pon1*, *Pon2*, and *Pon3*, increased mRNA and protein levels of COX2, and increased hepatic TBARS level in maternal HF offspring rats, suggesting that maternal HF increases hepatic inflammation and reduces antioxidant capacity, which potentially accelerates liver damage in these animals. In the future, we will perform “second hit” study to see how liver respond to different types of diets if they had a maternal HF diet and how cellular senescence involve in fatty liver development.

In summary, we have demonstrated that maternal HF diet during gestation and lactation results in the activation of markers of cellular senescence associated with a increase in the size of the lipid droplets in livers of offspring of HF-fed dams. Our study also demonstrates that alteration of antioxidant defense capacity might hasten the occurrence of chronic liver injury. Our results show the potential of maternal HF diet in triggering chronic liver diseases in the offspring. Further studies are needed to establish the precise mechanism behind this phenomenon and to determine how these molecular dysregulations affect later liver physiology and disease development.

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Table 3-1. Macro- and micronutrient composition of the diets in this study

| | Control | High Fat |
|--------------------------|----------------|-----------------|
| | g/kg | |
| Protein | 20 | 24 |
| Carbohydrate | 64 | 41 |
| Fat | 7 | 24 |
| Total energy, kJ/g | 16.75 | 19.68 |
| Ingredient | | |
| Casein | 200 | 200 |
| L-Cystine | 3 | 3 |
| Corn Starch | 437.2 | 72.8 |
| Maltodextrin | 100 | 100 |
| Sucrose | 102 | 172.8 |
| Cellulose | 50 | 50 |
| Soybean Oil | 25 | 25 |
| Lard | 47 | 177.5 |
| Mineral Mix ¹ | 10 | 10 |
| DiCalcium Phosphate | 13 | 13 |
| Calcium Carbonate | 5.5 | 5.5 |
| Potassium Citrate | 16.5 | 16.5 |
| Vitamin Mix ² | 10 | 10 |
| Choline Bitartrate | 2 | 2 |

Table 3-1. (cont.)

| | Control | High Fat |
|-------------------|----------------|-----------------|
| | g/kg | |
| Ingredient | | |
| Yellow Dye #5 | 0.025 | 0 |
| Red Dye #40 | 0.025 | 0.05 |

¹Mineral mix (AIN-93): (Research Diets, Inc., New Brunswick) Product# S10026 for rodent (Reeves et al., 1993).

²Vitamin mix (AIN-93): (Research Diets, Inc., New Brunswick) Product# V10001 for rodent (Reeves et al., 1993).

Table 3-2. Physiological outcomes of offspring of rat dams fed a HF diet or a C diet¹

| | C/C | HF/C |
|---------------------------------|---------------|----------------|
| Plasma TAG, mmol/L | 0.275 ± 0.027 | 0.468 ± 0.061* |
| Liver TAG, mmol/g ² | 7.86 ± 0.62 | 6.89 ± 0.76 |
| Liver NEFA, mmol/g ² | 0.106 ± 0.008 | 0.094 ± 0.005 |
| Plasma TBARS, µmol/L | 14.9 ± 2.0 | 16.7 ± 3.0 |
| Liver TBARS, µmol/L | 25.5 ± 0.9 | 29.9 ± 1.8* |

¹All values are Mean ± SEM, n = 9-10 rats. Asterisks indicated different from C/C:

*P<0.05.

²Values have been normalized to hepatic protein concentration.

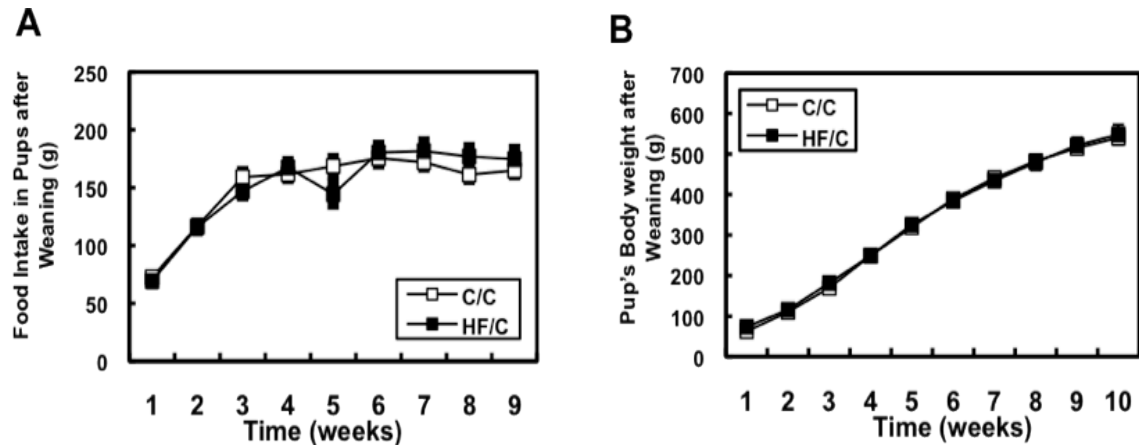
Table 3-3. Expression of hepatic genes in offspring of rat dams fed a HF diet or a C diet^{1,2}

| | C/C | HF/C |
|----------------------------|-------------|---------------|
| <i>Gpx1</i> | 1.25 ± 0.07 | 0.89 ± 0.10* |
| <i>Sod1</i> | 1.47 ± 0.11 | 0.98 ± 0.12** |
| <i>Sod2</i> | 1.15 ± 0.08 | 1.13 ± 0.16 |
| <i>Pon1</i> | 1.37 ± 0.09 | 0.97 ± 0.12* |
| <i>Pon2</i> | 1.16 ± 0.06 | 0.84 ± 0.11* |
| <i>Pon3</i> | 1.33 ± 0.07 | 0.90 ± 0.09** |
| <i>p16^{INK4a}</i> | 0.13 ± 0.03 | 0.60 ± 0.15** |
| <i>Cox2</i> | 0.12 ± 0.02 | 0.63 ± 0.15** |
| <i>p21^{Cip1}</i> | 1.24 ± 0.31 | 0.93 ± 0.28 |

¹All values are means ± SEM, n=9-10. *P<0.05, **P<0.01.

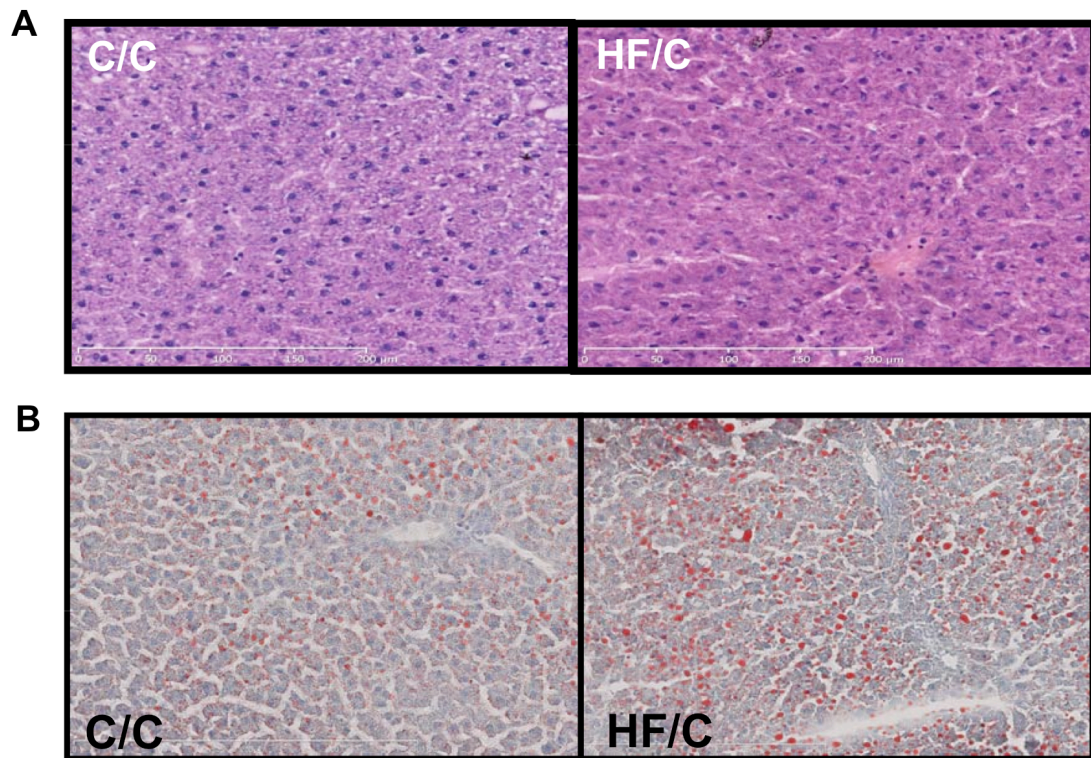
²Expression of mRNA is the ratio to the expression of L7a.

