AN INVESTIGATION OF THE ATHEROSCLEROSIS SUSCEPTIBILITY DIFFERENCE BETWEEN TWO STRAINS OF JAPANESE QUAIL BASED ON GENE EXPRESSION PROFILING

by

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ABSTRACT

Atherosclerosis is influenced by a large number of genetic and environmental factors. There is an urgent need for more research exploring the atherogenic process, so that more effective therapeutic approaches can be devised.

Japanese quail (*Coturnix japonica*) form very complex vascular lesions similar to those in humans. The atherosclerosis-susceptible (SUS) and resistant (RES) quail are particularly useful models. They were developed by divergent selection but there has been no genetic characterization of the strains. Genetic characterization of the differences between these two strains would not only improve the utility of this model, but also enable researchers to make more meaningful interpretation of the data generated using this model. This thesis research was conducted primarily to examine the differences between these two strains in terms of the expression of genes related to atherogenesis, but to also gain insights into the role cholesterol metabolism plays in atherosclerosis.

The first study investigated the expression of cholesterol synthesis genes HMGCR, FDFT1, SQLE, DHCR7 and cholesterol transporter genes ABCG5, ABCG8 and APOA1 in the liver. The results showed that HMGCR, FDFT1 and SQLE had significantly higher expression in the RES than in SUS. RES showed significantly higher expression of ABCG8 than did SUS. RES had significantly higher expression of ABCG5 than did SUS on a regular diet. Both strains had significantly increased APOA1 expression when challenged by dietary cholesterol. These results provided evidence to support the hypothesis that the RES were more resistant to atherosclerosis partly because "they metabolized and excreted cholesterol faster than SUS".

In the second study, the cDNA-AFLP technique was used to indentify gene expression differences between the SUS and the RES quail. AFLP reactions using *Mse* and *Eco*R primer combinations on liver tissues found one out of 1150 fragments being differentially expressed in the liver while there were no strain-related differences detected in the spleen. The results support the hypothesis that the divergent selection used to develop these two strains did not drastically altered their genotype. However, it is not possible to draw definitive conclusions because the small number of primer pairs used only covered 9% of the quail genome.

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LIST OF ABBREVIATIONS

ABC	Adenosine triphosphate-binding (ATP) cassette
ABCA1	ABC subfamily A, member 1
ABCG1	ABC subfamily G, member 1
ABCG5	ABC subfamily G, member 5
ABCG8	ABC subfamily G, member 8
AFLP	Amplified fragment length polymorphism polymerase chain reaction
APOA1	Apolipoprotein A1
APOB	Apolipoprotein B
ATP	Adenosine triphosphate
DHCR7	Delta-7-sterol reductase
ER	Endoplasmic reticulum
FDFT1	Farnesyldiphosphate farnesyltransferase / squalene synthase
FDPS	Farnesyl diphosphate synthase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HDL	High density lipoprotein
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase
HMGCS	HMG-CoA synthase
Insig	Insulin-induced gene
LDL	Low density lipoprotein
LXR	Liver X receptor
MVK	Mevalonate kinase
PCR	Polymerase chain reaction
PMVK	Phosphomevalonate kinase
RES	Atherosclerosis-resistant
RXR	Retinoid X receptor
S1P	Site-1 protease
S2P	Site-2 protease
SAGE	Serial analysis of gene expression
Scap	SREBP cleavage activating protein
SEM	Standard error of means
SLOS	Smith-Lemli-Opitz syndrome
SQLE	Squalene epoxidase
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
SUS	Atherosclerosis-susceptible
TG	Triglycerides
Th1	T helper cell 1

VLDL	Very low density lipoprotein
WHO	World health organization
M	Mole
µl	Microliter
µM	Micromole
hr	Hour
min	Minute
ml	Milliliter
mM	Millimole
nmole	Nanomole
pmol	Picomole
sec	Second

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CO-AUTHORSHIP STATEMENT

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Cardiovascular diseases

Cardiovascular diseases, particularly those heart diseases related to atherosclerosis, are the leading cause of morbidity and mortality world-wide according to a World Health Organization (WHO) report (1). WHO estimated that 20 million people will die from cardiovascular disease by 2015 if current trends continue (1). In Canada, cardiovascular disease has been the number one cause of death, even though the proportion has fallen in recent years (2).

The severity of atherosclerosis-related diseases has put the disorder under intensive epidemiological and pathological investigation for a long time. However, therapeutic strategies to remove cholesterol from atherosclerotic plaques have not been totally successful. Even the most commonly used cholesterol lowering drugs, the statins, are suggested to be not as beneficial or safe as originally thought (3-5). The relatively low impact of medicines and non-pharmaceutical interventions on atherosclerosis-related mortality emphasizes the urgent requirement of more research exploring mechanisms of the atherogenic process, so that more effective therapeutic approaches can be devised for the management of this widely prevalent disorder.

Atherosclerosis, caused by formation of multiple plaques in the inner wall of arteries, is a multifocal disease (6-8). One of the hallmark events in atherogenesis is the appearance of lipid-loaded foam cells containing cholesteryl esters in the arterial endothelium (6, 7). In 1989, Steinberg *et al.* (9) suggested that oxidation of LDL is an important event in atherogenesis.

Since then, a body of studies have supported the oxidative modification hypothesis which describes oxidative LDL (oxLDL) as the factor promoting foam cell formation through the scavenger receptor pathways. According to this hypothesis, the accumulated levels of LDL in the extracellular subendothelial space are believed to pose high risk of generating atherogenesis when oxidized to minimally modified LDL (mmLDL) through the action of endothelial cells (10). The modified LDL further induces vascular cells to produce molecules like monocyte chemotactic (MCP-1), colony-stimulating factors and adhesion molecules, which stimulates monocyte recruitment and differentiation to macrophages (9-11). The conversion from mmLDL to oxLDL can then be uptaken by the scavenger receptors. Scavenger receptors are expressed on macrophages, fibroblasts, endothelial cells and smooth muscle cells. The macrophage scavenger receptors recognize the presence of oxLDL and uptake them. The macrophage cells that absorb the cholesterol will become foam cells. Unlike conventional LDL receptors, scavenger receptors are not down-regulated by cholesterol levels (12). Thus, a great amount of cholesterol will be accumulated and such retention will contribute to the formation and progression of atherosclerotic plaques (12). In the absence of oxLDL, monocytes and macrophages (even with the presence of high concentrations of unoxidized LDL) would not be transformed to foam cells (9, 11). The macrophage LDL receptors are down-regulated by the LDL levels (13), and key enzymes in *de novo* cholesterol synthesis pathway will also be down-regulated by high cholesterol levels (13). Such events act to inhibit white blood cells internalizing circulating cholesterol. At the same time, accumulating evidence tends to suggest that cholesterol oxides, rather than native cholesterol, are responsible for the initiation of atheroma (9).

During this internalizing process, complex molecular and cellular changes have been involved. The early stages of fatty streaks eventually develop into fibrous plaques. In the more

advanced stages, fibrous plaques can lead to the occlusion of vessels, and more complex lesions and thrombi may form. During such stages, the atherosclerotic plaque can even lead to myocardial infarction, heart failure and stroke (7, 14-16). Another main problem of advanced plaque begins when the artery starts to enlarge and stretch to compensate for the narrowed vessel, and gradually, aortic swelling develops, aortic aneurysms can occur, and are associated with a great risk of rupture, resulting in many ischemic symptoms (17-19).

1.2. Risk factors for development of atherosclerosis

Atherosclerosis is a multifactorial disease, and various anatomic, physiological, behavioral and environmental factors have been identified as important risk factors that can initiate and promote the atherosclerotic lesion formation (7, 19) (Table 1.1).

1.3. The role of cholesterol in the development of atherosclerosis

Cholesterol is classified as a sterol, and is essential for the proper functioning of the entire body. It is a crucial component of cell membranes (20) and it is a precursor of vitamin D and all steroid hormones (21). However, it is also known to be involved in the pathogenesis of atherosclerosis and cholesterol-related metabolism determines the extent and clinical manifestations of this disease (6, 22, 23). Thus, it is important to understand the relationship between dietary cholesterol absorption, endogenous cholesterol synthesis and biliary cholesterol excretion as these regulate overall cholesterol homeostasis.

Table 1.1 Risk factors for development of atherosclerosis

Factors with a significant genetic component	Environmental factors
↑ Low density lipoprotein (LDL) (24)	Smoking (24)
↑ Very low density lipoprotein (VLDL) (24)	Lack of exercise (24)
↓ High density lipoprotein (HDL) (25)	High fat diet (24)
Systematic inflammation (17)	Infectious agents (26)
Hypertension (27, 28)	
Diabetes Mellitus (24, 29)	
Male gender (27)	
↑ Homocysteine (30)	
↑ Hemostatic factors (6)	
Metabolic syndrome (6)	
Insulin resistance (6)	
Obesity (24)	
Family history (8)	

1.3.1. Dietary cholesterol and cholesterol metabolism

Cholesterol comes from both exogenous and endogenous sources; the former comes from food and comprises about 25% of the cholesterol in the human body, whereas normal adults synthesize cholesterol at a rate approximately 3 times that of consumed cholesterol (1g/day to 0.3g/day) (22). With regard to the *de novo* production of cholesterol, about 80% is synthesized in the liver while about 10% in the intestine and about 5% in skin (31).

To maintain cholesterol homeostasis in the body, excess cholesterol is converted into bile acids, and is excreted in the feces (20, 32). If the cholesterol level is low, *de novo* synthesis

increases in the liver. It has been suggested that *de novo* synthesis acts as the primary controller of cholesterol homeostasis (21, 33).

1.3.2. *De novo* synthesis of cholesterol

Synthesis of cholesterol starts from the condensation of acetyl-CoA and acetoaceyl-CoA, catalyzed by HMG-CoA synthase (HMGCS). The product, hydroxymethylglutaryl-coenzyme A (HMG-CoA), is then catalyzed by HMG-CoA reductase (HMGCR), which is a transmembrane protein that catalyzes an early step in the mevalonate pathway (Figure 1.1) (4). The catalyzed reaction can produce both sterol (cholesterol and steroid hormone) and non-sterol metabolites; it is considered to be the rate-limiting enzyme for cholesterol biosynthesis and is a target for certain cholesterol-lowering drugs, e.g., the statins (3-5, 34-36).

This process will eventually lead to the key product, mevalonate. Mevalonate is then phosphorylated by ATP, yielding the phosphate derivative. Phosphomevalonate decarboxylase (PMVK) catalyzes ATP-dependent decarboxylation to produce 5-diphosphomevalonate, which is subsequently converted into isopentenyl diphosphate. Isopentenyl diphosphate is then converted into geranyl pyrophosphate by farnesyl diphosphate synthase (FDPS). Condensation with another isopentenyl pyrophosphate yields farnesyl diphosphate, catalyzed by squalene synthase (FDFT1) and leads to the formation of squalene. FDFT1 catalyzes the first step in the biosynthesis of sterols at the final branch point of the mevalonate pathway (Figure 1.1). Unlike HMGCR, the FDFT1 enzyme is only involved in the synthesis pathway of sterol and not in the non-sterol branch (37).

Squalene expoxidase (SQLE) catalyzes oxidation of squalene to form 2,3-oxidosqualene. After that, a series of reactions produces lanosterol. Finally, 19 reactions are involved to convert

lanosterol to cholesterol (32, 38). SQLE is one of the late-stage cholesterol biosynthesis enzymes; it catalyzes the first oxygenation step in the cholesterol biosynthetic pathway, converting squalene to 2,3-oxidosqualene. Like FDFT1, SQLE is also involved in the late branch of cholesterol biosynthesis (Figure 1.1) (36).

7-dehydrocholesterol reductase (DHCR7) is responsible for the final step in the production of cholesterol, converting 7-dehydrocholesterol to cholesterol (39) (Figure 1.1). The biochemical and pathological research about Smith-Lemli-Opitz syndrome (SLOS) showed that the deficiency of this enzyme causes severe dysfunction in several organs and systems (39).

1.3.2.1. Cholesterol synthesis feedback

The earliest cholesterol feedback research started in 1933 when Rudolf Schoenmier did an experiment measuring cholesterol balance in mice. He demonstrated that when a diet is deficient in cholesterol, the animal will produce endogenous cholesterol, and "with the administration of moderate amounts of cholesterol, synthesis decreases" (40). During the 1950's to 1960's, scientists demonstrated that the endogenous pathway occurs in the liver and the liver is subjected to negative feedback regulation with cholesterol feeding. Since then, a series of studies using cultured cells and research on familial hyercholesterolemia, a genetic disease characterized by elevated levels of cholesterol-rich LDL in plasma and premature myocardial infarction, led to the elucidation of the LDL receptor pathway and the negative relationship between "liberated" cholesterol and HMGCR level (35, 39). More recently, cholesterol feedback has been characterized at the molecular level. The discovery of transcript factor SREBP and its sterol-sensing partner SREBP cleavage activating protein (Scap) is the new milestone (4, 35, 39,40).

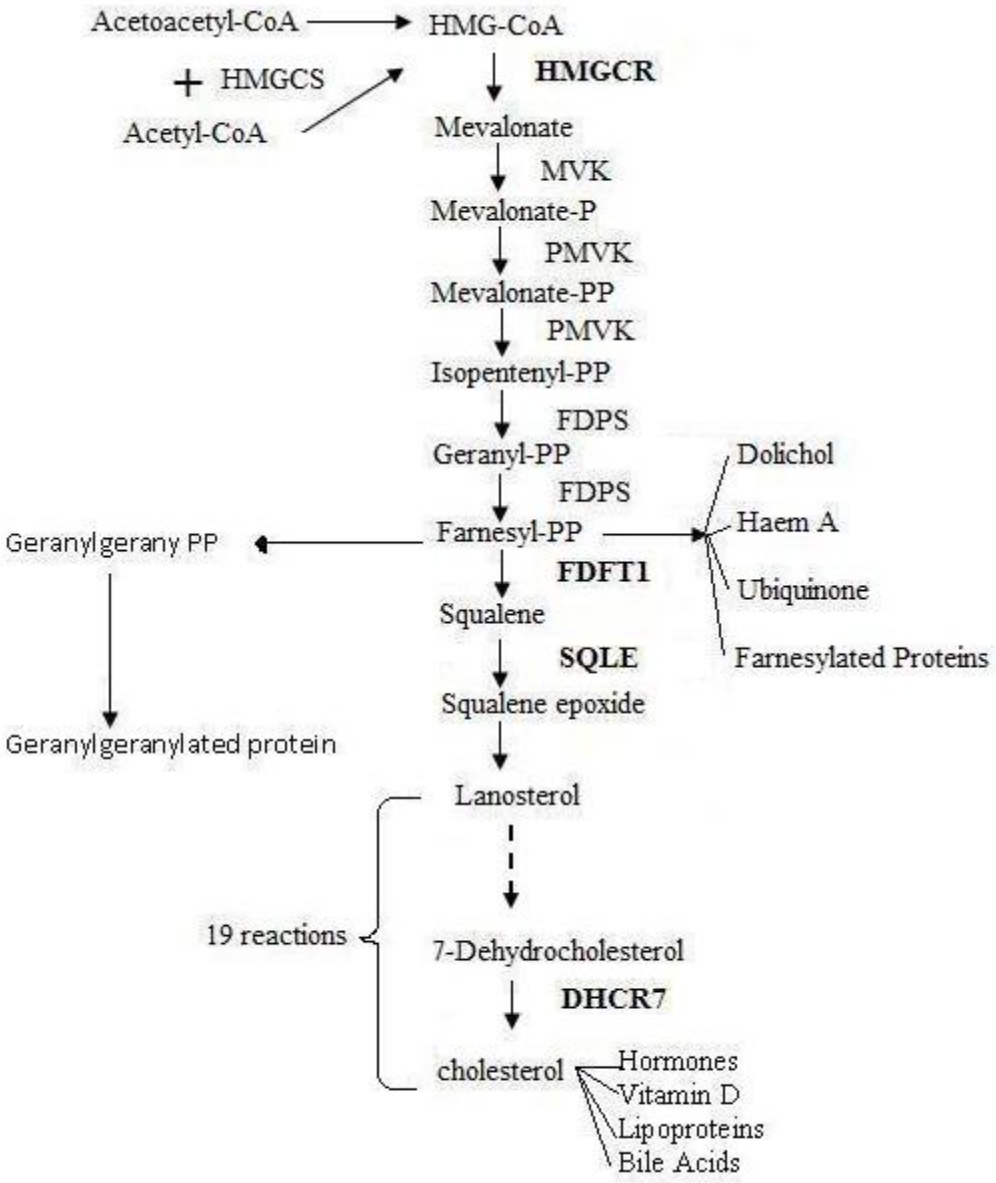


Figure 1.1 *De novo* cholesterol biosynthesis pathway (The Mevalonate pathway). HMGCS, HMG-CoA synthase; HMGCR, HMG-CoA reductase; MVK, mevalonate kinase ; PMVK, phosphomevalonate kinase; FDPS, farnesylpyrophosphate synthetase; FDFT1, squalene synthase; SQLE, squalene epoxidase; DHCR7, delta-7-sterol reductase; P, phosphate; PP, pyrophosphate.

