Chapter 1

Animal Models of Diet-induced Hypercholesterolemia

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Additional information is available at the end of the chapter

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1. Introduction

Cholesterol is a component of the cell membrane and metabolites of cholesterol, such as bile acids, steroid hormones and vitamin D, serve important biologic functions in vertebrates. Cholesterol is synthesized primarily in the liver and transported to cells throughout the body by lipoproteins via the blood, even though all nucleated cells in the body are capable of synthesizing cholesterol. Whole-body cholesterol homeostasis is determined by cholesterol absorption, cholesterol synthesis and cholesterol excretion, and losing control of any of these processes leads to an increase in plasma cholesterol. Liver and intestine are the major sites that control cholesterol homeostasis. The liver synthesizes cholesterol for secretion in nascent lipoproteins when blood levels of cholesterol are low, and removes excess cholesterol from the blood by taking up chylomicron remnants, high density lipoprotein (HDL), very low density lipoprotein (VLDL) and low density lipoprotein (LDL) particles. It converts cholesterol into bile acids, and secretes cholesterol and bile acids into bile for elimination from the body. The intestine regulates influx of cholesterol from the lumen and efflux of cholesterol back into the lumen to control the amount of cholesterol that enters the body [1].

Hypercholesterolemia is characterized by LDL cholesterol exceeding 159 mg/dl [2]. Many developed countries have a high prevalence of hypercholesterolemia. According to an estimate based on data from the 2005-2008 National Health and Nutrition Examination Survey, the Centers for Disease Control and Prevention reported that 33.5% of US adults aged 20 or older had high levels of LDL cholesterol [3]. Diets containing high levels of cholesterol and high levels of fat (HCHF) are frequently the culprit in causing hypercholesterolemia. In addition, genetic factors influence susceptibility to diet-induced hypercholesterolemia.

Hypercholesterolemia is a complex disorder often due to multiple genetic defects and rarely due to a single genetic defect as in the case of familial hypercholesterolemia [4]. Because

hypercholesterolemia is associated with risk of developing atherosclerosis, much research has been devoted to understanding the genetic variants and environmental factors that contribute to elevated blood LDL cholesterol. There are several challenges to investigating gene-diet interactions in humans. It is difficult to control the diet and environment of human subjects for long periods of time, and ethical constraints limit access to tissue samples. These problems can be circumvented by using animals to study the effects of diets on cholesterol homeostasis and atherosclerosis because animals can be fed the same diet and kept under the same laboratory conditions for long periods of time, and because access to tissue samples from animals is less restricted. Numerous animal species have been used as animal models for investigating hypercholesterolemia, including rabbits [5,6], mice [7], guinea pigs [8], minipigs [9], laboratory opossums [10] and nonhuman primates [11-15]. Nonhuman primates are more similar to humans than other animals, but ethical issues, facilities and high cost limit studies with nonhuman primates. Non-primate animal models have other limitations. However, extensive use of the collage of primate and non-primate models has provided considerable insights into the genes and molecular mechanisms that control plasma cholesterol in response to diets.

In this chapter, we discuss mouse, laboratory opossum and nonhuman primate models of hypercholesterolemia. Mice lipoprotein profiles differ from humans and they are resistant to developing hypercholesterolemia and atherosclerosis, but genetic engineering tools have been used effectively to alter their lipoprotein profiles. A commonly used mouse model in which the apolipoprotein E gene (*apoE)* is disrupted exhibits a lipoprotein profile similar to that of humans. In addition, *apoE* knockout mice become hypercholesterolemic and have a propensity to develop atherosclerosis [16,17]. Mice have also been used extensively to elucidate mechanisms that regulate cholesterol homeostasis. Cholesterol excretion is one of the major processes that can be targeted to reduce hypercholesterolemia. A nonbiliary pathway for disposal of cholesterol termed transintestinal cholesterol excretion (TICE) was first described in mice almost a decade ago [18]. A growing body of evidence for TICE has since been gathered using genetically modified and normal mice [19,20]. The discovery of TICE has opened a new avenue of research into the role of the intestine in cholesterol excretion [21].