Figure 3-1. Food intake and body weight graphs of HF/C and C/C rat offspring¹



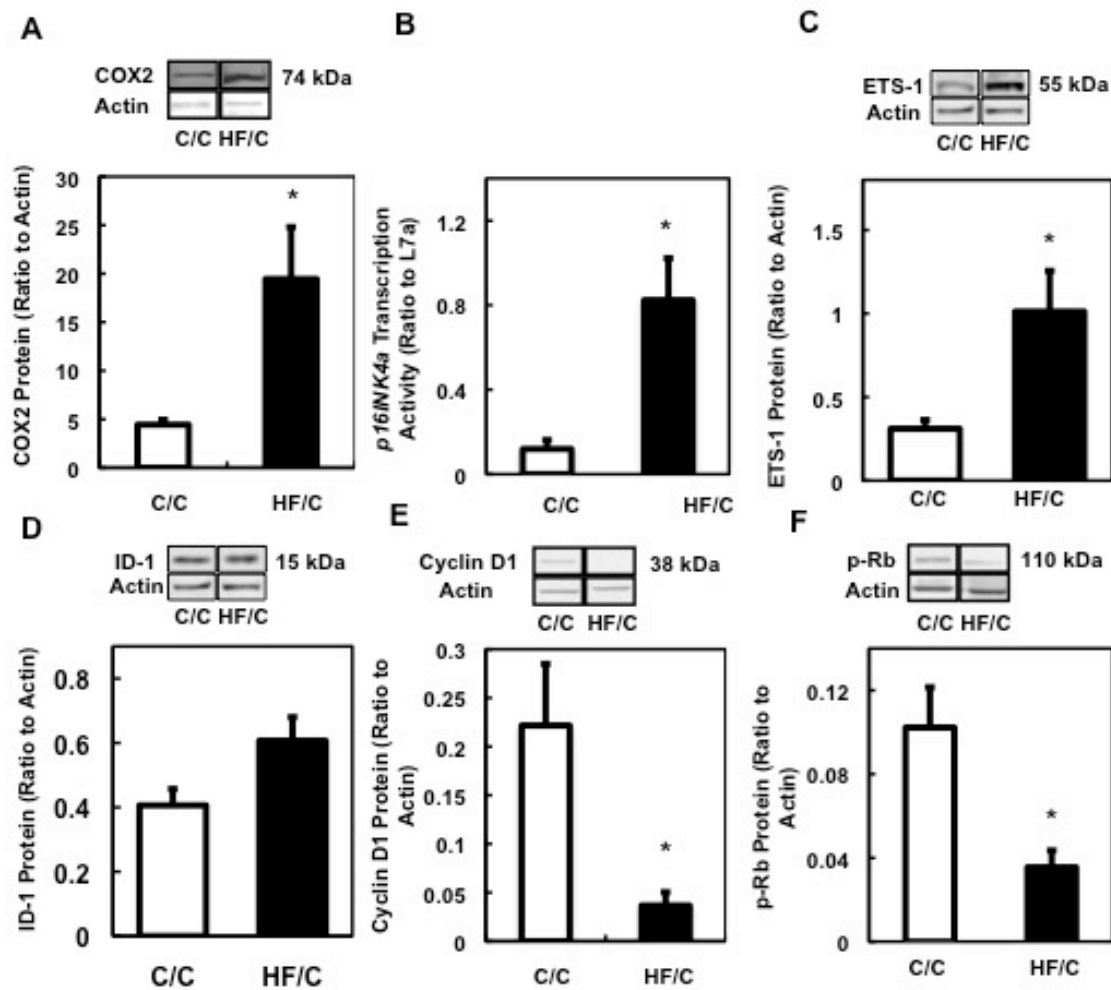
¹Food intake (A) and body weight (B) graphs of HF/C and C/C rat offspring. Values are Means \pm SEM, n=9/10.

Figure 3-2. Histological assays¹



¹Histological assays including H&E stain (A) and ORO stain (B) of livers of offspring of rat dams fed a HF diet or a C diet (20 \times).

Figure 3-3. Hepatic senescence pathway proteins and p16^{INK4a} transcription rate¹



¹Hepatic senescence pathway proteins including COX2 (A), Ets1 (C), Id1(D), Cyclin D1 (E) and phospho-Rb (F) and p16^{INK4a} transcription rate (B) in livers of offspring of rat dams fed HF or C diet during gestation and lactation. Values are means \pm SEM, n=9-10 or 4 (E,F). *Different from C/C, P<0.05.

APPENDIX A

LIST OF PRIMERS FOR REAL-TIME QUANTITATIVE PCR

Appendix A. List of primers for real-time quantitative PCR

| Gene name | Sequence and locations | Ensembl /NCBI ID |
|---|--|------------------|
| <i>p16^{INK4a}</i> (coding region) | Forward (p16+12F), 5'- TGC AGA TAG ACT | |
| | AGC CAG GGC A -3' | ENSRNOT00000006 |
| | Reverse (p16+76R), 5'- CTT CCA GCA GTG | 6011 |
| | CCC GCA -3' | |
| <i>p16^{INK4a}</i> (transcription rate) | Forward (p16+5829F), 5'- CGG GTC ACC GAC | |
| | AGG CAT AA -3' | |
| | Reverse (p16+5889R), 5'- TTG GAC CAC CCA | |
| | TGC TCA CC -3' | |
| <i>p21^{Cip1}</i> (coding region) | Forward (p21+5100F), 5'- CCG AGA ACG GTG | |
| | GAA CTT TGA C -3' | ENSRNOT00000000 |
| | Reverse (p21+5171R), 5'- GAA CAC GCT CCC | 0628 |
| | AGA CGT AGT TG -3' | |
| <i>Gpx1</i> (coding region) | Forward (GPX1+503F), 5'- AGT ACA TCA TTT | |
| | GGT CCC CG -3' | NCBI Gene ID: |
| | Reverse (GPX1+575R), 5'- TCT GGA CCT ACC | 24404 |
| | AGG AAC TTC T -3' | |

Appendix A. (cont.)

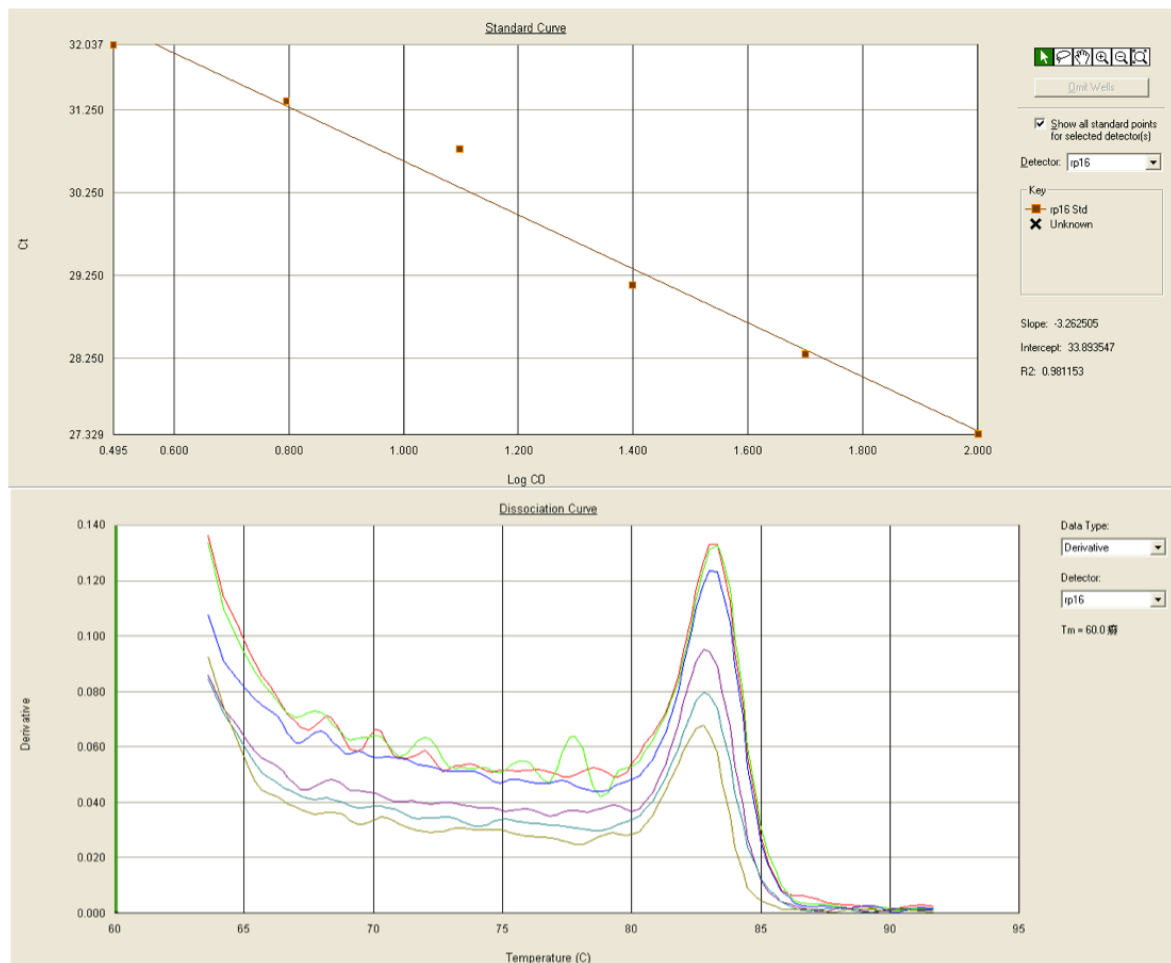
| Gene name | Sequence and locations | Ensembl /NCBI ID |
|-----------------------------|--|------------------|
| | Forward (COX2+729F), 5'- GGT CAT CGG | |
| <i>Cox2</i> (coding region) | TGG AGA GGT GTA TCC -3' | ENSRNOT0000000 |
| | Reverse (COX2+801R), 5'- GAC GTG GGG | 3567 |
| | AGG GTA GAT CAT GTC -3' | |
| | Forward (SOD1+239F), 5'- CAG CGG ATG | |
| <i>Sod1</i> (coding region) | AAG AGA GGC A -3' | ENSRNOT0000000 |
| | Reverse (SOD1+310R), 5'- ACA CAT TGG CCA | 2885 |
| | CAC CGT C -3' | |
| | Forward (SOD2+738F), 5'- GTT TGC AAG AAG | |
| <i>Sod2</i> (coding region) | TGA AGC -3' | ENSRNOT0000002 |
| | Reverse (SOD2+801R), 5'- ACT ACA AAA CAC | 5794 |
| | CCA CCA -3' | |
| | Forward (PON1+1149F), 5'- AGT GAG GCC | |
| <i>Pon1</i> (coding region) | ATC ATT TCA GCC -3' | ENSRNOT0000001 |
| | Reverse (PON1+1221R), 5'- ATT CGT TGG | 1823 |
| | TGA GCG GAG ATC -3' | |
| | Forward (PON2+66F), 5'- TGG CTC TGA GTT | |
| <i>Pon2</i> (coding region) | TGC TAG GCA -3' | ENSRNOT0000003 |
| | Reverse (PON2+137R), 5'- TAA GTC GAC TTC | 6460 |
| | TGA GCG CCA -3' | |