1.3.2.2. SREBP pathway- HMGCR, FDFT1, SQLE gene regulation

In mammals, there are three major SREBP isoforms, SREBP-1a, SREBP-1c and SREBP-2 (41). It has been shown that SREBP-1c and SREBP-2 are predominantly present in

the liver. SREBP-2 is the one mainly affecting cholesterol homeostasis while "SREBP-1c favors the fatty acid biosynthetic pathway" (41). It has been already elucidated that the mevalonate pathway genes activated by SREBP-2 include HMGCS, HMGCR, FDPS, FDFT1, and SQLE (4, 13, 42, 43). Two isoforms have been indentified in chickens, SREBP-1 and SREBP-2, which are functionally similar to human SREBPs (44-46).

In general, the activation of the different isoforms of SREBP is regulated by cholesterol via a common mechanism (47, 48). SREBP binds to the endoplasmic reticulum (ER) membrane or the nuclear envelope, combining with Scap to form a complex that functions as a sterol sensor. When cellular cholesterol levels are high, the protein insulin-induced gene-1 (Insig-1) and Insig-2 bind to Scap and prevent movement of the Scap-SREBP complex from the ER to the Golgi system, inhibiting transcription of responsive genes (33, 41, 49, 50). Experiments have shown that in cells depleted of cholesterol, Insig-1 and Insig-2 allow activation of the Scap-SREBP complex and its translocation to the Golgi apparatus, where SREBP is cleaved at two sites. Cleavage releases the amino-terminal basic helix-loop-helix domain, which enters the nucleus, functioning as a transcription factor that recognizes non-palindromic decanucleotide sequences of DNA called sterol regulatory elements (SREs) (33, 47, 48). Binding of the protein domain of SREBP to an SRE DNA sequence promotes transcription of the target genes. As listed in the previous section, the target genes include HMGCR, HMGCS, FDFT1, FDPS and SQLE mRNAs.

1.3.3. Cholesterol transport and regulation of secretion

Cholesterol homeostasis in the body is primarily controlled through *de novo* synthesis but it is also regulated in part by dietary cholesterol. Total cholesterol, both dietary and synthetic,

is finally utilized in the formation of membranes and in the synthesis of bile acids and steroid hormones.

1.3.3.1. Plasma lipoprotein transport

Dietary cholesterol is absorbed by the intestine. Because cholesterol is not soluble in water, it must be transported through the circulation in lipoproteins (51, 52). Chylomicrons are the largest and least dense lipoproteins, which consist predominantly of triacylglycerides and cholesterol. Chylomicrons have a relatively short residence time in the plasma, due to the lipoprotein lipase activity to hydrolyse triacylglycerides to free fatty acids and glycerol in adipose cells (53). The free fatty acids diffuse into the adipose cells where they are re-synthesized into triacylglycerides for storage until needed. When triacylglycerides are required, they are transported as free fatty acids through the action of cellular lipases. In the post-absorption stage or under fasting conditions, the majority of plasma lipids exist in VLDL, LDL and HDL cholesterol (21, 32, 54). Those lipoproteins are synthesized mostly by the liver, and some by the intestine; there are specific classes of apolipoproteins which dictate the metabolic pathway of transporting the lipids (21, 32, 54). Chylomicrons contain apolipoprotein A1, A2, C and B48 (53). The VLDL particles synthesized by the liver contain apolipoprotein B100, C and E. Apolipoprotein B100 is necessary for VLDL secretion and LDL recognition; it is also the single apolipoprotein in LDL particles. HDL particles contain apolipoproteins A1, A2, A4, C and E (51, 53). However, different species show variation of apolipoprotein species and amount. For example, birds generally lack apolipoprotein E (55); rat HDL contains all five apolipoproteins (56), whereas human lacks apolipoprotein E and C (53).

When triacylglycerides have been removed from the chylomicrons by lipases, the remaining unesterified cholesterol, phospholipids and apolipoproteins are removed from the

circulation by the liver (51). The transport of the remnants from the plasma is mediated by receptors on the surface of hepatocytes. The formation of VLDL by the liver is dependent on the synthesis or availability of triacylglycerides in hepatocytes. The VLDL particles are hydrolysed by lipoprotein lipase of adipose and muscle tissue to produce intermediate density lipoproteins (IDL). IDL remnants are either taken up by LDL receptors of liver or directly synthesized into LDL. LDL particles are taken up by hepatocytes via the LDL receptor pathway and normally down-regulated by cholesterol levels (7, 51, 57). As mentioned in section 1.1, a high cholesterol or lipid-contained diet can result in decreased numbers of LDL receptors, leading to hypercholesterolemia. In addition, when plasma LDLs invade the endothelium and become oxidized, they pose a great risk for atherosclerotic cardiovascular disease progressing. The process is worsened if there are insufficient HDLs, the lipoprotein particles that remove cholesterol from tissues and carry it back to the liver, which is called reverse cholesterol transport (21, 32, 54).

HDL is the smallest lipoprotein particle, and HDL-associated cholesterol is sometimes called "good" cholesterol because of its protective role in atherosclerotic development (58). During reverse cholesterol transport, HDL is responsible for carrying cholesterol from peripheral tissues back to liver. The cholesterol delivered to the liver can then be excreted into bile and eventually into intestine and feces (58, 59). It has been postulated that HDL is able to transport cholesterol from lipid-loaded macrophages of atherosclerotic artery walls to the liver for excretion, and this is considered as protective towards atherosclerosis (58-60).

1.3.3.1.1. Apolipoprotein A1 (APOA1)

APOA1 is the major protein component of HDL, which constitutes approximately 70% of HDL protein and is present on all HDL particles. APOA1 is synthesized in both intestine and the liver, while their contribution to plasma APOA1 is unknown.

The gene encoding APOA1 in common quail is 802 bp long. The coding sequence (CDS) of common quail has 94.5% homology with chicken and 63.8% with human (57). Because APOA1 (contained in HDL) can transport cholesterol to excrete from liver during reverse cholesterol transport process (59, 61, 62), APOA1 is considered to have a protective role in cardiovascular disease. Studies have shown that over-expression of the APOA1 gene in liver significantly increases HDL level and inhibits the progression and even can cause regression of atherosclerosis in mice (62).

1.3.3.2. Cholesterol excretion transporter

ABCG5 and ABCG8 are members of the adenosine triphosphate (ATP)-binding cassette (ABC) transporter family G, and both are expressed at high levels in liver and at lower levels in the small intestine and colon. The ABCG5 and ABCG8 transporters are involved in the control of total body sterol homeostasis and also in the regulation of lipid trafficking in macrophages, hepatocytes, and intestinal mucosa cells (63). The genes encoding these two transporters are arrayed in a head-to head manner, and when they are expressed, the two proteins form a heterodimer (63).

In 2000, Hobbs' group first found that in human's mutation in either of the two transporter genes leads to sitosterolemia and sterol accumulation (64, 65). Subsequent human and animal studies have elucidated the function of ABCG5 and ABCG8. It has been shown that

over-expression of human ABCG5 and ABCG8 genes in mice led to a supersaturation of cholesterol in bile, and these mice also had lower cholesterol absorption and lower plasma HDL (66); while knockout of either ABCG5 or ABCG8 led to a failure to secrete sterols and similar symptoms to those in sitosterolemia (67, 68). Deletion of ABCG8 in mice significantly increased the mass of intestinal cholesterol and sitostanol absorption, and reduced but did not eliminate hepatic secretion of cholesterol (69). A recent study showed that over-expression of ABCG5 and ABCG8 in the liver of mice reduces hepatic cholesterol and atherogenesis, promotes *de novo* hepatic cholesterol synthesis, produces lower levels of apolipoprotein B (APOB) (70). Such data showed that: 1). The ABCG5 and ABCG8 proteins are crucial for secreting cholesterol into the biliary lumen and regulating plant sterols as well, and 2). ABCG5 and ABCG8 are likely to function as obligate heterodimers.

It has been elucidated that ABCG5 and ABCG8 genes can be up-regulated through the liver X receptor (LXR) pathway (71, 72). It has also been shown that the knockout of ABCG5 leads to a sterol-poor bile, but sterol excretion seemed to be functioning normally upon LXR activation, e.g. by sterol feeding (71, 73). Similar experiments showed that the expression of ABCG5 and ABCG8 were increased in cholesterol-fed mice and it was concluded that ABCG5 and ABCG8 are regulated by the RXR/LXR heterodimer (RXR: retinoid X receptor) (74).

1.3.4. Cholesterol and plaque formation

As previously mentioned, atherosclerosis is initiated by cholesterol retention, oxidation, and modification. This occurs in the arterial wall, provokes chronic inflammation and gradually leads to thrombosis (7, 16, 27).

Initiating events in the development of a fatty streak lesion begin with LDL oxidative modification. Monocytes attach to endothelial cells and express cell adhesion molecules induced by modified LDL and inflammatory cytokines. Adherent monocytes migrate into the subendothelial space and differentiate into macrophages. Uptake of oxidative LDLs via scavenger receptors leads to macrophage foam cell formation and accumulation of extracellular cholesterol. The lesion then progresses wherein foam cells, T helper cell 1 (Th1) and T helper cell 2 (Th2) establish a chronic inflammatory reaction. Cytokines secreted by lymphocytes and macrophages exert both pro- and anti-atherogenic effects on each of the cellular elements of the vessel wall. Smooth muscle cells migrate from the medial portion of the arterial wall, proliferate and secrete extracellular matrix proteins that form a fibrous plaque. During the final stage, plaque rupture can expose the lipids and tissue factors to blood components, initiating coagulation and platelet adherence. The platelets and fibrin produce a blood clot, which directly contributes to thrombosis. The obstruction of the coronary artery by a thrombus can cause the stroke or myocardial infarction (6, 7, 16, 17). During the whole process, the step of oxidation of LDL and the inflammatory reactions are extremely crucial to the plaque formation and progression (7, 17, 27).

1.4. Genetic factors in atherosclerosis

Among the risk factors, a family history is a significant independent factor for the development of atherosclerosis (6, 8, 75). Before the 1970s, scientists already suggested heredity as a contributing factor that predisposes to the development of atherosclerosis (76, 77). Excluding environmental risk factors, various population groups and specific ethnic groups showed obvious variations in mortality and distinct cardiovascular disease rates. Marked sex

differences in cardiovascular disease mortality also indicate the role of heredity in the development of the disease (8, 78). At the same time, inheritance patterns in most families with a high incidence of coronary heart disease suggest that atherosclerosis susceptibility is not transmitted as a simple Mendelian trait (8).

1.4.1. Gene expression in aorta and spleen

Gene expression associated with the synthesis, metabolism and transport of cholesterol in the liver has been addressed in the previous sections. In this section, I will reviewed the literature on genetic factors and gene expression associated with atherosclerosis in the aorta and the spleen. Researchers have already identified many genes and genetic determinants influencing the development of atherosclerosis in aorta and relative immune response in spleen. Aorta is a tissue where atherosclerotic plaques are frequently found. Prior research has identified many genes involved in the progress related to oxidation, inflammation and apotoposis of aortic cells (Table 1.2). A very recent example is the canonical nuclear factor-kappa B (NF-kB) signalling pathway, which has been suggested to play a pathological role in atherosclerosis in several vascular cell types (68-70). Studies from human atherosclerotic tissues have found that NF-kB transcription factor is activated in those cells and this activity involves p65, p50, c-Rel and many other molecules and factors (Table 1.3) (68-70). The important feature is that NF-kB activation will lead to the up-regulation of several genes that initiate atherosclerosis (Table 1.3). In addition, NF-kB activation is also involved in atherosclerosis through innate immunity processes by toll-like receptors (TLR2 and TLR4). TLR2 and TLR4 initiate a series of signalling events including genes such as MYD88, MAL, IRAK1 and TAK1 (68, 70, 71). The progression of the inflammatory response is then augmented by more inflammatory cytokines

that promote plaque formation (TNF, IL-1. IL-4, IL-12, IFN). During this process, many other genes are actively involved, e.g. CX3CL1, CCL10, platelet factor 4, matrix metalloproteinase-9 PAI-1 and tissue factor (Table 1.3).

Spleen produces T cells and B cells, acting as part of the immune system and taking part in inflammatory reactions. Furthermore, some genes and new genetic determinants of the inflammation process have been shown to influence the development of atherosclerosis. Some of genes that can influence the severity of the disorder are listed in table 1.4.