The laboratory opossum is a model of diet-induced hypercholesterolemia developed at Texas Biomedical Research Institute that does not require genetic manipulation to knock out or overexpress specific genes to elevate LDL cholesterol [22,23]. Through many generations of inbreeding and selection for plasma cholesterol response to an HCHF diet, high and low responding strains of opossums were produced. All strains have normal levels of plasma cholesterol on a basal diet. However, high responding opossums exhibit an extremely high LDL cholesterol response when fed an HCHF diet compared to low responding opossums. Hypercholesterolemia in high responding opossums is caused by mutations in at least two genes. One of the causative genes has been identified as *ABCB4;* mutations in the *ABCB4* gene impair biliary cholesterol secretion [24]. The *ABCB4* gene has not been shown previously to be associated with hypercholesterolemia. The opossum model provides an opportunity to investigate genes that interact with *ABCB4* to regulate cholesterol homeostasis.

Nonhuman primates are utilized as models of hypercholesterolemia because of their physiologic and genetic similarities with humans [25]. In addition, nonhuman primates naturally develop hypercholesterolemia and atherosclerosis, both of which can be exacerbated by HCHF diets to mimic diet-induced hypercholesterolemia and atherosclerosis in humans [11,15,26]. Because of these characteristics, nonhuman primates including baboon (*Papio hymadryas*), rhesus macaque (*Macaca mulatta*), green monkey (*Chlorocebus aethiops*) and cynomolgus monkey (*Macaca fascicularis*) have been used as animal models for biomedical research aimed at understanding diet-induced hypercholesterolemia in humans. Nonhuman primates exhibit species and individual variations in plasma cholesterol in response to HCHF diets. Studies using pedigreed baboons and high-throughput sequencing technology have identified genetic factors that influence plasma cholesterol response to HCHF diets [13,27].

2. Mouse models

2.1. Mouse models of cholesterol metabolism

Mouse models are the most widely used animal models because of several advantages such as ease of breeding, large litter size, a short generation time of 9 months and economies of colony maintenance. An additional advantage of mice is the availability of tools to add exogenous genes to the germ line to create transgenic mice, or to disrupt endogenous genes by homologous recombination in murine embryonic stem cells to create knockout mice. Although there are important differences between mice and humans in lipoprotein and cholesterol metabolism, genetic manipulation has provided mouse models that resemble some aspects of hypercholesterolemia in humans.

Non-genetically modified mice have high levels of HDL cholesterol and low levels of LDL cholesterol, whereas humans have high levels of LDL cholesterol and low levels of HDL cholesterol. The difference in lipid profiles between mice and humans is due to absence of the cholesteryl ester transfer protein (CETP) in mice [28,29]. CETP is an enzyme that transfers cholesterol ester from HDL to VLDL and LDL in exchange for triglycerides [30]. In normal mice lacking CETP, more than 80% of plasma cholesterol is carried on HDL, so mice with high levels of HDL cholesterol are resistant to hypercholesterolemia and atherosclerosis. To overcome this problem to using mice as models for research aimed at understanding cholesterol metabolism in humans, several genetically engineered strains of mice were generated to alter the distribution of plasma cholesterol from HDL to VLDL and LDL. The genetically modified mice include CETP transgenic, apoE knockout and LDL receptor knockout mice.

CETP transgenic mice. Transgenic mice carrying human and cynomolgus monkey versions of the *CETP* gene were studied to investigate the effects of CETP on distribution of plasma lipoprotein cholesterol. Transgenic mice expressing high levels of human CETP showed a small decrease in HDL cholesterol and a small increase in VLDL and LDL cholesterol on the basal (chow) diet [29]. Transgenic mice expressing high levels of cynomolgus monkey CETP showed greater responsiveness than nontransgenic mice when challenged with an HCHF diet. Total plasma cholesterol of *CETP* transgenic mice averaged 250 mg/dl whereas those of

nontransgenic mice averaged 163 mg/dl. Furthermore, *CETP* transgenic mice showed that CETP activity was inversely associated with apoA-I, but positively associated with apoB [28]. These observations demonstrated that human and monkey CETP can interact with mouse lipoproteins to mediate its effects in lipoprotein metabolism.