Appendix A. (cont.)

| Gene name | Sequence and locations | Ensembl /NCBI ID |
|-----------------------------|---|------------------|
| | Forward (PON3+243F), 5'- TTT GCA CCA GAC | |
| <i>Pon3</i> (coding region) | AAG CCA G -3' | ENSRNOT00000001 |
| | Reverse (PON3+316R), 5'- TCC AGT GCT TGT 2050 | |
| | GCC TTT G -3' | |
| | Forward (L7a+64F), 5'- GAG GCC AAA AAG | |
| <i>L7a</i> | GTG GTC AAT CC -3' | ENSRNOT00000000 |
| | Reverse (L7a+127R), 5'- CCT GCC CAA TGC 6754 | |
| | CGA AGT TCT -3' | |

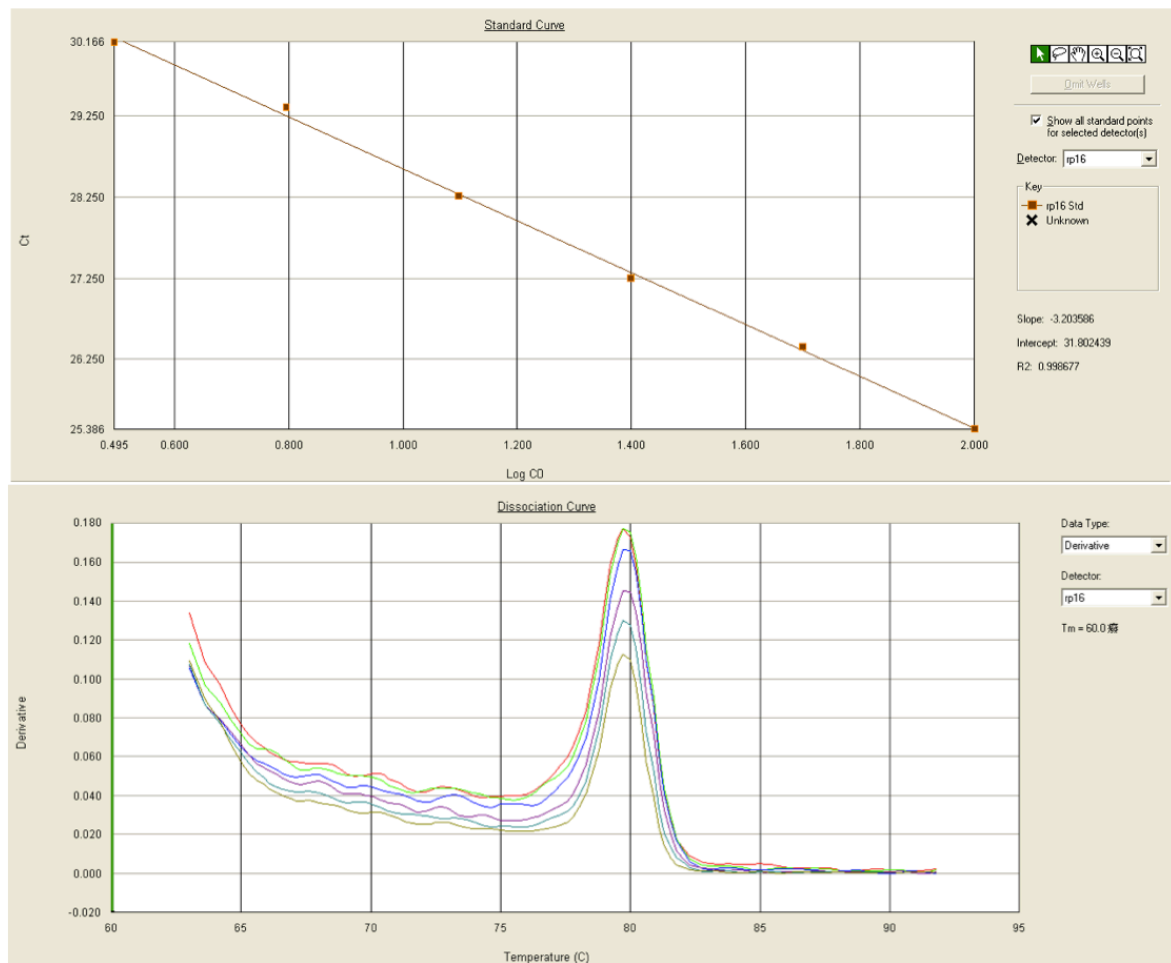
Appendix A. (cont.)