Table 1.2 Examples of gene expression that influence development of atherosclerosis in aorta

Gene name	Full name	Effects*	References
Acta2	actin, alpha 2, smooth muscle, aorta	+	(79)
VEGFA	vascular endothelial growth factor A	-/+	(80-85)
Pparg	peroxisome proliferator activated receptor gamma	-	(86, 87)
Tlr2	toll-like receptor 2	+	(88, 89)
TGFB1	transforming growth factor, beta 1	+	(90-92)
MPO	myeloperoxidase	+/no effect	(93-95)
Nos2	nitric oxide synthase2, inducible	-	(96)
Fabp4	fatty acid binding protein 4, adipocyte	+	(97, 98)
Esr1	estrogen receptor 1 (alpha)	+	(99, 100)
Gpr132	G protein-coupled receptor 132	-	(101-103)
PLAUR	plasminogen activator, urokinase receptor	+	(104-106)
MMP2	matrix metallopeptidase 2	+	(107-109)
MMP9	Martrix metallopeptidase 9	+	(107, 109, 110)
MMP1	matrix metallopeptidase 1	+	(109, 111)
Nos3	nitric oxide synthase 3, endothelial cell	-	(112-114)
Agtr1b	angiotensin II receptor, type 1b	-	(115)
Agtr2	angiotensin II receptor, type 2	-	(116-118)
Agtr1a	angiotensin II receptor, type 1a	+	(119)
Eln	elastin	+	(120)
Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b	-	(121)
Sod3	superoxide dismutase 3, extracellular	-	(122)
Col18a1	collagen, type XVIII, alpha 1	-	(123)
Hmox1	heme oxygenase (decycling) 1	-	(124)

* + = accelerated lesion development; - = retarded lesion development

Table 1.3 NF-kB pathway-related genes that influence development of atherosclerosis in aorta

Gene name	Full name (references)
ALOX5	5-lipoxygenase (125-127)
ALOX12	12-lipoxygenase (125-127)
COX2	Cyclooxygenase 2 (125)
MCP1	monocyte chemoattractant protein-1 (125)
ICAM-1	intercellular adhesion molecule (125-127)
VCAM-1	vascular cell adhesion molecule (125-127)
SELE	E-selectin (125)
SELP	P-selectin (125)
MCSF	macrophage colony-stimulating factor (125)
Pla2g2a	phospholipase A2, group IIA (platelets, synovial fluid) (125)
TLR2	toll like receptor 2 (88, 89, 125-128)
TLR4	toll like receptor 4 (125-127)
MYD88	myeloid differentiation primary response gene (125)
MAL	mal, T-cell differentiation protein (125)
IRAK1	interleukin-1 receptor-associated kinase 1 (125, 129)
IRAK4	interleukin-1 receptor-associated kinase 4 (125, 129)
IFN gamma	Interferon gamma (125)
MMP9	Matrix metallopeptidase 9 (125)
TRAF6	TNF receptor associated factor 6 (125)
TAK1(MAP3K7)	mitogen-activated protein kinase kinase kinase 7 (125, 129)
TNFR1	TNF receptor 1 (125)
TRADD	TNF R1 associated death domain (125)
RIP	receptor-interacting protein (125, 126, 130)
TRAF2	TNF receptor associated factor 2 (125, 126, 130)
TAB2	TAK1-associated Binding Protein 2 (125)
CX3CL1	chemokine (C-X3-C motif) ligand 1 (125)
CCL10	chemokine (C-C motif) ligand 10 (131)
PF4	platelet factor 4 (125-127, 130)
PAI-1	plasminogen activator inhibitor 1 (125-127, 130)

Table 1.4 Examples of gene expression in spleen that relat to atherosclerotic development

Gene name	Full name	Effects*	References
Il4	interleukin 4	N/C	(132-135)
Il6	interleukin 6	N/C	(136-139)
Il10	interleukin 10	+	(140-142)
Ifng	Interferon gamma	-	(143-146)
Nfkb1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	+	(147, 148)
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	+	(130)
Trp53	transformation related protein 53	-	(149)
Tgfb1	transforming growth factor, beta 1	-	(150)
TLR2	toll-like receptor 2	+	(128)
Tlr4	toll-like receptor 4	+	(151, 152)
Stat1	signal transducer and activator of transcription 1	+	(153)
Stat3	signal transducer and activator of transcription 3	+	(154)
Ccl2	chemokine (C-C motif) ligand 2	+	(140, 146, 155)
Ccl5	chemokine (C-C motif) ligand 5	+	(140, 152, 156)
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	+	(142)
Mfge8	milk fat globule-EGF factor 8 protein	+	(142)
Lta	lymphotoxin A	+	(157)
Lepr	leptin receptor	no effect/-	(158)
Plg	plasminogen	+	(159)
Itgb1	integrin beta 1 (fibronectin receptor beta)	+	(160)
Itgax	integrin alpha X	+	(161)
Itga1	integrin alpha 1	+	(162)
JAK2	Janus kinase 2	+	(163, 164)
Hspg2	perlecan (heparan sulfate proteoglycan 2)	+	(165, 166)
Csf2	colony stimulating factor 2 (granulocyte-macrophage)	-	(162)
Ucp2	uncoupling protein 2 (mitochondrial, proton carrier)	-	(167, 168)
Cd274	CD274 antigen	-	(169)
Tnf	tumor necrosis factor	+	(170-172)
Tnfs11	tumor necrosis factor (ligand) superfamily, member 11	+	(121)
Tnfrsf1a	tumor necrosis factor receptor superfamily, member 1a	+	(173, 174)
Ly6c1	lymphocyte antigen 6 complex, locus C1	+	(157)

* + = accelerated lesion development; - = retarded lesion development; N/C = not consistent

1.4.2. Tools in evaluating genetic factors

Over the past decades, genetic studies have investigated numerous genes and their functions. Methods in molecular genetics have provided valuable clues to elucidate the complex mechanisms of development and pathogenesis of disease. With the completion of the human genome as well as that of many other species, genetic studies have shifted from the identification of each gene each time to the genome-wide screen assay (175).

Before the early 1980s, genetic factors for a disease or trait could be tested directly aiming at single-locus alleles or genotype frequencies, usually through association studies. The most commonly used method involves case-control studies which compare one group who have a disease or trait with another matched group who don't have the disease or trait. The frequency of an allele or genotype of polymorphism will determine whether or not the genetic marker studied relates to a given trait. This method can result in a high number of false positives when the match of different groups is not well designed (176, 176, 177). A better method, linkage analysis, aims at finding the linkage position between genes or searching for a gene related to a certain trait relative to another known genetic marker. The use of restriction-fragment length polymorphism (RFLP) markers and microsatellite (short tandemly repetitive DNA) loci has led to the mapping of many disease-related loci successfully (178, 179). Then when DNA can be assayed directly and used as genetic markers in linkage studies, more efficient tools are able to narrow down the disease loci and their mutations by positional cloning. A great deal of molecular information related to Mendelian disease is clarified through position cloning (180, 181). However, linkage analysis is much more reliable to study Mendelian traits than non-Mendelian traits. For most human traits and diseases, the phenotype is controlled by multiple

genes and the interactions among them. Thus, the non-Mendelian traits require more reliable methods to be applied (175, 182)

1.4.2.1. Genome-wide expression assays

Nowadays, with the completion of some whole genome sequences and the availability of many partial sequences (183), measurements of candidate genes or studies of high-density genome screening have taken full advantage of the rapidly increasing body of sequence information (184). To measure gene expression at level of mRNA, Northern blots, reverse transcription of RNA (e.g. real-time PCR), nuclease protection, cDNA sequencing, clone hybridization, differential display, subtractive hybridization, cDNA fragment fingerprinting (e.g. cDNA-AFLP) and serial analysis of gene expression (SAGE), as well as the large-scale array-based technique have been recently put to good use to measure the expression levels of specific genes, characterize global expression profiles or to screen for significant differences in mRNA abundance (175).

1.4.2.1.1. cDNA-AFLP

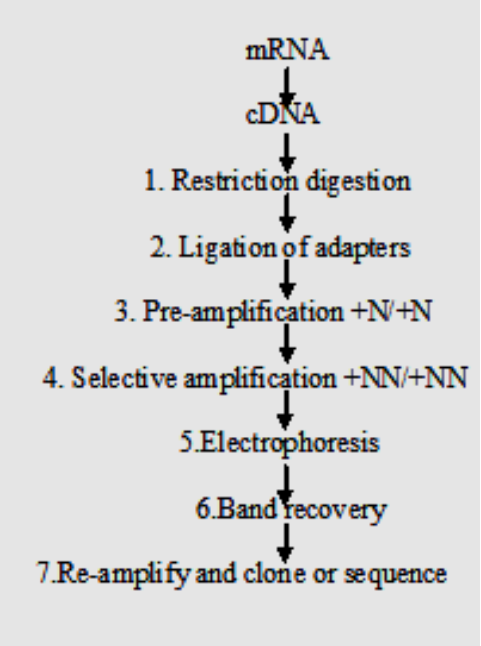
cDNA- AFLP is one of the genome-wide expression profiling methods capable of finding genes that have not yet been cloned or even predicted (185-188). In cDNA-AFLP, a complex cDNA mixture is divided into small subsets using restriction enzymes and ligated to specific adaptors according to the restriction fragments. The ligated DNA fragments are then selectively amplified with PCR primers that have the corresponding adaptor and restriction site-specific sequences. After multiplex PCR and the separation of amplified fragments on a gel matrix, band patterns can be visualised (189, 190). Detailed steps are demonstrated in figure 1.2.

Because each added nucleotide has four choices for each position, a great number of primer sets are needed to cover the whole genome. Although cDNA-AFLP with limited primer combinations performed has a low probability of finding the potential targets, the technique itself has numerous advantages as an expression assay: 1). Poorly characterized genomes can be analyzed. cDNA-AFLP is able to produce specific expression patterns even the primer sets are randomly picked up without knowing the sequence information. 2). Multiple transcripts responsible for the attribute being evaluated can be identified. 3). Transcripts that are either non-coding or contain no open reading frame can be analyzed. 4).The cDNA-AFLP method largely overcomes the limitations with reproducibility, difficulty in representing very rare messages, and generation of false positives and makes a simple and rapid verification of band identity possible (189, 191-193).

1.4.2.1.2. Other gene expression profiles

As gene expression techniques dramatically advanced, the ability to test thousands of genes in a single experiment is revolutionizing the study of identifying genes and pathways relevant in atherosclerosis development (175, 187). Many technologies such as differential display, DNA microarray and serial analysis of gene expression (SAGE), are now being applied to determine genome-wide responses of atherogenic stimulation (194) (195) or gene expression patterns associated with inherited genetic differences (195). Analysis of the data from these experiments will hopefully lead to the identification of new candidate genes that can be tested for roles in the development of atherosclerosis (27, 196). In principle, SAGE can provide quantitative data concerning gene expression; the mRNA sequences do not need to be known a priori, so genes or gene variants which are not known can be discovered. Using sequence databases, a researcher can usually determine, with some confidence, the original mRNA and

the corresponding gene. However, it is expensive and labour intensive when multiple sample points are to be compared. Microarray technology is extremely powerful in generating a broad view of gene expression. However, the design of oligo-nucleotide arrays requires comprehensive sequence knowledge, which at present is only available for a small number of organisms. Studies using these techniques assay tens of thousands of genes simultaneously, and they can provide an unbiased profile of expressed genes.



1. Restriction digestion with *Eco*RI and *Mse*I to generate fragments within a specific size range.
2. Adapter Ligation where known adapters are ligated to the ends of the cDNA fragments produced during restriction digestion to yield templates for amplification.
3. Pre-Amplification to increase the template available for selection is performed with primers complementary to the adapter sequences.
4. Selective Amplification is used to amplify subsets of the pre-amplified templates using primers containing .2 selective nucleotides at the 3' end of the amplification primers.
5. Electrophoresis of these products on a denaturing polyacrylamide gel yields distinctive patterns for analysis.
6. Extract fragments of interest.
7. Re-amplify fragments using the same primers and either clone or direct sequence, depending on the application.

Figure 1.2 cDNA-AFLP expression procedure. N stands for selective nucleotide.

1.4.2.2 Animal models

Because atherosclerosis is affected by multiple factors, the complexity of the disease has made it difficult to isolate particular genetic factors that may be linked to atherogenesis. In humans, the condition is the result of gene-gene and gene-environment interactions (6, 197), the incomplete penetrance in determining the phenotype (e.g. the effects of the presence of related

diseases, and differences in subject environmental exposure and lifestyle) and the large study group requirement for human genetic linkage research makes it very difficult to perform a good genetic analysis. As a result, animal models are extremely useful to analyze the genetic factors of the disease (198, 199).

The ability of animal models to reproduce a disease state that is similar to the human situation is variable. Certain animals can be useful in studying specific dyslipidemias. Often, the interaction of experimental treatment effects with the lipid and lipoprotein metabolism of animal models does not exactly mimic that of humans, so that the interpretation of results is difficult. Variations in feeding behaviour and dietary composition between rodent or avian models *versus* that of humans (56, 198, 200), differences in lipoprotein composition (27, 199), and susceptibility to atherosclerosis (199, 201) are some examples of species-related differences.

1.4.2.2.1. Laboratory mouse

In recent years, the use of mouse models to study atherogenesis has become increasingly common. From mouse studies, we have obtained a great deal of information about atherosclerosis.

Wild-type mice are naturally resistant to atherosclerosis. However, laboratory strains can be developed into useful models for studying atherosclerosis. One strain from the Jackson laboratory, the C57BL/6 strain, can develop vascular lesions when animals are fed a very high cholesterol diet containing cholic acid (202). Unfortunately, the lesions produced in this model differ from those in the human. Lesion formation in this model is largely limited to the aortic root after feeding the induced diet for 14 weeks to 9 months. The lesions are quite small, and do not show the fatty streak stage in smooth muscle cells and no progression to intermediate lesions (202-204). More useful models utilize genetically modified (GM) mice. The APOE knock-out

(ApoE KO) mouse is the most well-known and the earliest genetically modified (GM) model whose APOE gene has been inactivated (205). These animals have been shown to be very susceptible to atherosclerosis and have five times the plasma cholesterol levels of normal mice on a low-fat chow diet (199, 206). They develop the atherosclerotic lesion as early as 9 weeks especially when fed a "western-type" diet (21% fat by weight, 0.15% cholesterol) (18, 199, 206). Accordingly, ApoE KO mice are considered to be one of the most relevant models for studying atherosclerosis (18, 199). Since then, more GM mouse models have been generated and used in atherosclerosis-related research (Table 1.5).

Table 1.5 Transgenic and knockout mouse models

	Description	Reference
ApoE KO mouse	Apolipoprotein E null mouse, develop atherosclerotic lesions by feeding fat-rich cholesterol diet and have elevated plasma cholesterol even on low fat chow diet.	(199, 206)
LDLR-KO mouse	LDL receptor-deficient mouse, diet-responsive model: develop atherosclerosis under cholesterol-induced diet	(207, 208)
ApoB transgenic mouse	Human APOB transgenic mouse, develop atherosclerosis under cholesterol-induced diet	(209, 210)
ApoE/LDLr-DKO mouse	APOE and LDL-receptor double knockout mouse, develop marked atherosclerotic lesions on a regular chow diet	(211, 212)
apoE/eNOS double knockout mouse	Endothelial nitric oxide synthase gene and APOE double knockout mouse, chronic deficiency of eNOS increases atherosclerosis in ApoE KO mouse.	(213)

1.4.2.2.2. Japanese quail

Several avian species (e.g. chicken, pigeon and quail) have been used as experimental models in atherosclerosis research (214-216). The Japanese quail was first introduced into atherosclerosis research in the early 1960s (217), and many studies have been carried out with various strains of Japanese quail to obtain information on the development of

hypercholesterolemia and atherosclerosis (Table 1.6). The small body size, ease of maintenance and short life span make them a convenient animal model for laboratory use. Japanese quail are naturally deficient in apolipoprotein E (18, 55) and develop atherosclerotic plaques rapidly when they are placed on cholesterol-enriched diets (216, 218, 219). Previous studies also demonstrated that they develop hypercholesterolemia by dietary cholesterol supplementation and have atherosclerotic lesions in the aorta in a dose-dependent manner (218). Although under normal circumstances, the major fraction of cholesterol in quail plasma is HDL (whereas, in humans, LDL is the major cholesterol carrier in plasma) (218, 220); cholesterol feeding increases LDL levels to become the dominant lipoprotein in quail (218, 219, 221). The more important reason why the Japanese quail is a good model for atherosclerosis is that the etiology of plaque formation in quail is similar to what in humans, they form "complex" vascular lesions (focal haemorrhage, calcification, and fibrosis) that are very similar to those in humans (216, 218, 219, 222). Other similarities to the human disorder include the greater susceptibility of males than females to diet –induced atherosclerosis and the implication of viral infection as a possible initiator of lesion formation (216, 218, 219, 222, 223).

The atherosclerosis-susceptible (SUS) and resistant (RES) quail are particularly useful models. These are two strains of quail developed by divergent selection for susceptibility and resistance to atherosclerotic plaque formation induced by dietary cholesterol. Before selection, 8% of the random bred foundation population developed atherosclerosis when challenged with a high cholesterol diet (0.5% w/w) (216, 222). After divergent selection for 4 generations, 80% of the SUS males developed atherosclerosis in contrast to 4% of the RES males (216, 218, 219, 224, 225). SUS males show high susceptibility to atherosclerosis and can form visible lesions that are detectable as early as 4 weeks following the institution of cholesterol supplementation,

whereas the resistant birds are resistant to the disease and develop little atherosclerosis on the same high cholesterol diet (216, 218, 219, 222, 223). As described in Table 1.6, several Japanese quail models have been developed for atherosclerosis and hypercholesterolemia research. For example, the studies from HL and LL quail showed that after feeding 0.5% cholesterol, atherosclerotic plaque formation in the aorta in LL males was lower than those in HL males within 12 weeks (201, 226). It was also shown that dietary cholesterol is retained longer in plasma of HL than in LL quail and that bile acid excretion is reduced in HL quail (201). The CIA quail model provided important information on histological and biomedical investigation demonstrating that lesion composition and development in CIA quail is similar to human atherosclerotic lesions (227, 228).