ApoE deficient mice. ApoE is a 34 kD glycoprotein produced primarily in the liver and to a lesser extent in other tissues. With the exception of LDL particles, apoE is a structural component of all lipoprotein particles and chylomicrons. It binds to the LDL receptor and to the LDL receptor-related protein to remove VLDL and chylomicron remnants from the plasma [31]. Using gene targeting to disrupt the *apoE* gene, mutant mice developed severe hypercholesterolemia as expected from a defect in lipoprotein clearance from plasma. Total plasma cholesterol levels were highly elevated in homozygous apoE deficient (*apoE*-/-) mice (400-500 mg/dl) compared with normal mice (80 mg/dl) on a chow diet with 0.01% cholesterol and 4.5% fat. A more dramatic increase in plasma cholesterol was observed in *apoE*-/- mice (1800 mg/dl) fed an HCHF diet with 0.15% cholesterol and 20% fat. Plasma cholesterol concentrations in the VLDL and intermediate density lipoprotein (IDL) fractions were increased on both diets. The *apoE*-/- mice were highly susceptible to atherosclerosis, even on chow diet, as a result of the increase in plasma cholesterol concentrations [16,17].

LDL receptor deficient mice. The LDL receptor is a cell surface receptor expressed in many cell types. In the liver, the LDL receptor regulates plasma cholesterol by binding to apoB and apoE on the surface of lipoprotein particles and removes these particles from the plasma [32]. Patients with familial hypercholesterolemia [33] and Watanabe-heritable hyperlipidemic rabbits [34] develop elevated levels of LDL cholesterol due to mutations in the LDL receptor. Based on this knowledge, LDL receptor knock out (*LDLR*-/-) mice were generated to increase plasma LDL cholesterol concentrations. The loss of functional LDL receptors elevated total plasma cholesterol in *LDLR*-/- mice, but the effect was more moderate compared to *apoE*-/- mice. The mean total plasma cholesterol on a chow diet was 293 mg/dl and on a diet enriched with cholesterol (0.2%) and fat (19%) was 425 mg/dl. The increase in plasma cholesterol in *LDLR*-/- mice was attributed to increases in IDL and LDL cholesterol [35]. Compared with familial hypercholesterolemia patients (receptor-negative homozygotes) who have plasma cholesterol levels over 700 mg/dl, the effect of LDL receptor deficiency is less severe in mice. This is due to the fact that mice produce VLDL particles containing both apoB-48 and apoB-100, whereas humans only produce VLDL particles containing apoB-100 [36]. In mice, VLDL particles containing apoB-48 can be cleared from the plasma by the chylomicron remnant receptor in addition to the LDL receptor, so fewer VLDL particles are converted to LDL particles. Therefore, LDL receptor deficiency in mice does not increase plasma cholesterol to the same extent as in humans.

2.2. Mouse models of atherosclerosis

Disruption of the *apoE* gene causes hypercholesterolemic *apoE* knockout mice to develop atherosclerotic lesions spontaneously [16,17]. Foam cell lesions were observed in chow-fed *apoE*-/- mice 10 weeks after birth and the lesions progress to fibrous plaques by 20 weeks of age. An HCHF diet accelerates all stages of lesion formation and increases the size of lesions;

thus, formation of atherosclerotic lesions in *apoE-/-* mice is responsive to diet as in humans. Because of the short period of time for lesion formation in apoE-/- mice, they have been used extensively to study dietary and genetic factors affecting atherosclerosis and mechanisms of atherogenesis, as well as to assess efficacy of pharmacologic agents on lesion size. Progression of early lesions to advanced lesions in *apoE-/-* mice is similar to that in humans; lesions often develop at vascular branch points and progress rapidly to foam cells with fibrous plaques and necrotic lipid cores [37]. The major difference from humans is a low incidence of ruptured plaques that leads to thrombosis and arterial occlusion. Plaque rupture is the event that causes a heart attack in humans. A higher incidence of ruptured plaques was obtained by feeding a diet enriched with 0.15% cholesterol and 21% fat to *apoE-/-* mice. After 8 weeks of feeding the HCHF diet to *apoE-/-* mice, acute plaque ruptures were observed in the brachiocephalic arteries of more than half of the animals [38].