a. Validation for $p16^{INK4a}$ (coding region) primer



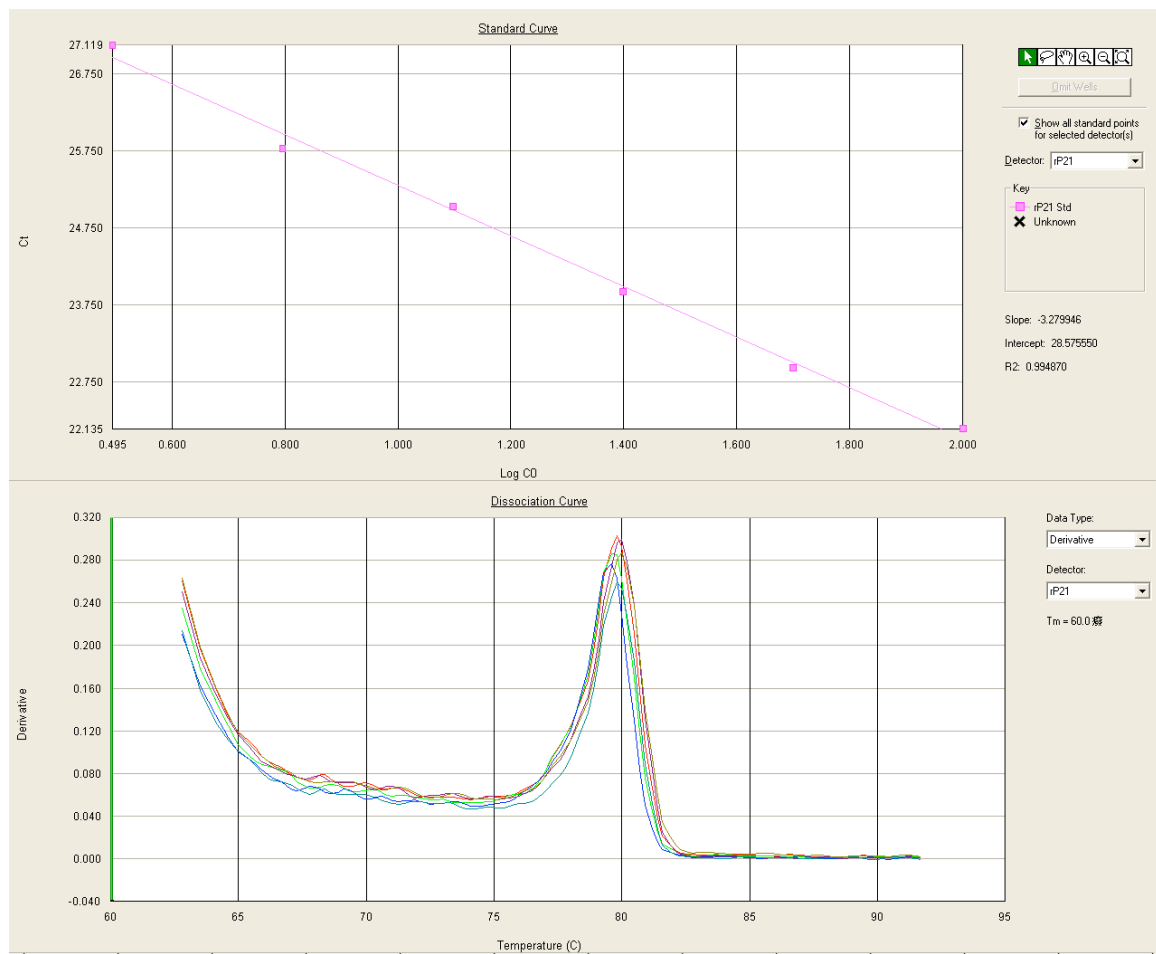
Appendix A. (cont.)

b. Validation for $p16^{INK4a}$ (transcription rate) primer



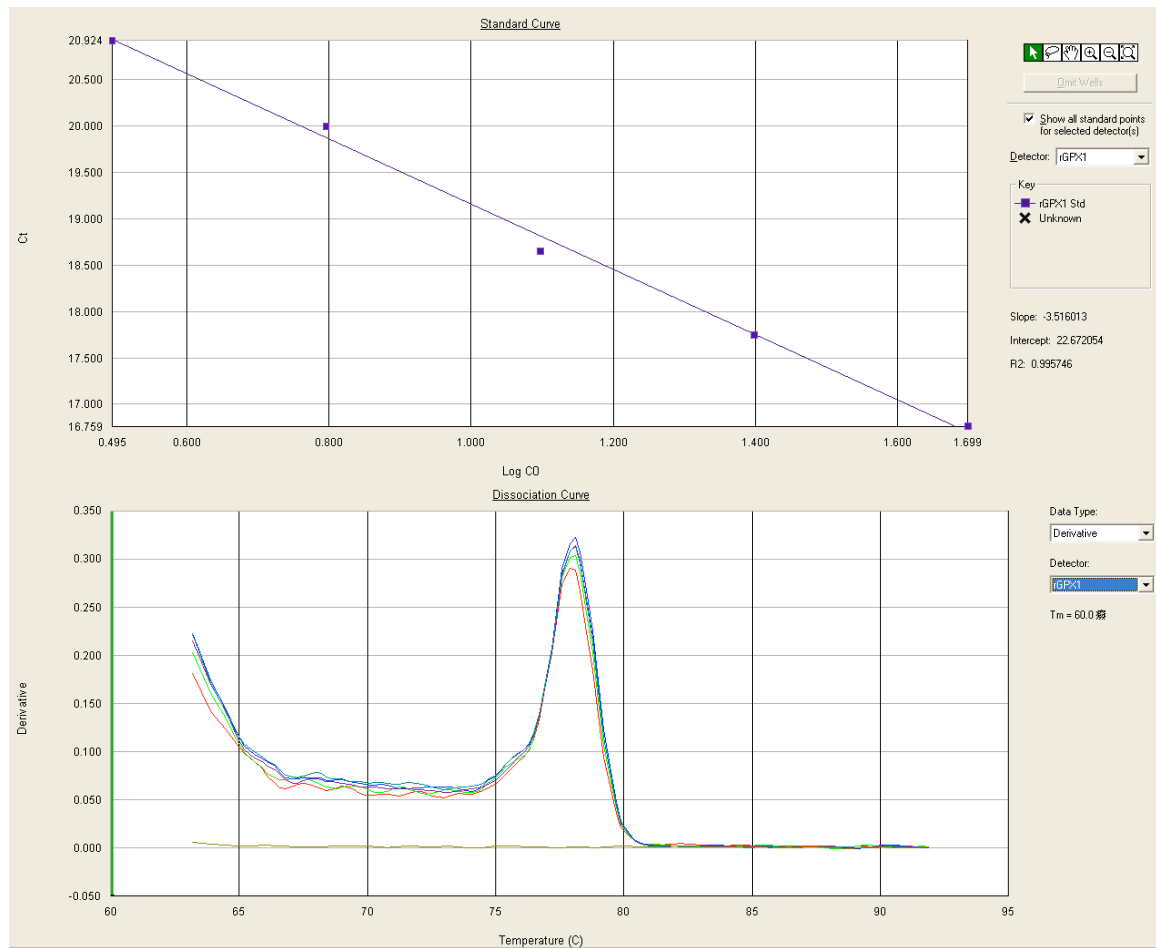
Appendix A. (cont.)

c. Validation for $p21^{Cip1}$ (coding region) primer



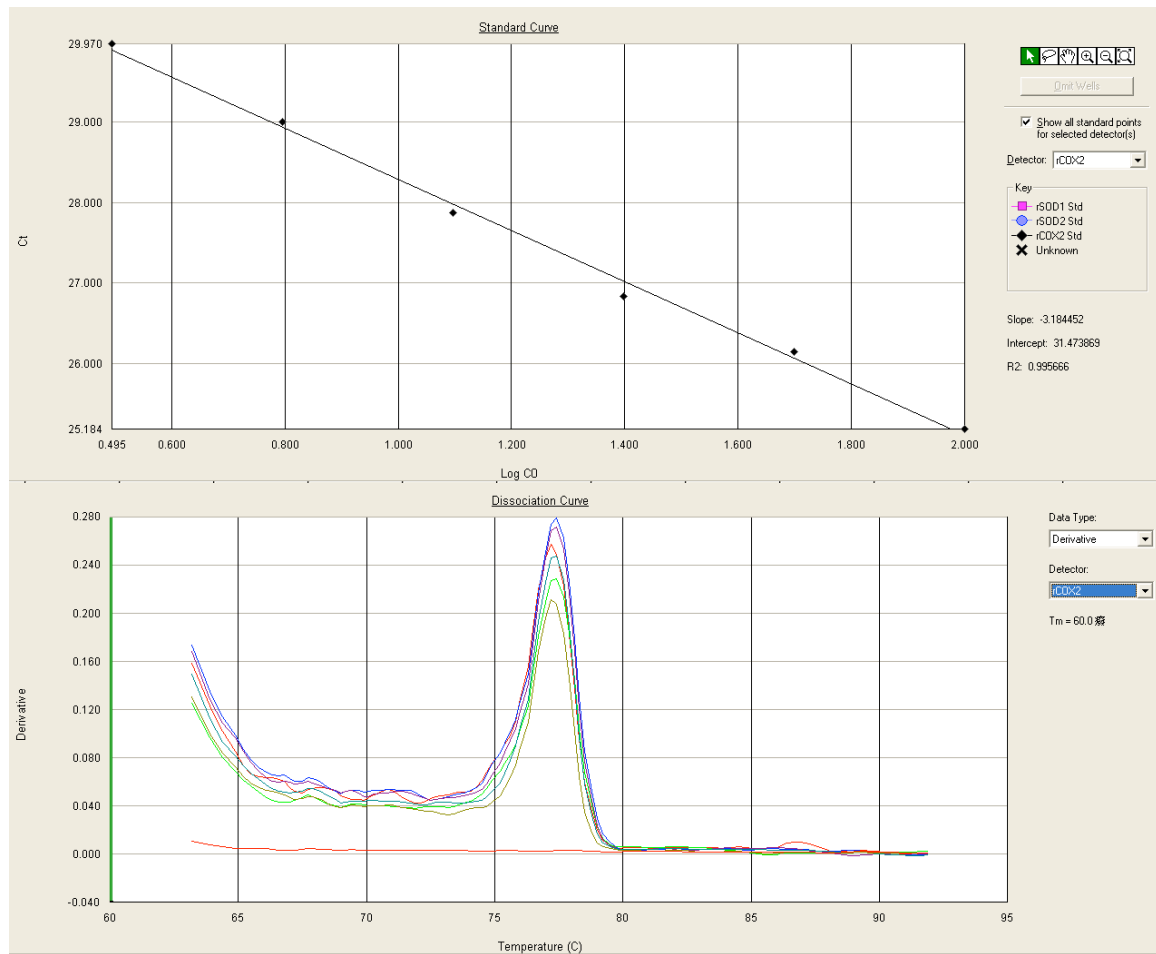
Appendix A. (cont.)