Table 1.6 Japanese quail models used in atherosclerosis related studies

model	Description	Reference
SEA	A selected line that is susceptible to the experimental induction of atherosclerosis (SEA), develop hypercholesterolemia and atherosclerosis when fed diets containing 1% cholesterol and 0.5% cholic acid.	(229, 230)
CIA	Cholesterol-induced atherosclerotic (CIA) Japanese quail. A model for the studying alterations in proteoglycan metabolism in atherosclerotic plaques induced by hypercholesterolemia.	(227, 228)
HL/LL	Two genetically selected lines: high cholesterol line (HL) and low cholesterol line (LL). They are divergently selected for high and low plasma cholesterol levels after injection with adrenocorticotropin.	(201, 226)
LAP	A line of Japanese quail susceptible to hyperlipidemia and atherosclerosis, known as hyperlipidemia- and atherosclerosis-prone (LAP) quail. (OMIA ID: 2003)	(231-234)
SUS/RES	Atherosclerosis-susceptible (SUS) and resistant (RES) quail are two lines of quail developed by divergent selection for susceptibility and resistance to atherosclerotic plaque formation induced by 0.5% dietary cholesterol.	(216, 218, 219, 222, 223)

OMIA ID is the Online Mendelian Inheritance in Animals identification number.

1.4.2.2.3. Other animal models for atherosclerosis

Numerous animal models have been used to in clinical research and pathogenesis studies. These include mice, rats, rabbits, pigeons, quail, chickens and some relatively large animals such as swine, cats, dogs and primates. Some well studied strains and breeds are listed in table 1.7.

Table 1.7 Animal models used in atherosclerosis related studies

Animal model	Description	Reference
Dog	A common form of arteriosclerosis in which deposits of yellowing plaques (atheromas) containing cholesterol and other lipid material are formed within the arteries. (OMIA ID: 2517)	(29, 235)
Domestic pigeon	Atherosclerosis-susceptible White Carneau and atherosclerosis-resistant Show Racer pigeons (OMIA ID: 2295)	(236, 237)
Pig	A strain of pig that spontaneously develops atherosclerosis, developed by selection. (OMIA ID: 173)	(238-240)
Rabbit	This phenotype is also known as Watanabe heritable hyperlipidemia, similar to that in human familial hypercholesterolmia	(200, 241)
Rhesus monkeys	Develop atherosclerosis by feeding an atherogenic diet.	(242, 243)
Hamster	Diet-induced atherosclerosis, similar lipoprotein profile to human	(244, 245)
Guinea pig	Develop atherosclerosis when challenged with hypercholesterolemic diets. A model for menopause, follow similar patterns to those observed in humans.	(246-248)

OMIA ID is the Online Mendelian Inheritance in Animals identification number.

1.5. Objectives of this thesis

The RES and SUS quail (see section 1.3.2.2.2.) were developed through divergent selective breeding from the same foundation population (216), and should be genetically similar except for that changed by selection. Except for some physiological characterization of the differences between these two strains, there has been no genetic characterization of these strains. Since the aorta, liver, and spleen play a key role in the development of atherosclerosis, we have focused our study on these organs. In particular, we examined the liver, where the expression of several cholesterol biosynthesis and transporting genes in this organ regulates cholesterol homeostasis. While gene expression is a phenotype, it will reflect more directly the genotypic

changes than morphological or physiological phenotypes. By comparing the gene expression in these two quail strains exposed to two different diets, I also hope to gain insights into the genetic x environment interaction in the development of atherosclerosis. A better description of these quail strains would allow more effective utilization of these birds as models to study atherosclerosis, and better interpretation of data from research utilizing these strains.

The first study investigated the expression of liver HMGCR, FDFT1, SQLE, DHCR7, ABCG5, ABCG8 and APOA1mRNAs in these two strains using real-time PCR. The objective was to determine whether or not there were differences in the expression of these genes due to strain differences and diet differences. This study was carried out in Dr. Patricia Schulte's laboratory (Department of Zoology) and under her supervision.

In the second study, we used a cDNA-amplified fragment length polymorphism PCR (AFLP) technique in an attempt to indentify genes in the aorta, liver, and spleen whose transcription patterns differed in these two quail strains under different diet regimes. This study was carried out in the Genetic Data Centre under the supervision of Dr. Carol Ritland.

This thesis research was carried out with the approval of the UBC Animal Care Committee; Certificate # A06-1473 (Appendix 1).

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CHAPTER 2: DIFFERENTIAL GENE EXPRESSION IN THE LIVER OF ATHEROSCLEROSIS-SUSCEPTIBLE (SUS) AND ATHEROSCLEROSIS-RESISTANT (RES) JAPANESE QUAIL, (*COTURNIX JAPONICA*)[1]

2.1. Introduction

Atherosclerosis is a complex pathological process that is affected by both environmental and genetic factors; it is a major cause of morbidity and mortality in industrialized societies (1, 2). Although surgical and medical treatments have progressed, current therapies to slow atherosclerotic plaque formation have not been totally successful (1, 2). Thus, investigation of the fundamental mechanisms of atherosclerosis remains a crucial way to formulate more effective treatment for the disease.

Cholesterol is essential for all animals, being an important component of the cell membrane and a precursor in several biochemical pathways. However, it is also involved in atherogenesis; cholesterol-related metabolism can determine the extent and clinical manifestations of this disease. It is therefore important to understand the relationship between dietary cholesterol absorption, endogenous cholesterol synthesis and biliary cholesterol excretion balancing cholesterol homeostasis (3-5). The liver plays a key role in regulating cholesterol homeostasis. It is the site of cholesterol biosynthesis, acting as the main site for lipid metabolism and it is also the location where bile salts are formed. It, therefore, became the target organ for our investigation.

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The mevalonate pathway (or HMG-CoA reductase pathway) is an important component of the endogenous cholesterol biosynthesis pathway (6) in the liver. During the process of converting mevalonate into cholesterol and other sterol isoprenoids, many important enzymes such as 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR or HMG-CoA reductase), squalene synthase (FDFT1) , squalene expoxidase (SQLE), mevalonate kinase (MVK) , phosphomevalonate kinase (PMVK) and 7-dehydrocholesterol reductase (DHCR7) are involved in regulating the whole process (6) (Figure 1.1). Many genes in this pathway are down-regulated by intracellular cholesterol via the SREBP pathway. FDFT1 gene expression, for example, can be regulated by SREBP-2, a SREBP isoform, negatively responding to sterol levels (7, 8). In humans, SQLE expression is regulated by intracellular cholesterol in a negative feedback loop through the SREBP pathway and nuclear factor Y (9, 10). Our study selectively quantified the gene expression of HMGCR, FDFT1, SQLE and DHCR7 mRNA – components involved in the mevalonate pathway.

ABCG5 and ABCG8, from the adenosine ABC transporter family, are cholesterol excretion transporters. Evidence from both animal models and human studies supports the important role of these two ABC transporters in regulating the secretion of sterols from liver and the efflux of dietary sterols from the gut (11). The major regulation of expression of ABCG5 and ABCG8 genes appears to be at the transcriptional level by the liver X receptors (12). Therefore, we also included these two genes in our study.

Apolipoprotein A1 is the major protein component of HDL in plasma. It is thought to have a protective effect against atherosclerosis by transporting hepatic cholesterol (13-16) considered as “good” cholesterol, and becomes one of the entities that we were interested in examining.

In the present study, two strains of Japanese quail (*Coturnix japonica*) - atherosclerosis-susceptible (SUS) and -resistant (RES) - were used to study gene expression differences following challenge with a high cholesterol diet.

2.2. Methods

2.2.1. Experimental birds

The SUS and RES quail were developed through divergent selective breeding from the same foundation population. These strains have been shown to differ in their response to a cholesterol diet with 80% of the SUS males developing atherosclerosis in contrast to 4% of the RES males (17-21).

The SUS and RES quail were originally acquired by the UBC Quail Genetic Resource Center from North Carolina State University in 1989, and have since undergone further divergent selection for susceptibility and resistance to atherosclerotic plaque formation induced by dietary cholesterol (0.5%w/w) (17-21).

2.2.2. Experimental design

After hatching, both SUS and RES males were fed a synthetic diet (See Appendix 2) prepared by the feed mill at the Agriculture and Agri-Food Canada Research Station at Agassiz, British Columbia, according to the NRC standard recommended for quail. At six weeks of age, 13 birds were euthanized to collect liver tissues. The remaining birds were divided into two dietary treatment groups and fed either a regular synthetic diet or a synthetic diet with added cholesterol (0.5%w/w; see Appendix 2) for another 6 weeks (Table 2.1). At twelve weeks of

age, 24 birds from two dietary treatment groups were euthanized and samples of liver tissue were obtained for further analysis.

Table 2.1 Dietary treatments (week 7 – week 12)

Diet Treatments	SUS	RES
Regular diet	6 males	6 males
Cholesterol diet (0.5%w/w)	6 males	6 males

2.2.3. Preparation of total RNA

Quail were euthanized by decapitation; livers were quickly dissected and stored in RNAlater reagent (Qiagen, Valencia, CA, USA) at -20 °C until use. Total RNA from livers was extracted using RNeasy mini columns (Qiagen, Valencia, CA, USA). The concentration and purity were checked by spectrophotometer. RNA was measured at wavelength of 260nm (OD 260nm), whereas protein was detected at OD 280nm. An OD260/OD280 ratio of 1.8-2.0 of RNA was selected for cDNA synthesis.

2.2.4. Synthesis of first-strand cDNA

cDNA synthesis was performed using SuperScript™ III RT (200 units/ µl) (Invitrogen Corporation, Carlsbad, CA, USA) at 50 °C using Oligo (dT) $_{18}$ primer (Fermentas Inc., Glen Burnie, MD, USA) according to the manufacturer's instructions. A 38 µl reaction volume contained 5µg of total RNA , 1 µl Oligo (dT) 18 primer (100 mM), 2 µl dNTP (10mM) (Fermentas Inc., Glen Burnie, MD, USA), 8 µl 5× first strand buffer, 4 µl DTT (0.1 M) and 2 µl SuperScript™ III RT. In addition, 1µl RiboLock™ RNase Inhibitor (40U/µl) (Fermentas Inc., Glen Burnie, MD, USA) was added in each reaction in order to inhibit RNA degradation during reverse transcription. The first-strand cDNA was stored at -20 °C for future use in real-time PCR.

2.2.5. Primer design

Primers for the selected genes were based on the mRNA information obtained from the National Center for Biotechnology Information (NCBI www.ncbi.nlm.nih.gov) GenBank data base. Four cholesterol biosynthesis genes and two cholesterol excretion gene sequences were chosen from the *Gallus gallus* (chicken) nucleotide data base; apolipoprotein A1 sequence was from the *Coturnix coturnix* (common quail) data base; and *Coturnix coturnix* glyceraldehyde 3-phosphate (GAPDH) sequences were previously published (22); GAPDH expression does not vary among strains, diets or ages and can be used as an internal control (22). Real-time PCR primers were designed using Primer Express version 2.0.0 (Applied Biosystems, Foster City, CA, USA) and were ordered from IDT (Integrated DNA technologies, Coralville, IA, USA).The primer information is listed in Table 2.2.

Table 2.2 Real-time PCR primer combination

Primer Name	Primer Sequence (5'— 3')	Species Gene ID
GAPDH Forward	GGCACTGTCAAGGCTGAGAAT	*C. coturnix*
Reverse	GCATCTCCCCACTTGATGTTG	Z19086
HMGCR Forward	GCAGAGGGCCTTACAAC	*G. gallus*
Reverse	GGAGGAGCAAGCCGTAT	NM_204485
FDFT1 Forward	GCC ATC ATG TAC CAG TAT GTG GAA	*G. gallus*
Reverse	GCT GCG TCT TGT TGG AGG AA	NM_001039294
SQLE Forward	GAG GTA GAA ATT CCT TTT CCA ACA TCT	*G. gallus*
Reverse	GCC GTG ATG GAA GGA CCT T	NM_001030953
DHCR7 Forward	GGG AAA GAT TGG AAA CGC TAC A	*G. gallus*
Reverse	CAG ATT CTG TGT CAG CCT TAA AAC A	XM_420914
ABCG5 Forward	ATT ACA AGA TCC CAA GGT CAT GCT	*G. gallus*
Reverse	GAG ACG ATC TGG TTT GCA GTC A	XM_419457
ABCG8 Forward	GCC TTC CAG CAT GTT TTT CAG	*G. gallus*
Reverse	CGC AAC CGT AGC TCT GCT ATT	XM_419458
APOA1 Forward	TCT GGT GCA GGA ATT CAA GGA	*C. coturnix*
Reverse	TCA TCC AGG AGG TCG ATC AAG	D85133

2.2.6. Real-time PCR

A portion of the prepared first strand cDNA templates was used to make up the standard curve cDNA template mixture. The remaining portion was diluted 1:1 and used as the cDNA templates. Real-time PCR was performed using an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA). The PCR was carried out in a reaction volume of 22 µl, containing 2 µl

cDNA template, 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM) (Integrated DNA technologies, Coralville, IA) and 10 µl SYBR Green universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and water added to make a final volume of 22 µl per reaction. Amplification and detection of the samples and the standards were directed using the following thermal cycling conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 15s and annealing and extension at 60 °C for 1 min. Fluorescence measurements were recorded using SYBR as the reporter dye and the results were normalized to the endogenous control, GAPDH. A standard curve was performed for all primers using serial dilutions of cDNA (2×, 1×, 1/2×, 1/4× and 1/8×). The 2× mixture was made by using the first-strand cDNA products (which had twice the concentration of template cDNA). The 7000 System Software was used for data analyses (Applied Biosystems, Foster City, CA, USA). Quantification of expression levels was determined by comparing the results of the standard curve produced by serial dilutions. Relative transcription amount was calculated as the ratio of the measured amount of target gene mRNA divided by the amount of GAPDH mRNA.

2.2.7. Plasma lipid assays

Plasma samples (N = 56) from 12 week old SUS and RES males on the two dietary treatments were sent to the Department of Pathology and Laboratory Medicine at St. Paul's Hospital (Vancouver, B.C.) and assayed for total cholesterol, HDL, and triacylglycerides using enzymatic methods on an ADVIA 1650 Chemistry System. HDL was assessed by the direct method without precipitation of apolipoprotein B (23-25). LDL values were determined by Friedewald's formula, using measured values for total cholesterol, HDL and triacylglycerides (26, 27).

2.2.8. Statistical analysis

Least squares analysis of variance was performed using JMP 8.0 (SAS Institute, North Carolina 2008). The statistical model for mRNA level was:

$$Y_{ijkl} = \mu + S_i + D_j + A_k + (SD)_{ij} + (SA)_{ik} + E_{ijkl}$$

where Y_{ijkl} was the measurement of the lth individual of the ith strain, jth diet from kth age group. S_i indicated whether the bird was RES or SUS. A_k represented the two age groups, 6-week or 12-week. D_j indicated whether the bird was on a regular diet or cholesterol diet. $(SD)_{ij}$ and $(SA)_{ik}$ were the interaction terms. E_{ijkl} was the error term. The results were reported as the least square mean values for each set data $\pm$ standard error of means (SEM) and the level of statistical significance was defined at $P < 0.05$. Student's t was used for mean separation.

For plasma lipid parameters, the following model was used:

$$Y_{ijk} = \mu + S_i + D_j + (SD)_{ij} + E_{ijk}$$

mRNA levels of the 7 candidate genes were also regressed on the plasma lipid parameters (JMP 8.0).

2.3. Results

2.3.1. Mevalonate pathway genes

2.3.1.1. HMGCR, FDFT1, SQLE

The expression of HMGCR, FDFT1 and SQLE was significantly (P<0.007, P<0.027 and P< 0.021, respectively) higher in RES than in SUS regardless of dietary treatment and age (Figure. 2.1). No other significant effects and interactions were found.