2.3. Mouse models of cholesterol excretion

Hepatobiliary cholesterol excretion. The human body cannot degrade cholesterol, but it can convert cholesterol to bile acids in the liver. Fecal excretion is the major route for the body to remove cholesterol as bile acids or neutral sterols (cholesterol and its metabolites formed by bacteria in the intestine). Diet-induced hypercholesterolemia can be mitigated by increasing the loss of cholesterol in feces. Fecal excretion of cholesterol and bile acids depends on transporting these molecules from the liver into bile. The ABCG5/G8 [39] and ABCB11 [40] proteins transport cholesterol and bile acids, respectively. The ABCB4 protein transports phospholipids (mainly phosphatidylcholine), which is essential for secretion of cholesterol into bile [41,42]. ABCB11 plays a central role in hepatobiliary secretion as bile flow is the driving force for biliary secretion of cholesterol and phospholipids [43].

Many studies have been carried out using transgenic and knockout mouse models to further our knowledge of the physiologic pathways and molecular mechanisms that control cholesterol excretion. Transgenic mice expressing the human *ABCG5* and *ABCG8* genes directed by their own regulatory DNA sequences (*hG5G8Tg* mice) had higher levels (5-fold) of biliary cholesterol and higher levels (3- to 6-fold) of fecal neutral sterol compared with nontransgenic mice, providing evidence that ABCG5 and ABCG8 function as cholesterol transporters [44]. Moreover, cholesterol absorption in these transgenic mice was reduced by 50% because the proteins encoded by *ABCG5* and *ABCG8* genes are also expressed in the small intestine and they transport cholesterol from the intestine into the lumen [44]. In another study, *Abcg5* and *Abcg8* double knockout (*G5G8-/-*) mice were created to investigate the effects of disrupting these genes on biliary cholesterol secretion. *G5G8-/-* mice were more diet-responsive than normal mice on a 2% cholesterol diet. Plasma cholesterol increased 2-fold in *G5G8-/-* mice, but not in normal mice. Hepatic cholesterol was markedly increased in *G5G8-/-* mice (18-fold) compared with normal mice (3-fold). Accordingly, expression of the cholesterol synthesis genes HMG-CoA synthase and HMG-CoA reductase was lower in *G5G8-/-* mice than normal mice. Fecal neutral sterol was reduced by 36% in *G5G8-/-* mice relative to that in normal mice. The findings using double knockout mice also supported ABCG5 and ABCG8 function in cholesterol transport [45].

Another series of experiments was conducted to investigate the role of phospholipids in secretion of biliary cholesterol. Disruption of the *Abcb4* (also known as *Mdr2*) gene in mice led to a severe liver disease. Phospholipids were undetectable in the bile of homozygous *Abcb4* knockout (*Abcb4*-/-) mice, while the levels in heterozygous *Abcb4* knockout (*Abcb4*+/-) mice were half of those in nontransgenic mice. Interestingly, *Abcb4*-/- mice secreted extremely low levels of cholesterol into bile, but cholesterol levels in flowing bile from *Abcb4*+/- mice were similar to those in nontransgenic mice [41]. Langheim et al. [46] investigated whether overexpression of ABCG5 and ABCG8 could restore biliary cholesterol secretion in *Abcb4*-/- mice by breeding *Abcb4*-/- mice with *hG5G8Tg* mice to generate *Abcb4*-/-;*hG5G8Tg* mice. The *Abcb4*-/-;*hG5G8Tg* mice also secreted very low levels of biliary cholesterol, which were similar to the levels in *Abcb4*-/- mice. Taken together, these results indicate that biliary cholesterol secretion requires a minimal concentration of phospholipids in the bile. Biliary phospholipid secretion in the liver serves two purposes. One is to protect the canalicular membrane of hepatocytes exposed to high concentrations of bile acids from damage by the detergent action of bile acids. Secretion of phospholipids by the liver into the bile reduces bile salt micelles to extract phospholipids from the membranes of hepatocytes. The other is to make phospholipids available for incorporation into pure bile salt micelles to form bile salt mixed micelles. Solubility of cholesterol in bile salt mixed micelles is greater than that in pure bile salt micelles, thus phospholipid secretion prevents formation of gallstones [47].