d. Validation for *Gpx1* (coding region) primer



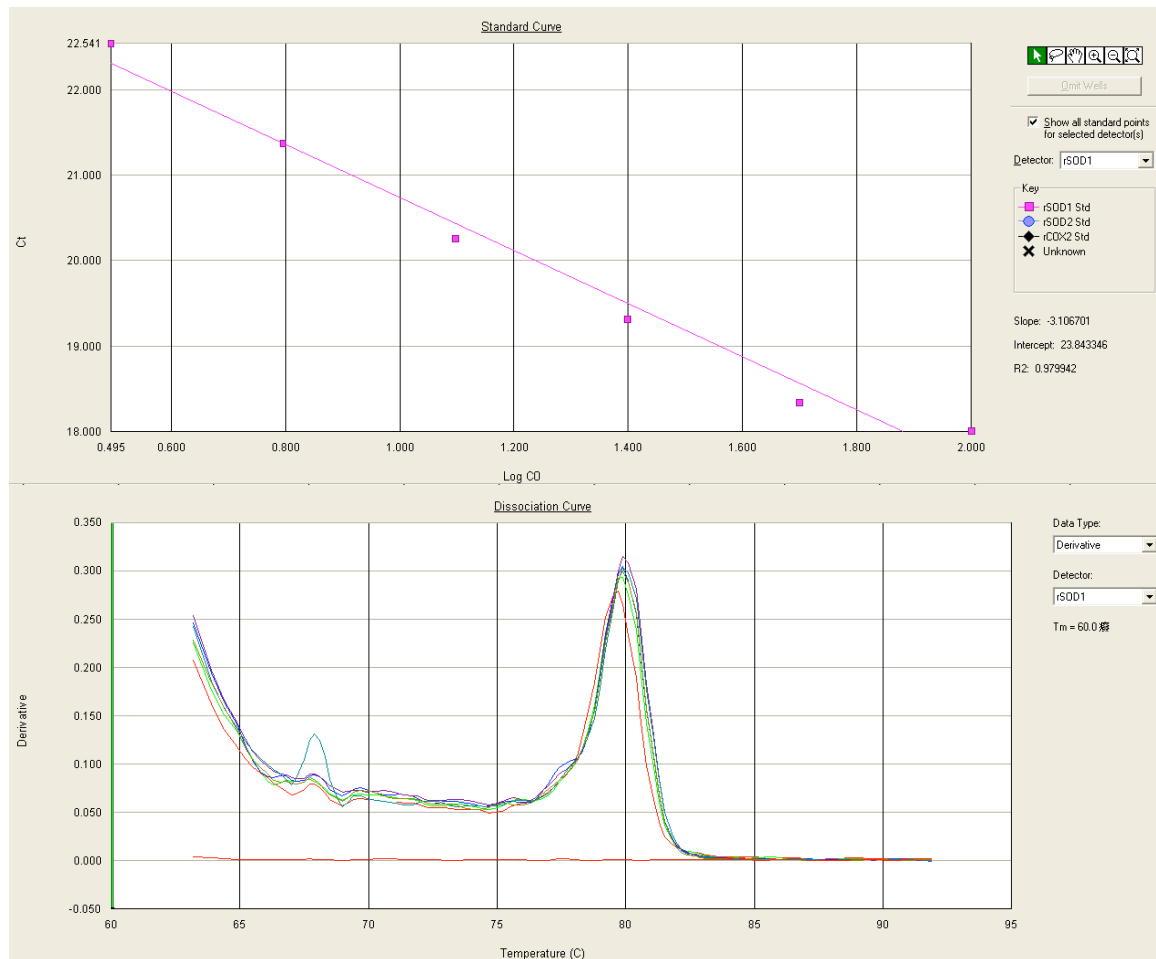
Appendix A. (cont.)

e. Validation for Cox2 (coding region) primer



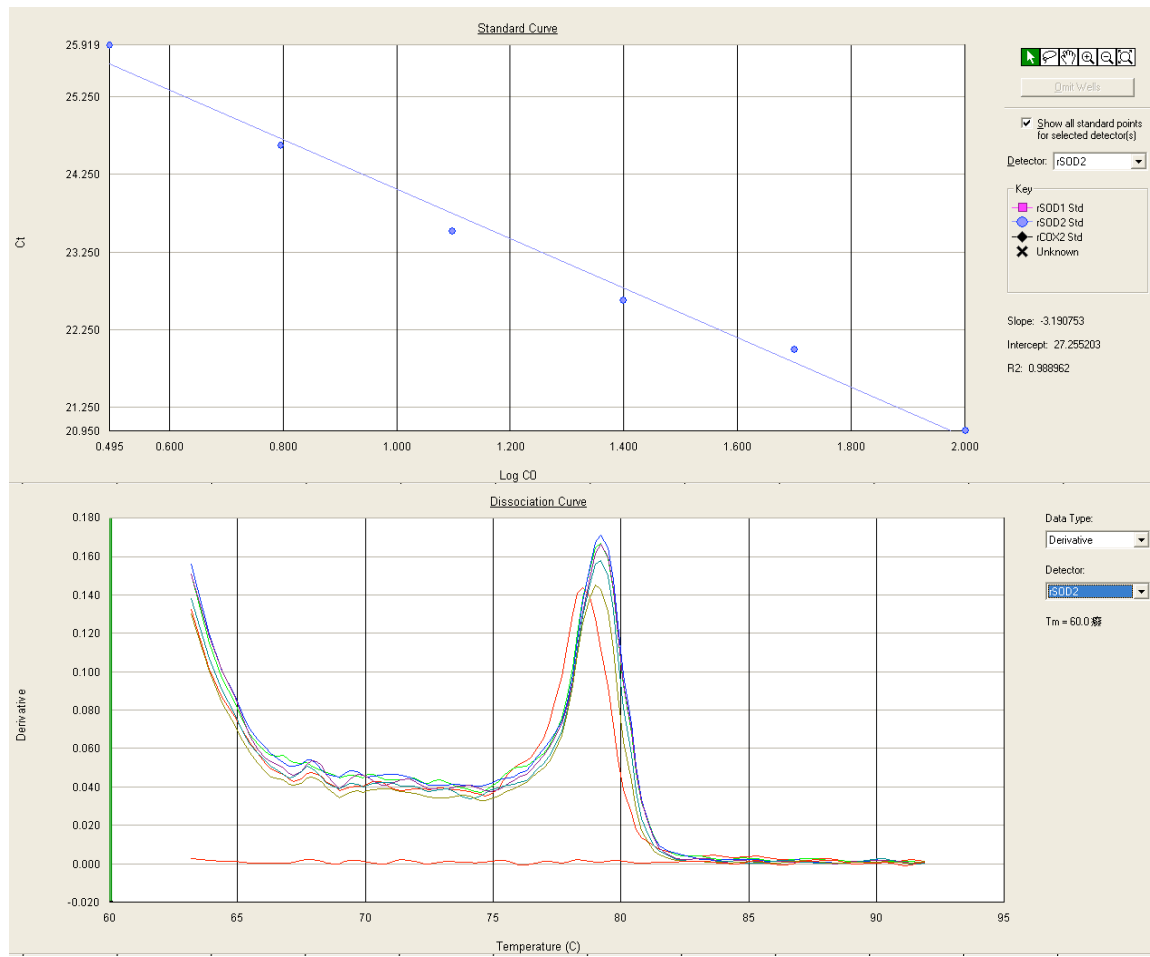
Appendix A. (cont.)