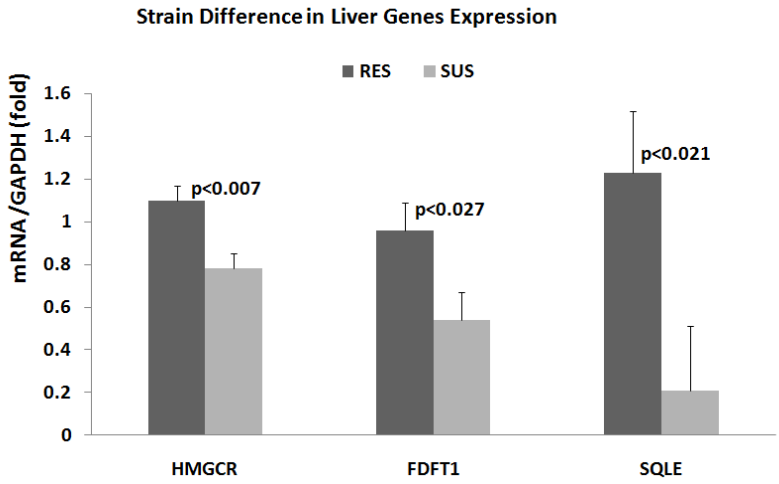


Figure 2.1 HMGCR, FDFT1 and SQLE mRNA levels in SUS and RES quail. All values indicate the gene of interest relative to GAPDH (arbitrary units). Levels of mRNA were determined by real-time PCR, and normalized to a standard curve generated from pooled samples. Values are the least square means ± SEM for birds in each treatment group.

2.3.1.2. DHCR7

The expression of DHCR7 was not affected by strain (P< 0.35) or dietary treatment (P< 0.74). However, 6-week old birds (1.36 ± 0.18 DHCR7/GAPDH) had significantly (P<0.03)

higher DHCR7 expression than 12-week old birds (0.88 ± 0.10 DHCR7/GAPDH). No significant interactions were found.

2.3.2. ATP-binding cassette transporter genes

2.3.2.1. ABCG8

The expression of ABCG8 was significantly ($P<0.004$) higher in RES (1.775 ± 0.1636 ABCG8/GAPDH) than in SUS (1.009 ± 0.1636 ABCG8/GAPDH) regardless of dietary treatment in 12-week old birds ($P< 0.35$ for strain × diet interaction). There was no significant diet effect ($P< 0.082$). 12-week old quail of both strains also had significantly ($P<0.015$) higher ABCG8 expression than 6-week old quail ($P< 0.07$ for strain × age interaction).

2.3.2.2. ABCG5

There was a significant ($P< 0.02$) diet × strain interaction in ABCG5 gene expression. When fed the regular diet, RES had significantly higher ($P< 0.05$) expression of ABGC5 than SUS. When challenged with the cholesterol diet, the expression level in RES remained the same but mRNA of SUS increased significantly ($P< 0.05$) and became similar to that of the RES (Figure 2.2A). There was a significant ($P< 0.0005$) strain × age interaction for birds on the control diet; the expression of ABCG5 significantly ($P<0.05$) increased with age in RES but that of SUS did not (Figure 2.2B).

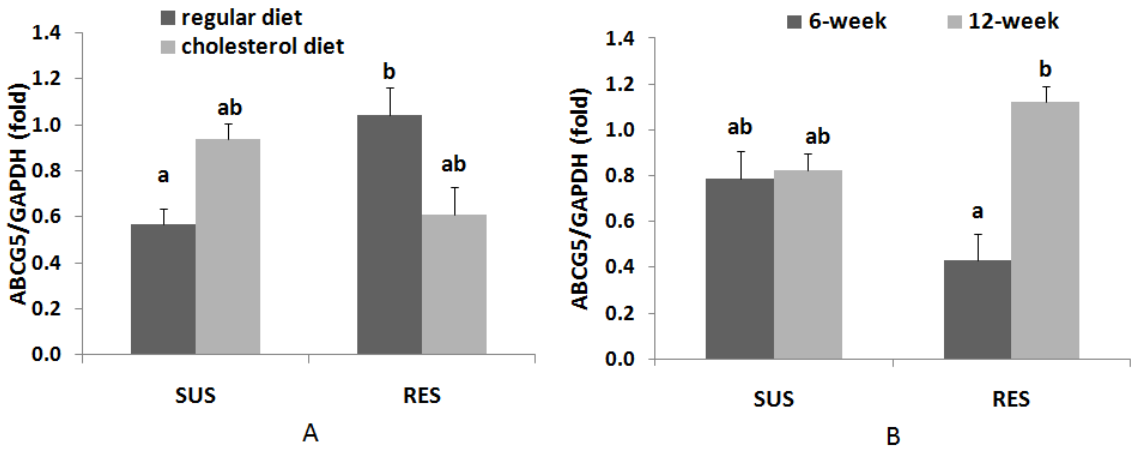


Figure 2.2 (A) ABCG5 mRNA levels of SUS and RES quail on regular or cholesterol diets. (B) ABCG5 mRNA levels of SUS and RES Japanese quail at 6 weeks and 12 weeks. All values indicate the gene of interest relative to GAPDH (arbitrary units). Levels of mRNA were determined by real-time PCR and normalized to a standard curve generated from pooled samples. Values are the least square means ± SEM for birds in each treatment group. For each graph, columns with different letters were significantly different (P<0.05).

2.3.3. APOA1

There was no significant (P<0.460) strain effect on the expression of APOA1. Both RES and SUS fed the cholesterol diet (1.40 ± 0.06 APOA1/GAPDH) had significantly (P< 0.011) higher expression compared to birds fed the regular diet (0.82 ± 0.05 APOA1/GAPDH) (P< 0.86 for strain × diet interaction). For both strains, APOA1 gene expression was also significantly (P<0.001) higher in older quail (1.26 ± 0.05 APOA1/GAPDH) than younger birds (0.67 ± 0.08 APOA1/GAPDH) (P< 0.39 for strain × age interaction).

2.3.4. Plasma total cholesterol, LDL, triacylglyceride and HDL levels

2.3.4.1 Plasma total cholesterol and LDL levels

There was a significant strain × diet interaction for plasma total cholesterol (P< 0.013) and LDL (P< 0.01) levels. Plasma total cholesterol and LDL levels in SUS and RES fed the

regular diet were not different. These plasma lipid levels were significantly (P< 0.0001) higher when the birds were fed the cholesterol diet, with levels in SUS being significantly (P< 0.05) higher than those in RES (Tables 2.3 and 2.4).

Table 2.3 Plasma total cholesterol levels (mmol/l)* in SUS and RES quail fed regular or cholesterol diets (N = 56; P<0.01)

Diet	SUS	RES
Regular	7.45 ± 2.58 c	6.15 ± 3.90 c
Cholesterol	42.43 ± 1.98 a	24.11 ± 4.21 b

*Means followed by different letters are significantly different (P<0.05) by the Student's t Test.

Table 2.4 Plasma LDL cholesterol levels (mmol/l)* in SUS and RES quail fed regular or cholesterol diets (N = 51; P<0.01)

Diet	SUS	RES
Regular	1.75 ± 2.03 c	1.64 ± 3.07 c
Cholesterol	32.82 ± 1.73 a	18.64 ± 3.32 b

*Means followed by different letters are significantly different (P<0.05) by the Student's t Test.

2.3.4.2. Plasma triacylglyceride levels

There was a significant (P< 0.02) strain × diet interaction in plasma triacylglyceride levels. Plasma triacylglyceride levels between SUS and RES fed the regular diet were not different. When the birds were fed the cholesterol diet, plasma triacylglyceride levels increased significantly (P<0.05) in SUS but not in the RES (Table 2.5).

Table 2.5 Plasma triacylglyceride levels (mmol/l)* in SUS and RES quail fed regular or high cholesterol diets (N = 56; P< 0.02)

Diet	SUS	RES
Regular	0.98 ±0.32 b	1.18 ±0.48 b
Cholesterol	3.32 ±0.24 a	1.54 ±0.52 b

*Means followed by different letters are significantly different (P<0.05) by the Student's t Test.

2.3.4.3. Plasma HDL cholesterol levels and LDL/HDL ratio

There was a significant (P< 0.028) strain × diet interaction in plasma HDL levels. Plasma HDL levels were significantly (P< 0.05) higher in SUS than in RES when birds were on the regular diet. Plasma HDL levels did not change significantly when the birds were fed the cholesterol diet; however, the difference between the SUS and RES became non-significant (Table 2.6). These small changes in the HDL level did not affect their LDL/HDL ratio (Table 2.7; compare with Table 2.4).

Table 2.6 Plasma HDL cholesterol levels (mmol/l)* in SUS and RES quail fed regular or high cholesterol diets (N = 56; P< 0.028)

Diet	SUS	RES
Regular	5.25 ±0.21 a	3.96 ±0.32 b
Cholesterol	4.81 ±0.16 ab	4.75 ±0.35 b

*Means followed by different letters are significantly different (P<0.05) by the Student's t Test.

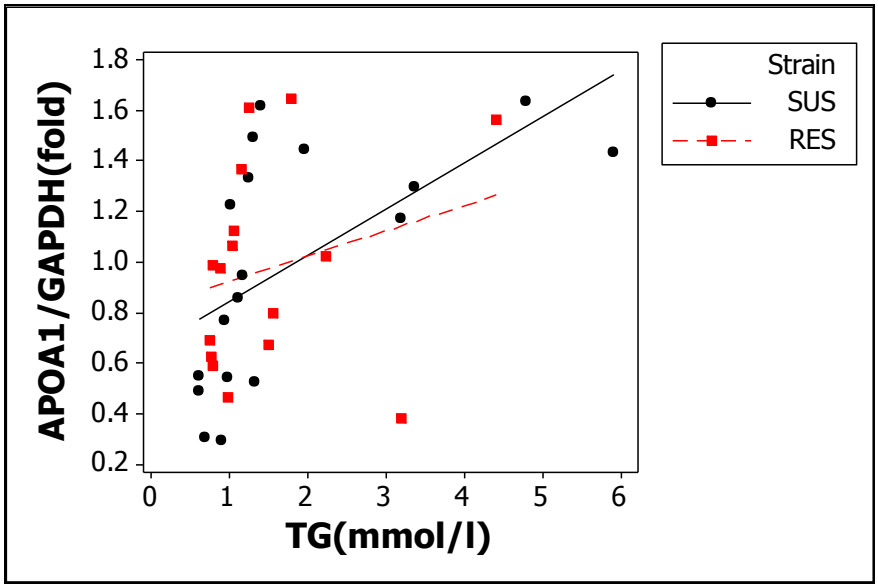
Table 2.7 Plasma LDL cholesterol/HDL cholesterol ratio in SUS and RES quail fed regular or high cholesterol diets (N = 51; P< 0.0042)

Diet	SUS	RES
Regular	0.34 ±0.43 c	0.42 ±0.66 c
Cholesterol	7.16 ±0.37 a	3.84 ±0.71 b

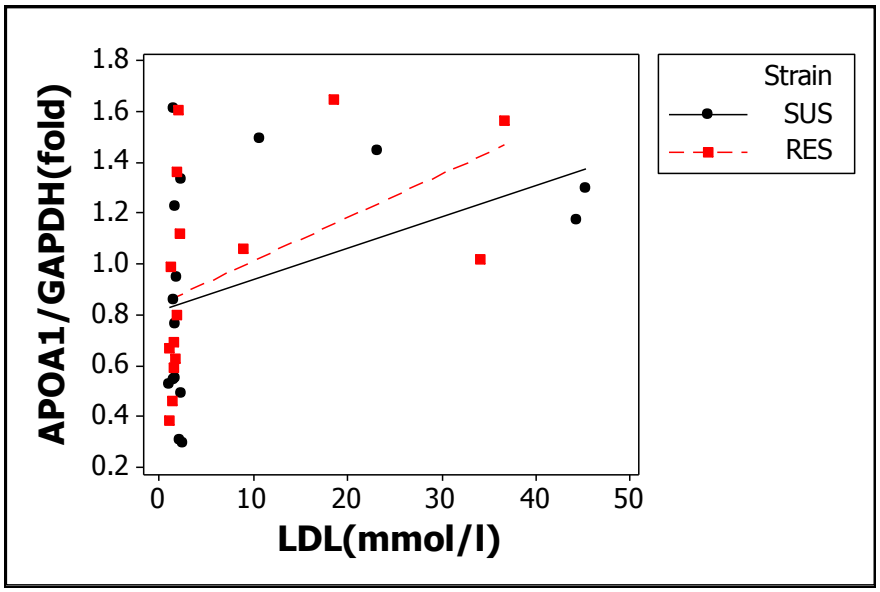
*Means followed by different letters are significantly different (P<0.05) by the Student's t Test.

2.3.4.4. Regression of gene expression on plasma lipid levels

When the SUS and RES data were pooled, APOA1 expression regressed significantly and positively on plasma triacylglyceride ($P<0.005$) and LDL ($P<0.01$) levels (Figure 2.3A, B). All LDL and triacylglyceride data were included except 3 samples where triacylglyceride could not be detected and 6 in which LDL was not determined; thus, N=31 for LDL and N=34 for triacylglyceride. When the two strains were analysed separately, APOA1 expression regressed significantly ($P<0.009$) on plasma triacylglyceride level in the SUS but not in the RES . APOA1 expression tended ($P<0.06$) to regress on LDL and significantly ($P<0.04$) regressed on the LDL/HDL ratio in RES (Figure 2.4).



A



B

Figure 2.3 APOA1 expression in SUS (Black) and RES (Red) liver (pooled) relative to plasma triacylglycerides (A) and LDL cholesterol levels (B)

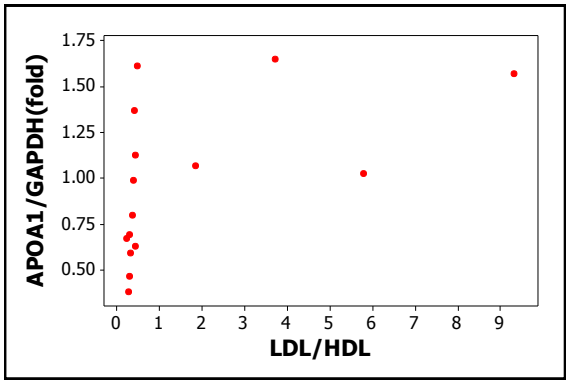


Figure 2.4 APOA1 expression in RES liver relative to LDL/HDL ratio.

In the SUS (N = 16), ABCG5 expression regressed significantly (P<0.008) and positively on plasma LDL level (Figure 2.5), whereas DHCR7 and SQLE expression regressed significantly (P<0.04 and P<0.02, respectively) and negatively on plasma triacylglyceride level (Figure 2.6 A, B).