Nonbiliary cholesterol excretion. Hepatobiliary secretion is known to be the main pathway for eliminating cholesterol from the body, but an increasing body of evidence suggests that plasma cholesterol is also eliminated by a nonbiliary pathway in mice. As mentioned above, *G5G8*-/- mice did not show a dramatic reduction in fecal neutral sterol excretion despite they had extremely low levels of biliary cholesterol. Normal or even increased fecal neutral sterol excretion was observed in other mouse models (*Abcb4*-/-, *Npc1l1*$^{\text{-LiverTg}}$) that are severely impaired in hepatobiliary cholesterol secretion [20]. This observation suggests the existence of an alternate route, known as TICE, which does not involve biliary cholesterol secretion. Plasma cholesterol is transported via blood to intestinal cells and eventually secreted into the intestinal lumen for disposal in feces [20]. It should be mentioned that a nonbiliary cholesterol excretion pathway has also been postulated to explain fecal neutral sterol loss in bile-diverted dogs [48, 49] and bile-diverted rats [50].

Intestinal perfusion studies [19] and *in vivo* stable isotope studies [51] in mice that have an intact hepatobiliary secretion and enterohepatic cycling system revealed that TICE accounted for ~30% of fecal cholesterol excretion. The site of action of TICE is the proximal small intestine [19,52]. It is thought that TICE involves plasma lipoproteins to deliver cholesterol to the intestine, then cholesterol is taken up by receptors at the basolateral membrane of enterocytes and traverses the enterocytes to the apical membrane where it is excreted into the intestinal lumen. There are still many gaps in our understanding of the molecular mechanism of TICE. HDL is ruled out as the lipoprotein that delivers cholesterol to the intestine because TICE was not diminished in *Abca*1 knockout mice having extremely low concentrations of plasma HDL [53,54]. Evidence to support VLDL remnants or LDL as the plasma cholesterol carrier came from studies using antisense oligonucleotides (ASO) to knockdown expression of proteins

critical for production of VLDL and alter plasma VLDL cholesterol concentrations. ASO-mediated knockdown of acyl-CoA:cholesterol acyltransferase activity 2 (ACAT2) in mice fed a high cholesterol diet resulted in an increase in both VLDL cholesterol and fecal neutral sterol excretion [55]. Conversely, ASO-mediated knockdown of microsomal triglyceride transfer protein resulted in a decrease in both VLDL cholesterol and fecal neutral sterol excretion [56]. As for the receptor that takes up cholesterol at the basolateral membrane, it is not likely to be the LDL receptor nor scavenger receptor BI (SR-BI) because neutral sterol excretion was not affected in *LDLR*-/- mice [57] and SR-BI-/- mice [58,59]. It may involve other members of the LDL receptor or a novel receptor. Lastly, Abcg5/Abcg8 and Abcb1 participate in the secretion of cholesterol from enterocytes into the intestinal lumen [57].

A study in patients with complete biliary obstruction revealed they still excreted substantial amounts of neutral sterol into feces [60]. Data from these patients showed that ~20% to 30% of neutral sterols were excreted by TICE, which is similar to that excreted by TICE in normal mice. However, the relevance of TICE for disposal of cholesterol in humans without biliary obstruction has yet to be established. A recent *ex vivo* study showed that human and mouse intestinal (duodenual) explants mounted on Ussing chambers were capable of effluxing cholesterol, providing evidence for the activity of TICE in humans [57].

3. Laboratory opossum model

3.1. Characteristics of laboratory opossums

The gray short-tailed opossum (*Monodelphis domestica*) is a docile, nocturnal marsupial native to Brazil and adjacent countries. It is the only marsupial species that has adapted to breeding in captivity to produce large numbers of animals [61]. In addition to being able to breed in captivity throughout the year, *Monodelphis* have a short generation time of 6 months and produce large litters (typically 6-13). Breeding colonies have been established in the United States, Brazil, Germany, United Kingdom, Japan, and Australia. Opossums in captive colonies are quite different genetically from their wild counterparts due to selection that has undoubtedly taken place while the animals were bred in isolated colonies for many generations; therefore laboratory stocks of this species are referred to as laboratory opossums. Adult laboratory opossums weigh 70-150 g, which is intermediate between mice (20-30 g) and rats (250-300 g). They are maintained in polycarbonate rodent cages, and the standard laboratory diet is commercial pelleted fox food provided *ad libitium*. Owing to its physical characteristics as a laboratory animal and economic production in captivity, *Monodelphis domestica* has become the most widely used marsupial in biomedical research. Furthermore, it was the first marsupial species to have its genome sequenced and analyzed [62]. The availability of genome sequence data has accelerated progress on genetic aspects of research involving *Monodelphis*.