f. Validation for *Sod1* (coding region) primer



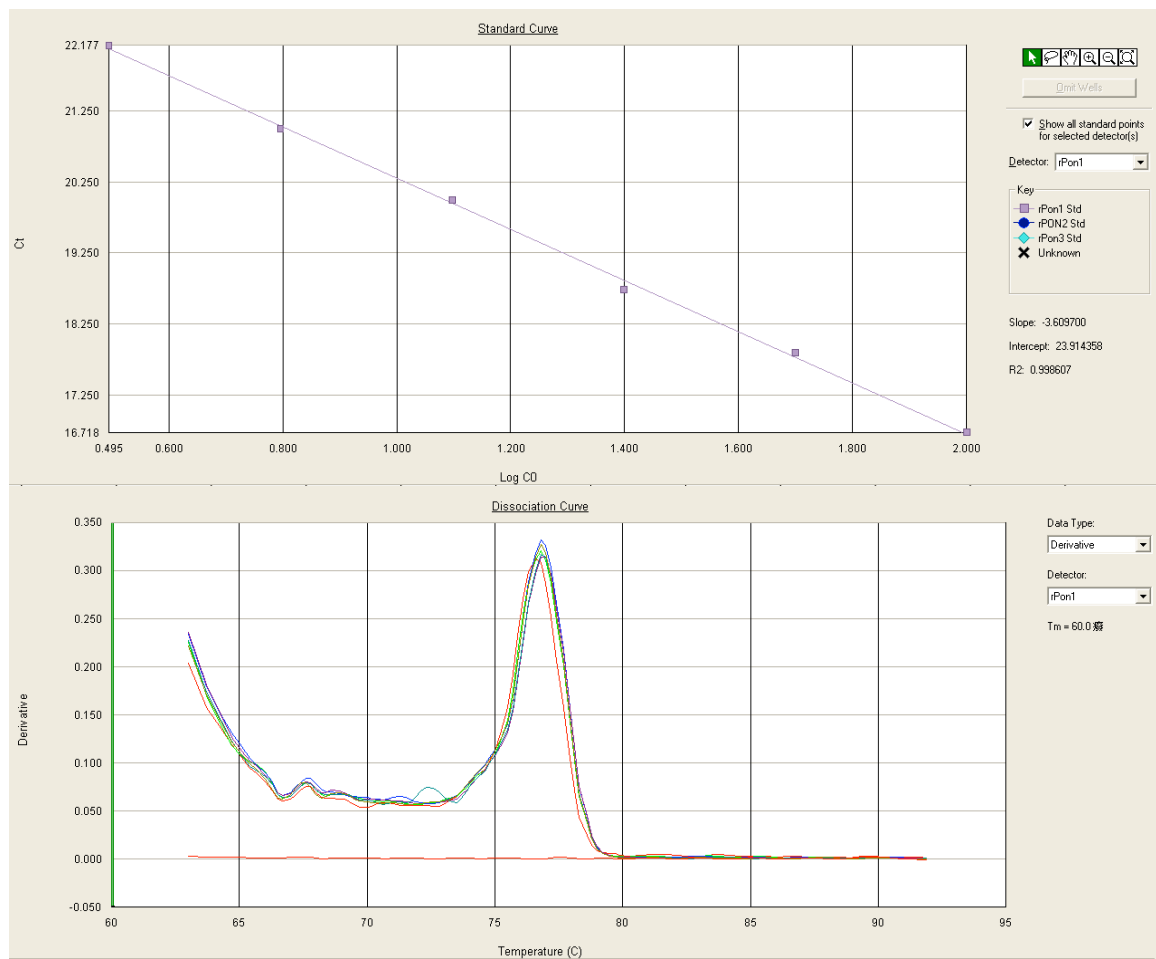
Appendix A. (cont.)

g. Validation for *Sod2* (coding region) primer



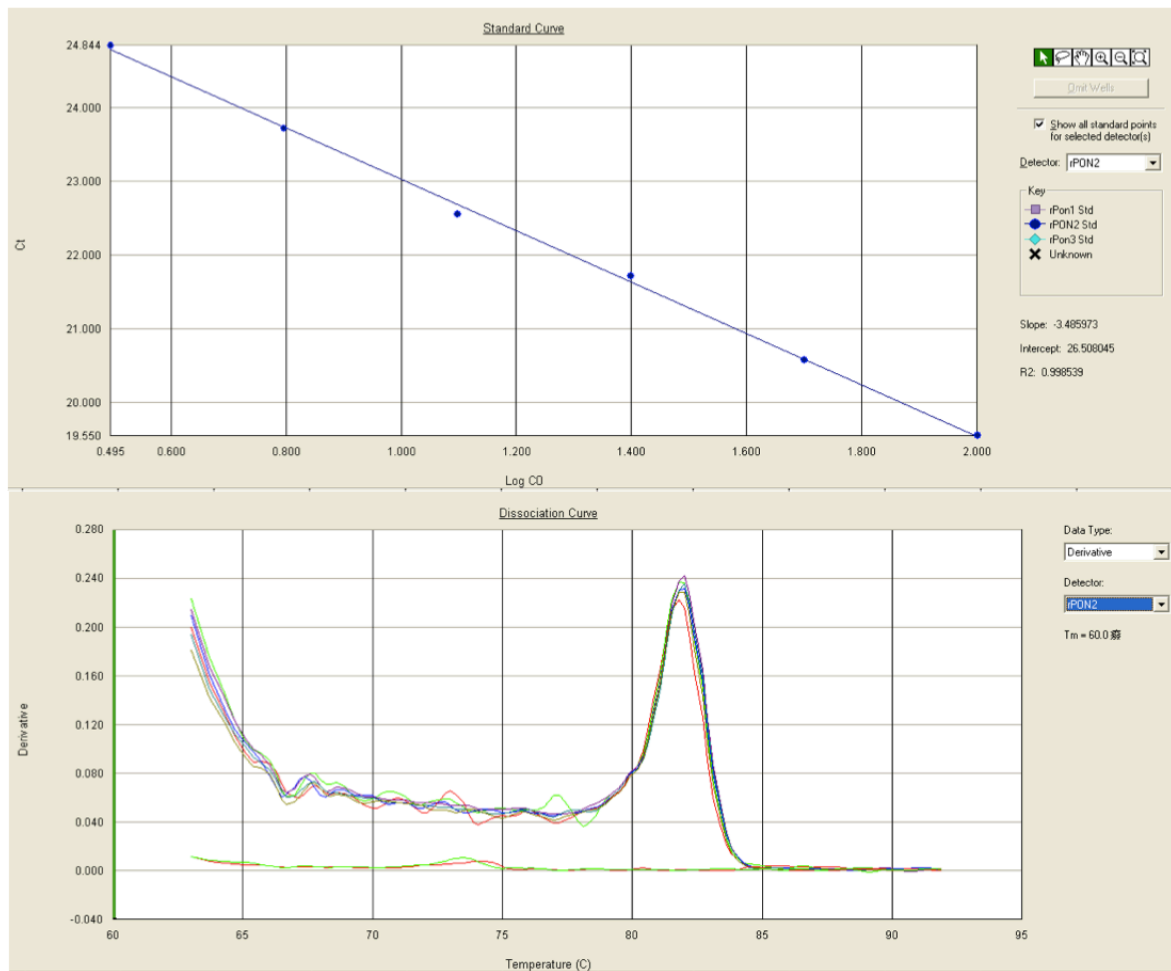
Appendix A. (cont.)

h. Validation for *Pon1* (coding region) primer



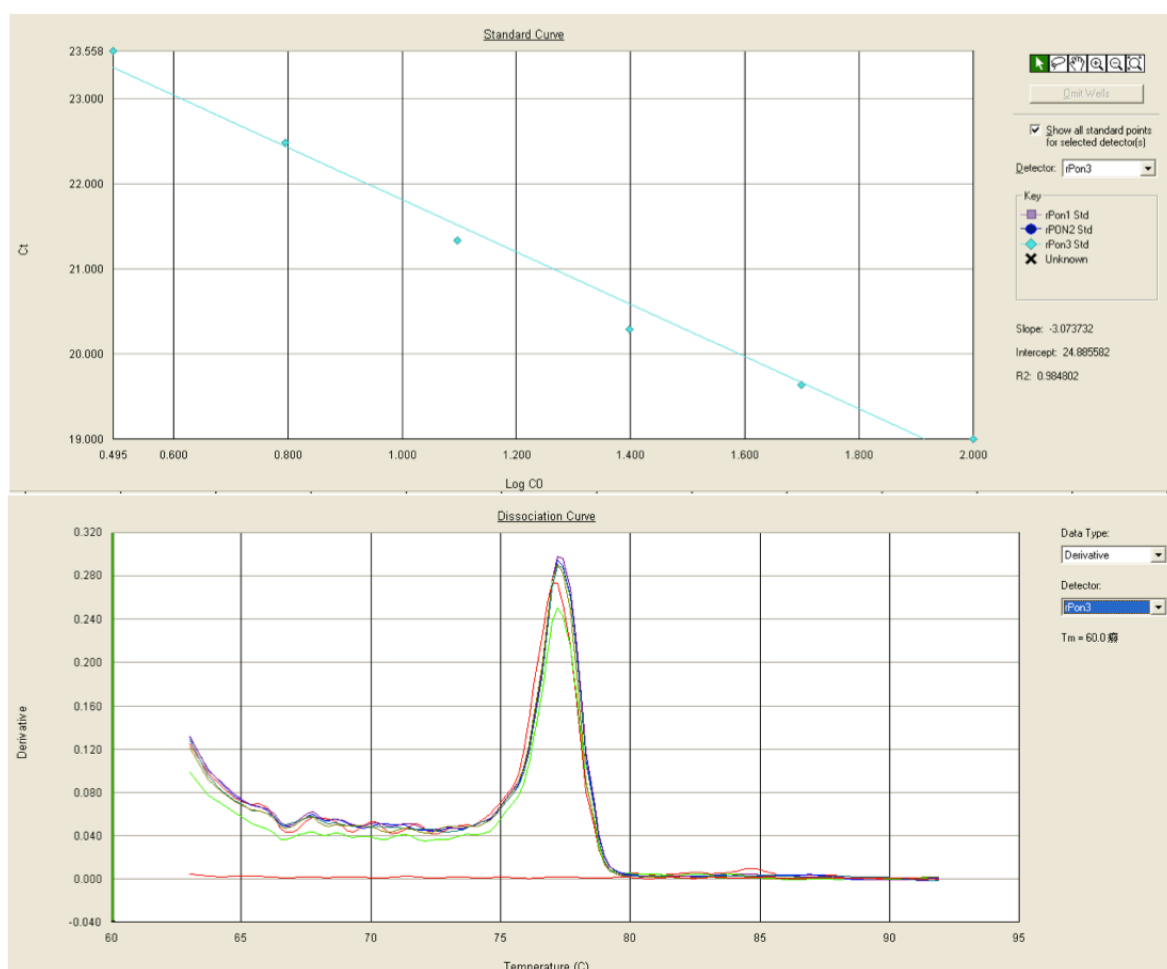
Appendix A. (cont.)