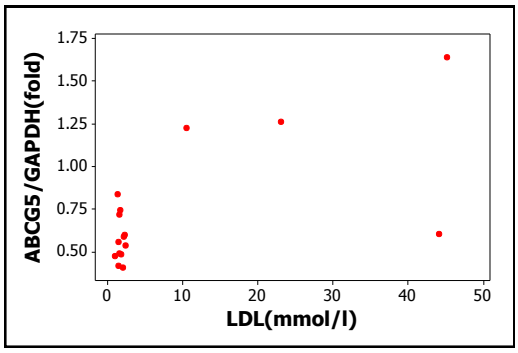
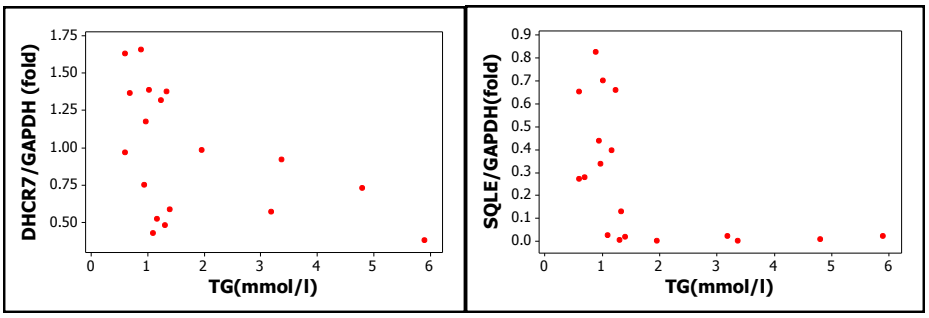


Figure 2.5 ABCG5 expression in SUS liver relative to plasma LDL cholesterol levels

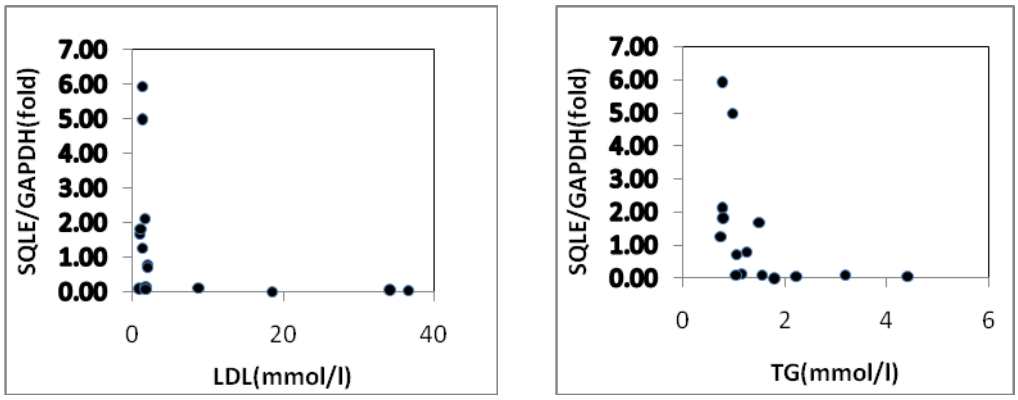


A B

Figure 2.6 DHCR7 (A) and SQLE (B) expression in SUS liver relative to plasma triacylglycerides

No other significant regressions of gene expression on plasma lipid levels were found in the RES. However, it is interesting to note that in both the SUS and RES, SQLE expression plasma triacylglycerides or LDL reached a threshold level (Figure 2.7).

RES Quail



SUS Quail

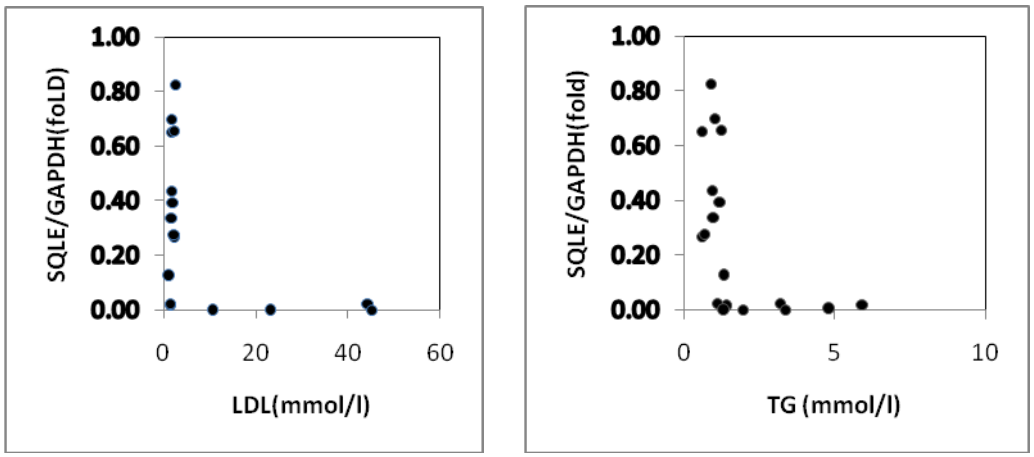


Figure 2.7 SQLE expression in SUS and RES liver relative to plasma triacylglycerides and LDL cholesterol levels.

2.4. Discussion

The RES and SUS quail were developed through divergent selective breeding from the same foundation population (17), and should be genetically similar except for that changes induced by selection. Other than the identification of some physiological differences between these two strains, there has been no genetic characterization of these strains. Since the liver plays a key role in regulating cholesterol homeostasis, acting as the main site for lipid metabolism and where bile salts are formed, we have focused our initial study on this organ - in particular, on the expression of several cholesterol biosynthesis and transporting genes in this organ. While gene expression is a phenotype, it will reflect more directly the genotypic changes than morphological or physiological phenotypes.

We also chose these genes because it was previously reported that one of the observable differences between RES and SUS was that after the feeding of cholesterol, elevated plasma cholesterol levels were sustained in the SUS but not in the RES (17).

2.4.1. Strain differences in gene expression

RES had significantly higher levels of ABCG8 expression in the liver than SUS under all dietary conditions, and higher ABCG5 expression under some dietary conditions. Evidence from both animal models and human research supports the important role of these two ABC transporters in regulating the excretion of sterols from the liver via bile to prevent the accumulation of dietary sterols (11). In human beings, mutations in either of these genes causes sitosterolemia, a disorder that is characterized by intestinal hyper-absorption of all sterols and impaired ability to excrete sterols into bile. Patients develop tendon and tuberous xanthomas, accelerated atherosclerosis, and premature coronary artery disease (11)(28, 29). On the other

hand, enhancing the ABCG5/8 pathway has been shown to protect against atherosclerosis by eliminating more cholesterol in the bile and reducing plasma cholesterol levels (30). For example, a mouse study has shown that over-expression ABCG5 and ABCG8 in an atherogenic model decreases diet-induced atherosclerosis, in association with reduced liver and plasma cholesterol levels (28). Another study of a partially inbred strain of opossums (*Monodelphis domestica*) showing low expression level of ABCG5 and ABCG8 was associated with an elevation in diet-induced VLDL and LDL cholesterol (31). In our study, the lower ABCG8 and ABCG5 expression in the SUS may be at least partially responsible for the susceptibility of this strain to diet-induced atherosclerosis (31).

In the RES, ABCG5 expression remained high regardless of dietary treatment, whereas in the SUS, an increased level of ABCG5 expression was induced by dietary cholesterol. The selection for susceptibility may have altered the regulation of the ABCG5 gene in the RES. Mouse studies provided evidence for the direct control of ABCA1, ABCG5, and ABCG8 mRNA expression by the liver X receptors. These gene expressions were up-regulated by cholesterol feeding (10, 12). RXR/LXR heterodimer transcript factor acts as an intracellular sterol sensor (12, 32). Accordingly, the selection for resistance may not have altered the expression of ABCG5 but rather altered the expression of some of these X receptor genes (12, 33, 34) enabling the ABCG5 expression to remain at an up-regulated state in the RES.

On the other hand, while we have essayed plasma cholesterol and triacylglycerides levels, we have not essayed intracellular cholesterol and triacylglycerides levels. The LDL receptor gene is regulated by the sterol regulatory element-binding protein (SREBP) pathway via negative feedback (35-37). When intracellular cholesterol level is high, the LDL receptor gene is down-regulated (37, 38). With fewer receptors, the liver takes up LDL less efficiently

from blood, and plasma LDL level increases. It is possible that there is a differential expression of the LDL receptor gene in the SUS and RES. The SUS, being less efficient in removing excess cholesterol from the liver, may have down regulated the expression of the LDL receptor gene and causing a higher level of dietary cholesterol to remain in circulation. It is therefore important to examine the expression of the LDL receptor gene in these quail under different dietary treatments.

The three cholesterol biosynthesis genes, HMGCR, FDFT1and SQLE, had lower expression in SUS than in RES, regardless of diet and age. These mevalonate pathway genes are also regulated by intracellular cholesterol via SREBP pathway (35-37). HMGCR, for example, has been shown to be regulated by sterol and non-sterol metabolites derived from mevalonate in a negative feedback loop (38-43). When cellular cholesterol levels are high, the proteins Insig-1 and Insig-2 (Insulin-induced genes) bind to Scap (SREBP cleavage-activating protein) and prevent movement of the Scap-SREBP complex from the ER to the Golgi, inhibiting transcription of the HMGCR gene. When cholesterol levels decrease, SREBP is released from the membrane by proteolysis and migrates to the nucleus - the activated SREBP binds to SRE and transcription is promoted. As well, non-sterol metabolites such as dolichol and ubiquinone have been shown to be required to fully suppress HMGCR activity by negative feedback (39, 40). Similar to HMGCR, the transcriptional regulation of FDFT1 and SQLE is also through the SREBP pathway (10). We have also found that the expression level of some of these genes regressed negatively on plasma LDL and triacylglyceride levels. A very high level of plasma cholesterol has a strong negative effect on SQLE expression. Since SQLE enzyme is the secondary rate-limiting enzyme in the cholesterol biosynthesis pathway (31), the absence of SQLE can be a protective action to turn off the endogenous cholesterol synthesis.

Thus, it is reasonable to hypothesize that the high intracellular cholesterol level in the liver cells of SUS is related to the sub-normal functioning of their transporter genes ABCG8 and ABCG5 and the down-regulation of the mevalonate pathway genes. This may be an ineffective attempt to normalize intracellular cholesterol levels in the SUS liver cells.

2.4.2. Age-related differences in gene expression

It has been well established that in human beings (41, 42) and in other animal species that have been studied (43, 44), older individuals are more susceptible to the development of atherosclerosis than younger individuals. In our study, three genes, APOA1, ABCG8 and DHCR7, showed age-related differences in gene expression. APOA1 and ABCG8 had higher expression in older than in younger quail of both strains. DHCR7 had the opposite trend. APOA1 is the major protein component of plasma HDL. This protein promotes cholesterol efflux from tissues to the liver for excretion. Our results indicate that older birds seemed to be more efficient in moving cholesterol from tissue to the liver for excretion (APOA1), more efficient in promoting biliary excretion of cholesterol (ABCG8), and less efficient in cholesterol biosynthesis (DHCR7). All three age-related differences do not account for the increased susceptibility in older individuals.

Colins *et al.* (2009) reported that in laboratory mice, age-related atherosclerosis correlated with the failure of up-regulating antioxidant genes (45). Previous studies in our laboratory with the SUS quail also found older birds exhibited complex time-dependent alterations in antioxidant status when placed on an atherogenic diet (20, 21). As a result, the oxidized modification hypothesis (as well as other alternative hypotheses) may be the main contributor to age-related atherosclerosis.

2.4.3. Plasma lipids and gene expression

Previous studies have reported rapid aortic plaque development in SUS quail fed a 0.5% cholesterol-supplemented diet (17-19). The visual scoring method has been used to assess the severity of atherosclerosis and the method has been validated by scanning electron microscopy (17-19). Those studies have demonstrated that increased plasma cholesterol level is a reliable method for the induction of atherogenesis.

The elevated LDL cholesterol in Japanese quail is similar to the hypercholesterolemia reported in atherogenic pigeons, which was attributed to the expression of non-functional LDL receptors rather than LDL-receptor down- regulation (38). Such observations indicate a possibility of defective receptor-mediated LDL clearance with hypercholesterolemia, which can be tested in future studies.

APOA1 is the major protein component of HDL and is responsible for transporting cholesterol out of the liver for excretion. We found that APOA1 regressed positively on plasma LDL and triacylglycerides levels (Figure 2.3). APOA1 may therefore be up-regulated by intracellular cholesterol to increase the efficiency of excreting cholesterol. However, it has been established that the relative contributions of various tissues to overall fatty acids and cholesterol synthesis vary among animal species (4). All of fatty acid synthesis takes place in the liver of chickens and humans, whereas in rats and swine, adipose tissue is a major contributor (46-48). Thus, the APOA1 may have an additional function in bird species (49, 50).

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CHAPTER 3: CDNA-AFLP DIFFERENTIAL DISPLAY IN THE LIVER AND THE SPLEEN OF ATHEROSCLEROSIS-SUSCEPTIBLE (SUS) AND ATHEROSCLEROSIS-RESISTANT (RES) JAPANESE QUAIL, (*COTURNIX JAPONICA*)[2]

3.1. Introduction

Atherosclerosis is a complex disease resulting from the interaction of a large number of genetic and environmental factors. Expression array analysis of hyperlipidemic ApoE KO mouse liver and adipose tissue has identified a large number of quantitative trait loci (QTLs) that co-localized with candidate genes involved in the development of atherosclerosis (1). However, we maintain that the divergent selection in Japanese quail to develop the SUS and RES strains (2) did not involve the change in expression of a large number of genes. 8% of randomly selected Japanese quail develop atherosclerotic plaques when fed a cholesterol-enriched diet (0.5% cholesterol w/w). Selection for resistance did not further improve the resistance significantly, as seen in the RES strain. Selection for susceptibility elicited a rapid response within 4 generations (2) and the response plateaued after a few more generations of selection. This is an indication that a few genes with major effects were involved rather than many genes with minor and epistatic effects. In Chapter 2, we studied the expression of 7

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candidate genes involved in lipid metabolism and cholesterol biosynthesis in the liver and found differences in expression related to strain in 4, with a 5th one caused by strain x diet interaction.

In this study, we used the cDNA-amplified fragment length polymorphism PCR (AFLP) technique in an attempt to identify genome-wide transcript patterns that were distinguishable between the SUS and the RES quail. cDNA-AFLP is one of the genome-wide expression profiling methods capable of finding genes that have not yet been cloned or even predicted (3-6).

3.2. Methods and materials

3.2.1. Experimental animals

Two strains of Japanese quail (*Coturnix japonica*), atherosclerosis-susceptible (SUS) and -resistant (RES), were used for cDNA-AFLP expression analysis. The SUS and RES quail were developed through divergent selective breeding. These strains have been shown to differ in their response to a cholesterol-enriched diet with 80% of the SUS males developing atherosclerosis in contrast to 4% of the RES males (2, 7-10).

The SUS and RES quail were acquired by the UBC Quail Genetic Resource Center from North Carolina State University in 1989, and have since undergone further divergent selection for susceptibility and resistance to atherosclerotic plaque formation induced by dietary cholesterol (0.5%w/w) (2, 7-10).

3.2.2. Experimental design

After hatching, both SUS and RES males were fed a regular diet (See Appendix 2) prepared by the feed mill at the Agriculture and Agri-Food Canada Research Station at Agassiz,

British Columbia, according to the NRC standard recommended for quail. At six weeks of age, some birds were sacrificed to obtain liver tissues. The remaining birds were divided into two dietary treatment groups and fed either a regular diet or a cholesterol-enriched diet for another 6 weeks (Table 2.1). At twelve weeks of age, 24 birds from two dietary treatment groups were euthanized and samples of liver tissue were obtained for further analysis.

3.2.3. Preparation of total RNA

3.2.3.1. Preparation of liver total RNA

Six quail from each treatment were euthanized by decapitation; livers were quickly dissected and stored in RNAlater reagent (Qiagen, Valencia, CA, USA) at -20 °C until use. Total RNA from livers was extracted using RNeasy mini columns (Qiagen, Valencia, CA, USA). The concentration and purity were checked by spectrophotometry. RNA was measured at OD 260nm, whereas protein was detected at OD 280nm. RNA with an OD260/OD280 ratio of 1.8-2.0 was considered sufficient quality for cDNA synthesis.

3.2.3.2. Preparation of spleen total RNA

After sacrificing the quail, spleens were also dissected and stored in RNAlater reagent (Qiagen, Valencia, CA, USA) at -20 °C until use. Total RNA from spleens was extracted using TRIzol® Reagents (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. The concentration and purity were checked by spectrophotometry (See section 3.2.3.1.) before being used for cDNA synthesis..

3.2.3.3. Preparation of aorta total RNA

After sacrificing the quail, aortae were dissected and stored in RNAlater reagent (Qiagen, Valencia, CA, USA) at -20 °C until use. Total RNA from aortae was isolated using TRIzol® Reagents (Invitrogen, Breda, The Netherlands), according to the manufacturer's instructions. The concentration and purity were checked by spectrophotometry (See section 3.2.3.1.) before being used for cDNA synthesis. However, after repeated trials, we were not able to obtain sufficiently good quality RNA from the aortic tissue and therefore cDNA was not synthesized from aortic tissue RNA.