i. Validation for *Pon2* (coding region) primer



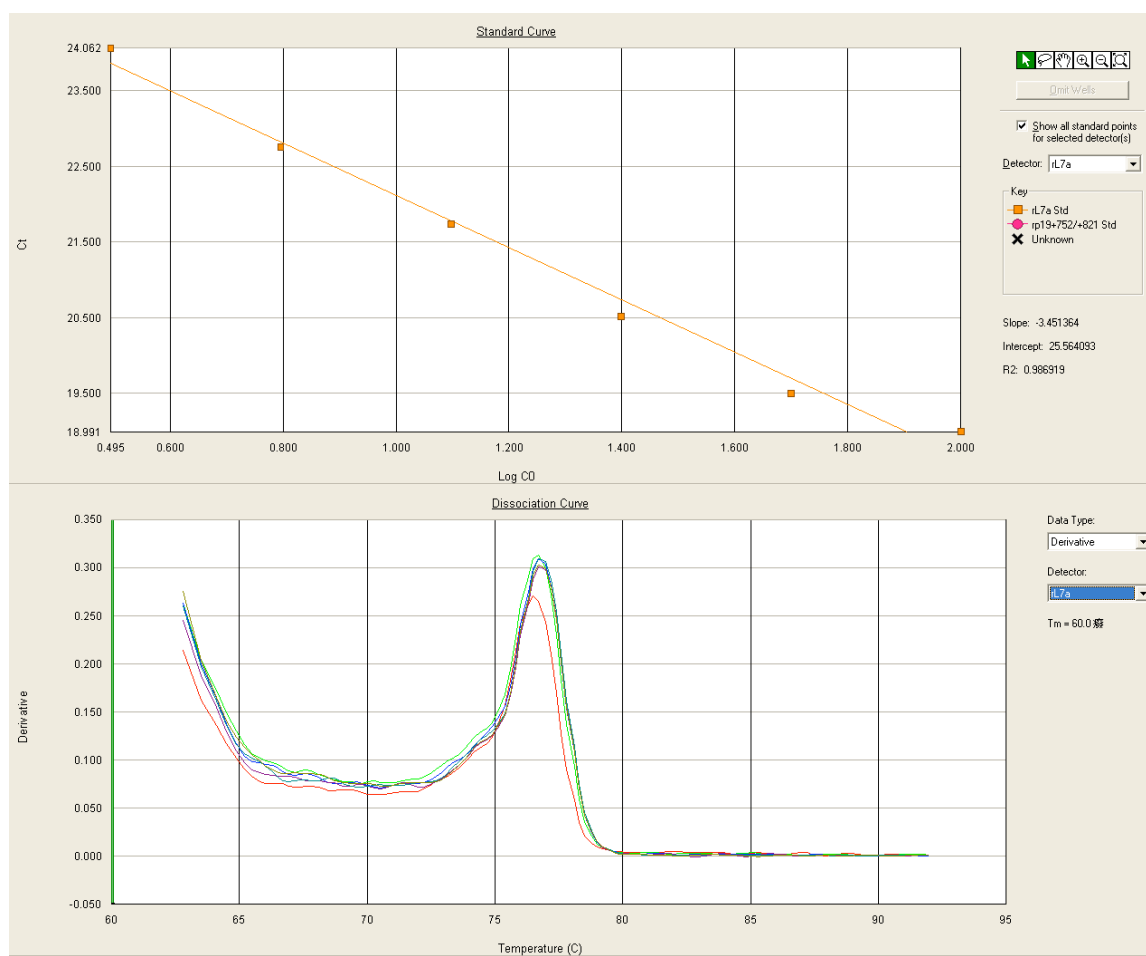
Appendix A. (cont.)

j. Validation for *Pon3* (coding region) primer



Appendix A. (cont.)

k. Validation for *L7a* primer



APPENDIX B

EXPERIMENTAL PROTOCOLS IN THIS STUDY

a. Triacylglyceride (TAG) Measurement

Reagent preparation

1. Triglyceride Reagent
Thermo Infinity (TR22421)
2. A and B standards
Matrix Plus Chemistry Reference Kit (9500 Verichem Lab Inc.)
3. Phosphate Buffer Saline (PBS)
4. 0.9% Saline
5. 1% deoxycholate

Sample preparation

1. Weigh out approximately 100 mg of ground tissue into a 1.5 ml centrifuge tube
2. Add 300 μ L of 0.9% Saline
3. Quick frozen in liquid nitrogen and store in -80°C until measurement
4. Quick thaw the frozen samples in 37°C water bath and mix with 1.2 mL of 0.9% Saline
5. Mix 20 μ L of diluted sample with 20 μ L of 1% deoxycholate.

Standard preparation

1. 245mg/dL – **B Matrix Plus** (no dilution)
2. 200 mg/dL – 81.6 μ L B Matrix Plus + 18.4 μ L 1X PBS (Use for next 3 dilutions)

3. 150 mg/dL – 37.5 μ L of #2 dilution + 12.5 μ L 1X PBS
4. 100 mg/dL – 20 μ L of #2 dilution + 20 μ L 1X PBS
5. 50 mg/dL – 8.8 μ L of #2 dilution + 26.3 μ L 1X PBS
6. 10 mg/dL – **A Matrix Plus** (no dilution)
7. Reference blank – 1X PBS

Performing the assay

1. Thaw samples
2. Vortex each sample, then add 5 μ L of each sample/standard into each well (triplicate)
3. Add 200 μ L of TAG reagent into all wells
4. Incubate 5 min at 37°C
5. Assay at wavelength=562 nm.

b. Non-Esterified Fatty Acid (NEFA) Measurement

Reagent preparation

1. NEFA-HR reagent (Wako Diagnostics, Inc.)
2. 0.9% Saline
3. 1X PBS

Performing the assay

1. Pipet 5 μ L of standard, control, or patient sample into well
2. Add 200 μ L Color Reagent A solution to each well
3. Incubate plate in 37°C water bath for 5 min
4. Use ELISA reader to measure absorbance at 550 nm of each sample
5. Add 100 μ L Color Reagent B Solution, then repeat steps 5 & 6.

6. Calculate results by plotting an linear model x y scatter plot of standards
- c. Thiobarbituric Acid Reactive Substances (TBARS) Assay

Reagent preparation

1. Thiobarbituric Acid (TBA)

Commercial product from *Sigma-Aldrich*, item number: T5500

2. Acetic Acid

Dilute 20 mL glacial acetic acid with 80 mL ddH₂O

3. Sodium Hydroxide (10 mol/L)

4. Malondialdehyde (MDA) Standard

5. SDS solution (10%)

6. To prepare color reagent

Weigh 1060 mg TBA and dissolve with 100 mL diluted acetic acid.

Adjust the pH of this liquid to 3.5 with 10 M NaOH (1.5 mL approximately). Make the total volume to 200 mL with ddH₂O.

Sample preparation

1. Weigh out approximately 25 mg of ground tissue into a 1.5 ml centrifuge tube
2. Add 250 µl of RIPA buffer with protease inhibitors of choice
3. Sonicate for 25 times at power setting at 2 over ice
4. Centrifuge the tube at 1,600 x g for 10 minutes at 4°C. Use the supernatant for analysis. Store supernatant on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for one month
5. Tissue homogenates do not need to be diluted before assaying.

Standard preparation

Dilute 250 μL of the MDA standard with 750 μL of ddH₂O to obtain a stock solution of 125 μM . Take eight 1.5 mL micro-centrifuge tubes and label them A-H. Add the amount of 125 μM MDA stock solution and water to each tube as described in the following table.

| Tube (number) | MDA (μL) | ddH ₂ O (μL) | MDA concentration (μM) |
|---------------|-----------------------|--------------------------------------|-------------------------------------|
| A | 0 | 1000 | 0 |
| B | 5 | 995 | 0.625 |
| C | 10 | 990 | 1.25 |
| D | 20 | 980 | 2.5 |
| E | 40 | 960 | 5 |
| F | 80 | 920 | 10 |
| G | 200 | 800 | 25 |
| H | 400 | 600 | 50 |

Performing the assay

1. Label 15 mL plastic tubes with standard number or sample identification number. Add 100 μL of sample or standard to appropriately labeled 15 mL tube
2. Add 100 μL of SDS solution to tube and swirl to mix
3. Add 4 mL of the color reagent forcefully down side of each tube
4. Cap tubes and bound tubes to keep the tubes upright during boiling
5. Add tubes to vigorously boiling water. Boil tubes for one hour
6. After one hour, immediately remove the tubes and place in ice bath to

stop reaction. Incubate on ice for 10 minutes

7. After 10 minutes, centrifuge the tubes for 10 minutes at 1,600 x g at 4°C
8. Tubes are stable at room temperature for 30 minutes
9. Load 150 µl (in duplicate) from each tube to either the clear plate
10. Read the absorbance at 530-540 nm.

Calculation

1. Calculate the average absorbance of each standard and sample
2. Subtract the absorbance value of the standard A (0 µM) from itself and all other values (both standards and samples). This is the corrected absorbance
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of MDA concentration
4. Calculate the values of MDA for each sample from the standard curve.

$$MDA(\mu M) = \left[\frac{(\text{Corrected absorbance}) - (y - \text{intercept})}{\text{Slope}} \right]$$

d. H&E Staining

Performing the staining

1. 7 µm thickness of frozen liver was sectioned and attached to a polarized glass slide
2. 70% of ethanol was used for 1 min to fix and remove the bedding media
3. dH₂O was use for 30 s until the slides look clear
4. Hematoxylin was use to stain for 1 min
5. Clarifier 2 was used to remove the extra hematoxylin for 30 s
6. Slides were subjected to running water for 30 s

7. Eosin Y was used to stain for 20 s
8. Three times of 100% ethanol were used for 30 s each
9. Three times of Xylene were used for 30 s each
10. Permount mounting media was used to mount the glass slide with the coverlips
11. Scan slides under microscope (Nanozoomer, Hamamatsu).

e. Oil-Red-O Staining

Performing the staining

1. 7 μ m thickness of frozen liver was sectioned and attached to a polarized glass slide
2. Wash section gently in water
3. Rinse in 60% isopropanol
4. Stain for 10 min in working Oil-Red-O
5. Wash very well in 60% isopropanol
6. Counterstain for 20 s in hematoxylin
7. Wash slides in water to blue
8. Mount from water with Permount mounting media
9. Scan slides under microscope (Nanozoomer, Hamamatsu).

f. mRNA Isolation

1. Add 1 mL Tri-reagent to frozen ground sample
2. Add 0.2 mL 1-Bromo-3-Chloropropane (BCP) per 1 mL of Tri-reagent used

3. Vortex for 15 s or mix by inverting several times, incubate at RT for 15 min
4. Spin at 12000x g for 15 min in beer box
5. Transfer aqueous phase to a fresh eppendorf tube
6. Add 500 μ L isopropanol per 1 mL of Tri-reagent used
7. Vortex and incubate at room temperature for 5 min
8. Spin at 12000x g for 10 min in beer box and remove the supernatant
9. Add 1 mL 75% ethanol per 1 mL of Tri-reagent used
10. Vortex and spin at 7500x g for 5 min in beer box
11. Discard ethanol and air dry RNA pellet for 5-10 min at room temperature
12. Add RNase-free H₂O to resolve RNA and make sure the concentration of RNA is between 500-1000 μ g/ μ L
13. Take 48.5 μ L of the resolved RNA and incubate with 0.5 μ L DNase I + 1.5 μ L 0.1 mol/L MgCl₂ at 95°C for 20 min
14. Measure the concentration of resulting RNA and use RNase free water to adjust the concentration to 200 μ g/ μ L.

g. Reverse Transcription (RT)

Prepare the RT master mix use High Capacity cDNA kit from ABI

1. Allow kit components to thaw on ice; mix and spin down every tube before use
2. Calculate the volume of components needed to prepare the 2x RT reaction using the RNA calculator. Mix enough for all the samples to be done.

| Component | Volume (μ L)/rxn | Total needed (rxns) |
|---|--------------------------|-------------------------|
| 10X Reverse Transcription Buffer | 2 | |
| 25X dNTPs (100mM) | 0.8 | |
| 10X random primers | 2 | |
| MultiScribe Reverse Transcriptase, 50U/ μ L | 1 | |
| Nuclease-free H ₂ O | 4.2 | |
| Total per reaction | 10 | |

Prepare the reaction

1. Pipette 10 μ L of 2x RT master mix into each well
2. Pipette 10 μ L RT template into each well. Pipette up and down two times to mix
3. Close the caps
4. Place the strip or plate on ice until you are ready to load the thermal cycler.

Thermal Cycler Program

| | Step 1 | Step 2 | Step 3 | Step 4 |
|-------------|--------|---------|--------|--------|
| Temperature | 25°C | 37°C | 85°C | 4°C |
| Time | 10 min | 120 min | 5 sec | hold |

Storage of the reaction

1. Do not store the reaction in the reaction strip or plate
2. Spin down the strips or plates in the rotor for microplate

3. Transfer the RT product to nuclease-free microfuge tubes and dilute with nuclease free TE (pH 8.0). For cDNA standards, dilute the RT product to 100 μ L final volume. For unknown samples, dilute to 400 μ L final volume
4. Store the reaction at 4°C.

h. Quantitative Real-Time PCR

Prepare master mix for real time PCR

| Component | Volume μ L/ reaction | Total needed |
|------------------------------|-----------------------------|--------------|
| | | |
| 2X SYBR Green PCR Master Mix | 10.0 | |
| Sense primer (5 μ M) | 1.0 | |
| Antisense primer (5 μ M) | 1.0 | |
| H ₂ O | 3 | |
| Total per reaction | 15 | |

Template preparation

1. The final concentration for a given unknown sample after the RT is 5 ng/ μ L, that is 25 ng per well for PCR
2. For Standard curve: do several regular RT and combine together for a good stock to start with. Combine and dilute to 40 ng/ μ L cDNA, that's 20 μ L RT product to 50 μ L final volume. Typically start a standard curve with 100ng, 50ng, 25ng, 12.5ng, 6.25ng per well.

Assemble PCR reaction for each well

1. Use plates or strips designed for real time PCR machine.

2. Pipette 15 μ L of prepared master mix into each well first
3. Pipette 5 μ L template to each well. Mix well by pipetting up and down 2-3 times.

| | Step 1 | Step 2 | Step 3 | Step 4 | Step 5 | Step 6 |
|-------------|--------|--------|--------|--------|--------|--------|
| Temperature | 95°C | 95°C | 60°C | 95°C | 55°C | 95°C |
| Time | 10 min | 15 s | 1 min | 15 s | 1 min | 15 s |

i. Cell Cycle Analysis

1. Weigh out 50 mg ground frozen sample and homogenize with 1 mL PBS containing 5% Fetal Bovine Serum (FBS) until clear
2. Spin at 100 rpm for 2 min and transfer the supernatant to a fresh 15 mL tube
3. Repeat step 3, add 3 mL PBS containing 5% FBS to resulting liquid
4. Wash with PBS containing 5% FBS twice, spin at 800 rpm for 5 min
5. Count cells under microscope and adjust concentration to 1×10^6 cells/mL using PBS with 5% FBS
6. Take out $0.5-1.0 \times 10^6$ cells and collect by centrifuge at 800 rpm for 5 min
7. Use 0.5 mL cold PBS to resuspend the cell pellet and add 0.5 mL cold 100% ethanol slowly to fix
8. Store at -20°C until analysis
9. Before analysis, pellet cells by spinning at 1000 rpm for 5 min in beer box and discard the liquid, let the ethanol evaporate

10. Add 0.5 mL PBS, 5 μ L 100 μ g/mL RNase A and 10 μ L 250 μ g/mL 250 μ g/mL Propidium Iodide
11. Mix well and incubate at room temperature in dark for \geq 20 min
12. Analyze by flow cytometry.