3.2.4. cDNA synthesis

3.2.4.1. First-strand cDNA synthesis

cDNA reactions were performed using SuperScript™ III RT(Invitrogen, Breda, The Netherlands) at 50 °C , following standard procedures. Oligo $(dT)_{18}$ primer (MBI Fermentas Inc. , Hanover, MD, USA) was used for the synthesis according to the manufacturer's manual. A 38 µl reaction volume containing 5mg of total RNA , which was mixed with 1 µl Oligo$(dT)_{18}$ primer of a 100 mM concentration, 2 µl dNTP (10mM each), 8 µl 5 first strand buffer, 4 µl DTT (0.1 M) and 2 µl SuperScript™ III RT. In addition, 1 µl RiboLock™ RNase Inhibitor (40U/µl, MBI Fermentas Inc., Hanover, MD, USA) was added in each reaction mixture in order to inhibit RNA degradation during *in vitro* transcription.

The first-strand cDNA was used to make second-strand cDNA for AFLP.

3.2.4.2. Second-strand cDNA synthesis and cDNA-AFLP PCR

Second-strand cDNA was made using DNA polymerase I, *E.coli* (MBI Fermentas Inc. , Hanover, MD, USA), incubated at 15 °C for two hours and heated to 70 °C for 10 minutes to stop the reaction. The total volume of the reaction mixture was 198 µl, with 6ul DNA polymerase I, *E.coli* (10U/µl) (MBI Fermentas Inc., Hanover, MD, USA), 0.4 µl RNase H, *E.coli* of concentration 5U/µl (MBI Fermentas Inc., Hanover, MD, USA) and 38 µl first-strand cDNA already made.

Double-stranded cDNA was used for AFLP analysis as previously described (11). The concentration and quality of double stranded cDNA was checked by spectrophotometry using wavelengths in the range 200nm to 350nm. cDNA was detected at OD 260nm, whereas protein was detected at OD 280nm. Double stranded cDNA with an OD260/280 ratio of 1.7 or higher was selected to be diluted into 100ng/µl. 20 µl cDNA from liver and spleen was used in the double restriction enzyme digestion reaction; 1.2 µl *Eco*R 1 (12U/µl) and 0.8 µl *Mse* 1(4U/µl) enzymes (NEW ENGLAND BioLabs Inc.) were used to digest the samples and the total volume was 30 µl; the digestion was 2-3 hours at 37 °C. To check total digestion, 10 µl of digested DNA can be viewed on 0.8% agrose gel, looking for smears between 100-1200bp.

After digestion, the adapters were ligated to the DNA sequences. In each reaction, 0.5 µl *Eco*R 1 adapter (50pmol/µl) (Table 3.1), 0.5 µl *Mse* 1 adapter (50pmol/µl) (Table 3.1); 0.5 µl ATP (10mM), 0.5 µl T4 ligase (0.5 Weiss Unit) and 20 µl digested cDNA from last step were used. The total volume was 25 µl. The ligation was incubated for 3-16 hours at 37 °C.

Table 3.1 *Eco*R adapter and *Mse* adapter

Adapter name	Primer Sequence
*Eco*R (50pMol/µl)	5'-AAC GAC GAC TGC GTA CC-3’
	3’-CTG CTG ACG CAT GGT TAA-5'

Mse (50pMol/µl)	5'-GAC GAT GAG TCC TGA G-3' 3'-TA CTC AGG ACT CAT-5'

The pre-amplification PCR reactions were performed in a 15µl volume. Following ligation, the cDNA was diluted to a 1:10 ratio and 5µl was used in each reaction; the rest was stored at -20 °C for long- term. 0.6µl of each *Eco*R + N primer and *Mse*+N primer (50ng/µl) (Table 3.2), 0.8 µl dNTP mix (10×) and 0.24 µl Taq polymerase (5U/µl, Paq) was used. All amplifications were performed in a MJ thermocycler (MJ Research Products, Waltham, MA, USA) as follows: denaturation for 1 minute at 94 °C, followed by 28 cycles (30 seconds at 94 °C, 30 seconds at 60 °C, 60 seconds at 72 °C) and extension for 5 minutes at 72 °C. The pre-amplification product was viewed on 0.8 % agrose gel.

Table 3.2 *EcoR*/ *Mse* primer+N and pre-amplification primer

Pre-amplication primer		
Primer +N		
*Eco*R primer		
5'-GAC TGC GTA CCA ATT C-3'	+N	+G
		+C
		+A
Mse primer		
5'-GAT GAG TCC TGA GTA A-3'	+N	+C
		+A

The final amplification PCR used *Eco*R/*Mse* + NN primers (6ng/µl) (Table 3.3); the first nucleotide added was the same as the pre-amplification nucleotide. In addition, M13 primer (1pMol/µl) was used to label the PCR product. DNA from the previous step was diluted to a 1:40 ratio, 2.5µl was used in the reaction; the rest was stored in -20 °C for long-term. 0.3µl M13 labelled primer, 0.42µl *Eco*R primer, 0.42µl *Mse* primer, 0.4µl dNTP mix (10×) and Taq polymerase (5U/µl, Paq) were used, the total volume was 10µl. The PCR reaction was light-

sensitive and all reaction mixtures were covered with aluminum foil during the reaction and for storage.

The final amplification reaction was performed in a MJ thermocycler (MJ Research Products, Waltham, MA, USA) as follows: denaturation for 1 minute at 94 °C, followed by 4 cycles (30 seconds at 94 °C, 30 seconds at 65 °C, 60 seconds at 72 °C), cycle 4-15 was lowering annealing temperature 0.7 °C each cycle (30 seconds at 94 °C, 30 seconds from 65 °C -0.7 °C /cycle, 1 minute at 72 °C) , from cycle 16 to cycle 38, each cycle ran 30 seconds at 94 °C, 30 seconds at 56 °C, 60 seconds at 72 °C , then 5 minutes at 72 °C for extension.

Table 3.3 Final-amplification primer combination. Each row is one final-amplification primer combination: *Eco*R prime and *Mse* primer plus two selective bases in 3'position. Combinations in bold letters detected differential expression (See Sections 3.3.2. and 3.3.3.)

*Eco*R primer+NN	+GT	*Mse* primer+NN	+CT
	+GT		**+CC**
	+GT		+CG
	+GA		+CA
	+GA		+CT
	+GA		+CG
	+GA		+CC
	+GA		+AC
	+GA		+AG
	+CT		+AG
	+CT		+AA
	+CT		+AT
	+CT		+AC
	+CA		**+CT**
	+CA		+AT
	+CA		+AC
	+AG		+AG
	+AG		+AA
	+AG		+AT

+AG	+AC
+AC	+AG
+AC	+AA
+AC	+AT

3.2.5. cDNA-AFLP analysis

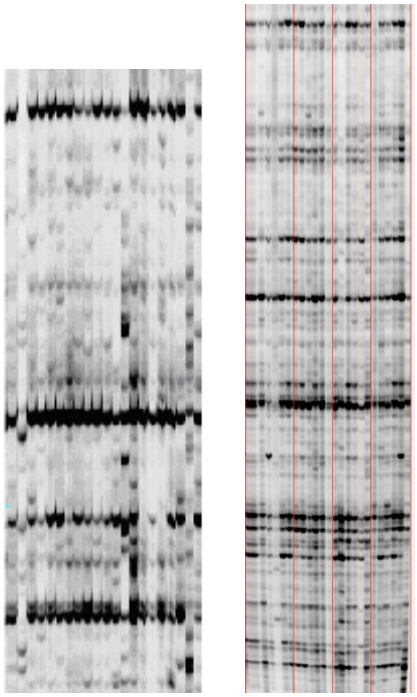
Samples were run on a LiCor 4200 auto-sequencer (LI-COR Biosciences, Nebraska, US) using a 25cm plate with a 7% Long Ranger gel. The spacer and comb thickness was 4nm and the comb was a 48 sharktooth comb. The running conditions were as follows: Speed=4, voltage=2000V, Amperage=35mA, Power=70W, Heat=50 °C, Time=5:00hours.

Differentially transcript-derived fragments were selected as those differentially expressed among treatment groups and at least four individuals showed the same pattern (with band or without band) within each treatment group.

3.3. Results

3.3.1. AFLP markers

In theory, *EcoR*/*Mse*+NN can produce 256 primer combinations to cover the whole genome. We have examined 23 primer combinations (Table 3.3) or 9% of the total coverage. Of the primer combinations that we have examined, poor quality primer combinations (which either showed unscorable band patterns even after optimization (e.g. Figure 3.1 A) or no variations in all of the individuals (e.g. Figure 3.1 B) were eliminated . Only two primer combinations (Bold in Table 3.4) were considered as effective markers, allowing the discovery of the transcripts that were differentially expressed in the livers and the spleen.



A B

Figure 3.1 Unscorable cDNA-AFLP examples (A) Spleen AFLP amplified by *Eco*R+GT/*Mse*+CC. The figure shows both random pattern among individuals and also some bands that were smeared and could not be scored. (B) Liver AFLP amplified by *Eco*R+CT/*Mse*+GA. The figure does not show much variation among individuals.

3.3.2. Differential expression between SUS and RES

AFLP reactions were performed using *Mse* and *Eco*R primer combinations on liver cDNA, resulting in the detection of about 50 cDNA fragments of which 2 were differentially expressed between RES and SUS (these being consistent over both dietary treatments). Both fragments were amplified by primer combination *Eco*R+GT/*Mse*+CC, an over 700bp band and a 460bp band (Figure 3.2).

The 460bp fragment was present in 11 of the 12 12-week SUS (both dietary treatment groups: SUS RD wk12 and SUS CD wk12) but not present in 11 of 12 12-week RES birds

(Figure 3.2 and Figure 3.3). This fragment was present in 1 of 6 6-week SUS on the regular diet and none of the 6 6-week RES on the regular diet.

The 700^{+}bp fragment was present in 9 of the 12 12-week SUS (both dietary treatment groups: SUS RD wk12 and SUS CD wk12) but not present in 8 of 12 12-week RES birds (Figure 3.2 and Figure 3.3). This fragment was not present in any of the 6-week old birds.

AFLP reactions using the same primer combinations did not reveal any significant differential expression in the spleen.

In a repeatability trial 3 months later using stored cDNA, only the 460bp fragment was consistent with the first trial, whereas the 700^{+}bp fragment showed considerable deterioration and was weakly expressed (Figure 3.3).

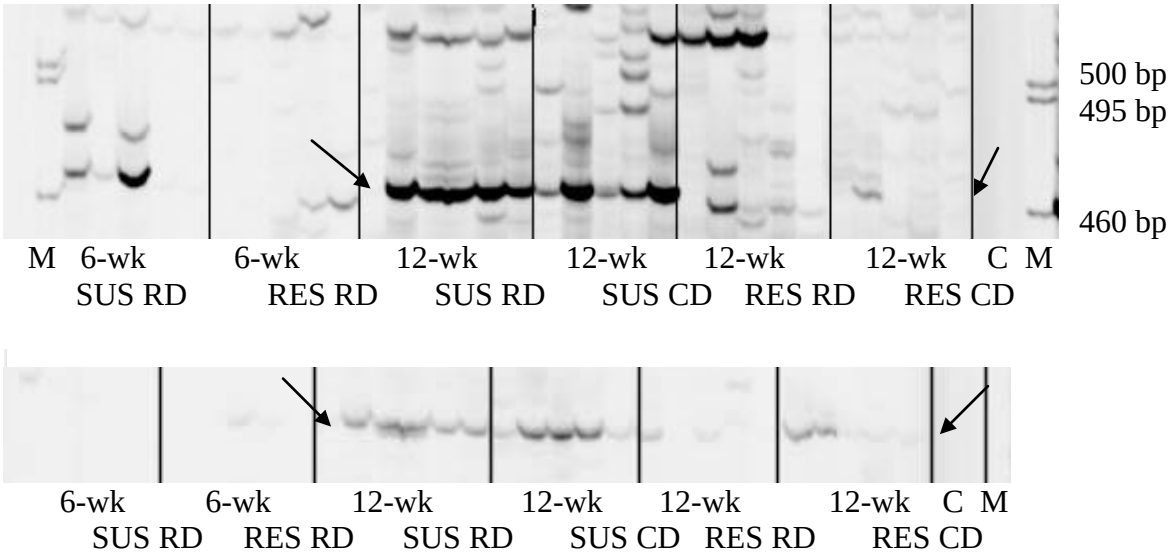


Figure 3.2 cDNA-AFLP analysis with primer combination *Eco*R+GT/*Mse*+CC of liver of 6 or 12 weeks old SUS and RES Japanese quail on regular (RD) or cholesterol diets (CD). M=molecular weight marker, C=no template control Upper gel shows the 460bp band variation and the lower gel: shows the 700+bp band variation.

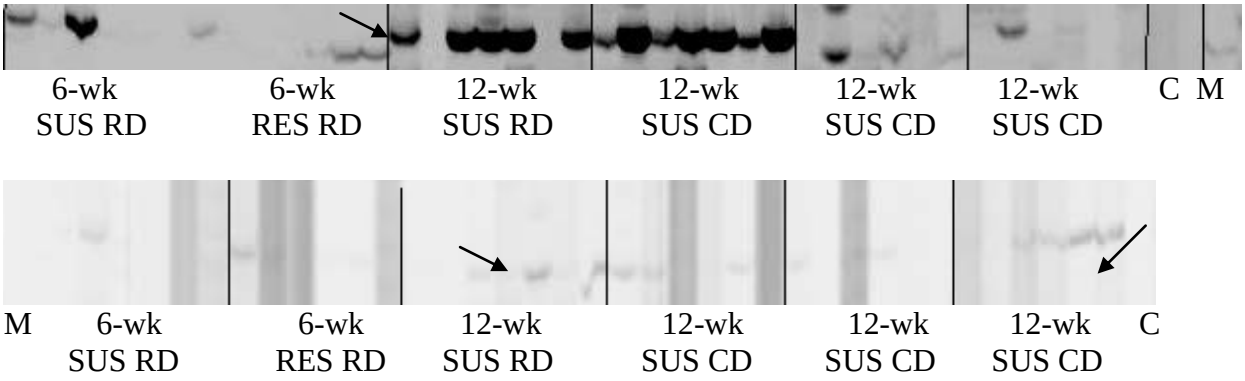


Figure 3.3 Repeatability trial of primer combination EcoR+GT/*Mse*+CC analysis of liver of 6 or 12 weeks old SUS and RES Japanese quail on regular (RD) or cholesterol diets (CD), M=molecular weight marker, C=no template control. Upper gel shows the same 460bp band variation as in Figure 3.2, and the lower gel shows the deteriorated 700+bp band

3.3.3. Differential expression related to diet

We detected one differential expression in spleen cDNA-AFLP, amplified by *Eco*R+CA/*Mse*+CT, that seems to be related to a difference in diet treatment, Overall, the primer combination resulted in more than 40 bands and only a 365bp band was found to show differences in expression in birds on different diets. The fragment was present in all the 6-wk SUS, RES RD quail, four out of six 12-wk SUS RD quail and five out of six 12-wk RES RD quail. It was not present in any of the 12-week RES CD and four of six 12-wk SUS CD (Figure3.4). The repeatability test results were consistent with the initial trial (Figure 3.5).

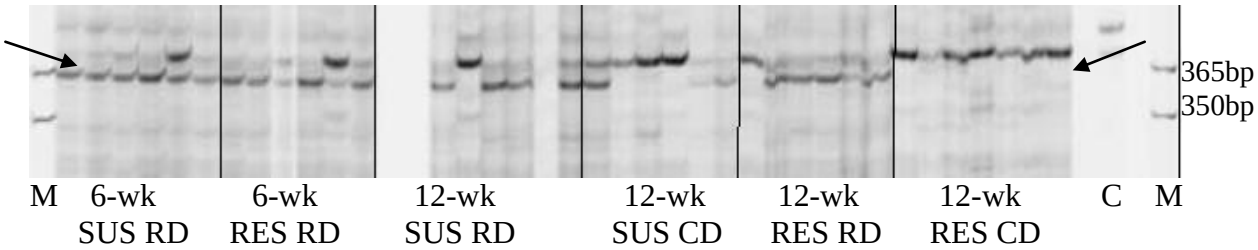


Figure 3.4 cDNA-AFLP analysis with primer combination *Eco*R+CA/*Mse*+AT of spleen of 6 or 12 weeks old SUS and RES Japanese quail on regular (RD) or cholesterol diets (CD). Gel shows the 365bp band variation

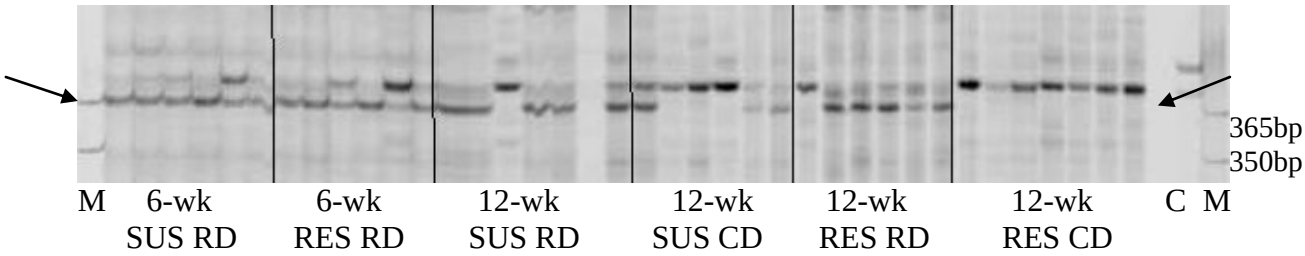


Figure 3.5 Repeatability trial of primer combination *Eco*R+CA/*Mse*+AT analysis of spleen of 6 or 12 weeks old SUS and RES Japanese quail on regular (RD) or cholesterol diets (CD). Gel shows the same 365bp band variation as in Figure 3.4

AFLP reactions using the same primer combinations did not detect any significant differential expression in the liver.

3.4. Discussion

Our study employed the AFLP technique to detect differential gene expression in the liver and spleen of the SUS and RES quail in order to test our hypothesis that the divergent selection to develop these two strains, while resulting in large differences in their phenotypes, has not drastically altered their genotype. We have developed the hypothesis based on the following observations:

1) The two strains originated from the same foundation population and therefore their ancestral genotypes were very similar (2).

2) The selection criteria were robust, based on visual classification of the severity of the lesions and not by histological, biochemical, or molecular evaluation (2, 7, 8, 12). The selection was for diet-induced atherosclerosis. The resistance and susceptibility were only expressed when the birds were challenged by a high cholesterol diet. Quail of either strain fed the regular diet did not express this trait. This genotype by environment interaction should slow down

genetic changes caused by the selection (13-15). The diet-induced atherosclerosis was only expressed in males and therefore selection has been on males only. This sex-limited trait should also slow down genetic changes (15-17).

3) Given the limitations mentioned in the above paragraph, the response to selection was rapid, and so were the plateaus. This is an indication that selection was effective in altering the expression of a small number of genes with major and additive effects, rather than a large number of genes with non-additive (dominance and epistasis) or minor effects (14, 18, 19).

Results of AFLP reactions performed using *Mse* and *Eco*R primer combinations on liver cDNA indicated that about 50 cDNA fragments per primer were detected, of which only one showed differential expression between the SUS and RES quail. AFLP reactions using the same primer combinations detected more than 40 cDNA fragments per primer in the spleen, but none of these showed differential expression related to strain. At first glance, these results seem to support our hypothesis that there were no major differences in gene expression between the two strains. However, several factors need to be considered before any conclusions can be drawn:

1) Unlike genome-wide expression arrays, AFLP reactions using 23 primer combinations covered only a small portion of the genome. Experimental results from other organisms also indicated that cDNA-AFLP is not highly efficient in finding target products. For example, in a *Arabidopsis thaliana* cDNA-AFLP experiment, 228 primer combinations were used generating 16,000 highly reproducible banding patterns (average 70 bands/primer), but only 60 band differences were detected (0.4%) (20) . In a study investigating potato tuber formation, the authors found that about 2% out of the 200 cloned fragments appeared to be derived from the related developmental process (11). Another similar study involving *Globodera rostochiensis* tested 40 primer combinations, resulting in about 4500 transcript

fragments. Then eight primer combinations were used to generate about 230 “discrete” bands (5%) (21).

2) Unlike genome-wide expression arrays or real-time PCR, AFLP reactions can only detect all-or-none differences and cannot quantify less obvious differences (22, 23). The absence of a band is an indication that no mRNA product was produced, which may lead to the absence of the protein (22). Everything else being equal, the total lack of expression would have more drastic effects than a decrease or increase in the level of expression. An extreme example would be a knock-out gene which is permanently disabled (24, 25). We therefore do not expect many of this type of gene to exist in the genome to be detected by AFLP reaction assays.

3) We have only examined the liver and the spleen, two organs that are removed from the site of atherosclerotic lesions. Unfortunately, we were not able to examine the aorta and the macrophages, where more expression differences are expected (26-32).

4) We would expect to find a diet x strain interaction in the expression difference but we did not.

3.5. Conclusion

In conclusion, we have detected only one gene expression difference in the liver and spleen of the two divergently selected quail strain. However, for the various reasons given above, even this low rate (0.14%) of occurrence could not make us reject our null hypothesis.

Genome-wide gene expression profiles assay tens of thousands genes simultaneously; they provide an unbiased profile of expressed genes, which may lead to insights into previously unknown molecular interactions (5, 33, 34). Currently, high-throughput techniques such as serial analysis of gene expression (SAGE) and hybridization-based microarray are widely used

for large-scale monitoring of gene expression. SAGE can provide quantitative data concerning gene expression, mining sequence databases to determine the original mRNA (and therefore which gene) tag. Microarray technology is extremely powerful in generating a broad view of gene expression. While these tools could not be utilized within the time frame of this MSc thesis, it can be utilized in future research to follow up on our initial attempt to find support for our hypothesis.

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CHAPTER 4: GENERAL DISCUSSION

Despite progress in its management and identification of risk factors, atherosclerotic cardiovascular disease remains a major cause of morbidity and mortality worldwide (1, 2). Genetic factors contribute to the development and clinical course of the disease through multiple pathways (3). Gene expression in any individual is a temporal and spatial process, and disease processes are affected by such changes in gene expression (4, 5). We therefore hypothesize that under controlled environmental conditions, differences in susceptibility to atherosclerosis reflect differences in genetic makeup, and these differences can be reflected by gene expression patterns that are temporally related to the development of disease. This thesis reports the results of two studies that were undertaken to examine the susceptibility differences to develop atherosclerosis at the transcriptional level, using two strains of quail known to exhibit susceptibility difference.

The selectively bred atherosclerosis-susceptible (SUS) and atherosclerosis-resistant (RES) quail have been shown to differ in their response to a diet supplemented with 0.5% (w/w) cholesterol with 80% of the SUS males developing atherosclerosis in contrast to only 4% of the RES males (6-10). Histological study of atherosclerotic lesions of the quail aortae revealed a close similarity to lesions of human atherosclerosis (6, 7). Other comparative studies using these two strains have provided related information on physiology and pathogenesis (6-10). However, because of the differences in lipoprotein composition, the variations in diet between quail and human and the interaction of experimental conditions and lipid and lipoprotein metabolism (7, 10-12), animal models may provide insights to similar or alternative pathways of the course of atherosclerosis.

Cholesterol is the crucial component for all animals, but it is also suggested to be involved in atherogenesis and pro-atherosclerotic pathways (13-15). Chapter 2 compared the gene expression level of cholesterol metabolism and transport genes to investigate the genetic basis of the susceptibility difference between the two strains. The results demonstrated that strain-related differences in susceptibility to atherosclerosis correlated with gene expression differences of some cholesterol biosynthesis gene and cholesterol excretion transporter gene in liver tissue. SUS had significantly lower expression of the cholesterol transport genes ABCG8 and ABCG5 than RES. This is an indication that the SUS quail are not as efficient as the RES in excreting the excessive ingested cholesterol. Three cholesterol biosynthesis genes, HMGCR, FDFT1 and SQLE had significantly lower expression in SUS than in RES, suggesting that in the SUS, the biosynthesis of endogenous cholesterol has been down-regulated and even turned off – presumably because of the excessive retention of exogenous cholesterol. Previous studies (6) have reported that following cholesterol feeding, plasma cholesterol levels remained high for a significantly longer time in the SUS than in the RES. The SUS birds were also found to have fatty livers and higher amounts of liver cholesterol (6) . Shih *et al.* hypothesized that the reason the RES strain is more resistant is because "they metabolized and excreted cholesterol faster than SUS strain" (6). Our study may have provided an explanation for this observed difference in susceptibility.

LXR acts as an oxysterol sensor and activates transcription by various oxidized cholesterol derivatives (oxysterols) or LXR agonists (16-18). The natural ligand for LXR is oxysterol, normally formed in amounts proportional to cholesterol amount in the cell (16-18). It has been demonstrated that both ABCG5 and ABCG8 mRNAs are up-regulated through the LXR pathway. LXRs are also involved in regulation of many other cholesterol transport and

metabolic processes by affecting the gene expression of the target mRNA, e.g. cholesterol 7alpha-hydroxylase (CYP7A1), cholesteryl ester transfer protein (CETP), and ABC transporters (ABCG1 and ABCA1) (19). Through LXR stimulation, these target genes can be regulated to minimize excessive cholesterol accumulation by inhibiting cholesterol absorption, promoting cholesterol efflux from cells to HDL, converting cholesterol to bile acids, and facilitating biliary excretion (19). Our study showed that such systems may have been compromised in the SUS and the accumulation of intracellular cholesterol is at least one of the causes of their increased susceptibility to atherosclerosis. Kushwaha *et al*. reported that baboons with high and low cholesterolemic responses to dietary lipids differed in intestinal cholesterol absorption and hepatic cholesterol metabolism (20). Their hepatic CYP7A1, ABCG5 and ABCG8 gene expression were higher in low-responding baboons than in high-responding ones. In opossum, it has been found that CYP27A1 (sterol 27-hydroxylase), ABCG5, ABCG8 and SLC10A1 (solute carrier family 10 member1) genes were expressed at lower levels in those that showed high levels VLD and LDL when fed a high-cholesterol and low-fat diet compared with low-responding opossums on the high-cholesterol and low-fat diet (21, 22). Further studies on the differential expression of the LXR-regulated genes in the RES and SUS would seem warranted.

An effective up-regulation of ABCG5 and ABCG8 could provide a potential therapeutic approach in the treatment of atherosclerosis. LXR agonists that induce the ABCG5, ABCG8 expression without blocking the cholesterol biosynthesis pathway may be an effective treatment. Drugs such as statins have already been demonstrated to have certain protective functions as anti-atherosclerotic agents (25, 26). Statins, however, lower cholesterol levels by inhibiting HMGCR, which not only reduces cholesterol levels but also non-cholesterol steroidal products (23, 24). This may result in adverse side-effects (23, 24). A good agent requires the desired

function without such side effect (19). LXR agonists seem to fit the bill and might be potential hypolipidemic drugs.

Another finding is that in both strains, older birds (12 weeks) had significantly higher ABCG8 and APOA1 expression and lower DHCR7 expression than young birds (6 weeks). Japanese quail have a relatively short generation interval. They became sexually mature when 4 weeks old and are considered "old" at 20 weeks of age. Humans and many animal species show age-related increases in susceptibility to atherosclerosis (10, 27), but results from our quail study would suggest that this may not be due to age-related changes in sterol absorption and/or excretion ability (Chapter 2).

Our real-time PCR study could only measure the expression level of a few candidate genes in the liver. However, it could not give a broad picture about the process of disease development and could not provide information on other regulatory mechanisms or systems contributing to the pathogenesis of atherogenesis. Thus, a time-course studies would be the logical next step. In addition, protein level confirmation should be included in future study in order to verify the relationship between transcription to translation and even post-translation (28-30).

This study is the first attempt to understand the genetic basis of susceptibility differences in this model. SUS and RES have been under investigation for physiological and biochemical differences related to their susceptibility (6, 7, 9, 10, 31), but there has not been much information on their genetic differences. The assumption was that since these strains were developed through divergent selective breeding and showed differences in their phenotype, there must be genotypic differences. The nature and extent of genetic differences were not addressed by the physiologists and pathologists who were utilizing these animal models. Our premise was

that the lack of genetic information would hinder the interpretation of data generated with these models, and therefore we felt it was important to study these differences. For example, the knock-out mice proved to be useful models because the genetics are known, and the ramifications of the effects of the knocked-out genes have been well studied. Although little is known about the genome of quail, the closely related phylogenetic relationship between quail and chicken provided the good opportunity for cross-species amplification in real-time PCR. With the completion of the chicken genome sequence, large-scale genome-wide array based gene expression analysis becomes possible and will benefit quail studies from many aspects. A chicken multi-tissue cDNA microarray with 13007 features has been available since 2005 (32) and is believed to be a promising resource for quail microarray in future.

While real-time PCR allowed us to use existing genetic information to test our hypothesis, we had to resort to a genome-wide transcriptional analysis to target the unknown cDNA fragments that may represent the genotype differences. Particularly, cDNA-AFLP enabled us to discover transcriptional level expression differences without prior knowledge of any genomic information (33-36). Thus, in Chapter 3, gene expression profiles from the two quail strains were generated with cDNA-AFLP before and after challenge with a high cholesterol diet. Unfortunately, with the limitations of time and resources, we were only able to examine 23 primer combinations (See Table 3.3) which amounted to only 9% of the total genome coverage. We also limited our study to an investigation of gene expression in the liver and the spleen. Of the primer combinations that we have examined, only two sets detected transcripts that were differentially expressed in the livers and the spleen of the two strains. Although it is not possible to draw conclusions based on such a small percentage of the coverage and for the reasons mentioned in Chapter 3, our results did give us some indication

that there were no wide-ranging genetic changes that caused the difference in cholesterol metabolism observed in these two strains of quail.

For future studies along this line, other powerful and well informative genome-wide techniques, such as microarray and SAGE, should be used to explore more systematic information. It will be important to also conduct a time-course study with the aorta and other vascular tissues.

In summary, we have taken the first steps in examining the differences in gene expression between the atherosclerosis-resistant and the atherosclerosis-susceptible quail. We have detected expression differences in genes in the liver that relate to cholesterol metabolism and synthesis. Our results suggest that further studies along this line would be worthwhile.

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APPENDIX 1: ANIMAL CARE CERTIFICATION

THE UNIVERSITY OF BRITISH COLUMBIA

Xinrui Li

has successfully completed the online training requirements of the Canadian Council on Animal Care (CCAC) / National Institutional Animal User Training (NIAUT) Program

Chair, Animal Care Committee

Veterinarian

Certificate #: 2104

Date Issued: November 24, 2006

APPENDIX 2: INGREDIENTS OF REGULAR AND CHOLESTEROL DIETS

Ingredient		Regular diet	Cholesterol diet
Soy protein flour (50% protein)	(g/kg)	340	340
Corn starch	(g/kg)	400	390
Limestone	(g/kg)	50	50
Mineral premix	(g/kg)	5	5
Monofos	(g/kg)	30	30
Sucrose	(g/kg)	20	20
Alphacel	(g/kg)	70	70
Vitamin premix	(g/kg)	5	5
D-L methionine	(g/kg)	4	4
Choline chloride	(g/kg)	3.8	3.8
Tallow	(g/kg)	50	50
Vegetable oil	(g/kg)	30	30
Cholesterol	(g/kg)	0	5
Cholic acid	(g/kg)	0	2.5