The opossum model has advantageous characteristics for understanding cholesterol homeostasis. *Monodelphis* is omnivorous like humans, and its natural diet includes cholesterol derived from the consumption of insects and small vertebrates. Therefore, laboratory opossums and humans are likely to have many similarities in lipoprotein and cholesterol metabolism. Unlike

genetically modified (transgenic and knockout) mouse models, the opossum model provides an opportunity to identify naturally occurring variants of genes and to study how interactions among gene variants lead to development of hypercholesterolemia. Some partially inbred strains of opossums have inbreeding coefficients in excess of 0.95, and strains with high or low responses to an HCHF diet have inbreeding coefficients in excess of 0.8. Since more than 80% of the genes in each partially inbred strain have alleles that are identical by descent, the work required to identify genes that cause diet-induced hypercholesterolemia in the opossum model is substantially reduced.

3.2. Development of partially inbred strains

Nine wild-caught animals were imported from Brazil into the United States in 1978 by the National Zoo in Washington, D.C. The founders of the breeding colony of laboratory opossums at Texas Biomedical Research Institute were comprised of 20 first and second generation descendants of those nine founders, together with 17 additional wild-caught opossums from Brazil and two from Bolivia. After several generations of inbreeding, some individuals were fed a high cholesterol (0.6% by weight) and high fat (17% by weight) diet for 6 months, and total blood cholesterol was measured after an overnight fast. Low and high responses were observed among opossums, with few animals exhibiting an intermediate response, i.e. the phenotypes are clustered at the high and low ends of the range. Low responding opossums had blood cholesterol levels ranging from 62-171 mg/dl whereas high responding opossums had levels of 215-932 mg/dl [22]. Furthermore, analysis of lipoprotein particles by gradient gel electrophoresis showed elevated levels of LDL particles in high responding opossums [22]. Subsequently, inbreeding and selection for either low responsiveness or high responsiveness to the HCHF diet led to development of two related low responding partially inbred strains (designated ATHE and ATHL) and a high responding partially inbred strain (designated ATHH) that show extreme difference (>5-fold) in plasma cholesterol concentrations in response to the HCHF diet. These strains are being used to identify genetic variants and molecular mechanisms that cause diet-induced hypercholesterolemia.

3.3. Hypercholesterolemia in high responders

Dietary challenge and plasma cholesterol response. Studies were conducted to compare lipoprotein characteristics of low responders from the ATHE strain and high responders from the ATHH strain. The standard laboratory diet is the basal diet, which contains 0.04% cholesterol and 8.1% fat, by weight. Plasma cholesterol concentrations of low and high responders do not differ on this diet. Most of the plasma cholesterol is carried by HDL, and ~30% of total plasma cholesterol is carried by LDL [10]. After consuming the HCHF diet for at least 4 weeks, total plasma cholesterol increases slightly (< 2-fold) in low responders, but dramatically in high responders (>5-fold). HDL cholesterol levels of low and high responders show small (< 2-fold) but significant increases. The increase in plasma cholesterol in high responders is mainly due to an increase in VLDL and LDL (V+LDL) cholesterol such that the percentage of total plasma cholesterol carried by V+LDL is increased to ~85% in response to diet. The increase in V+LDL cholesterol on the HCHF diet alters the plasma lipid profile of high responding opossums to

resemble more closely that of humans. Plasma triglyceride concentrations are relatively low in low and high responders on the basal diet, and they are not responsive to dietary challenge. The major lipoprotein is apoA-I and the minor lipoprotein is apoE in HDL particles from low and high responders on both diets. ApoB is the major lipoprotein in V+LDL particles from low and high responders on the basal diet and low responders on the HCHF diet. The V+LDL particles from high responders on the HCHF diet are more heterogeneous, and they carry apoE in addition to apoB [10].

Cholesterol absorption. Additional studies were conducted to determine whether low and high responders differ in cholesterol absorption, which is one of the physiologic processes that govern cholesterol homeostasis. Cholesterol absorption was measured by the fecal isotope ratio method. On the basal diet, the percentage of cholesterol absorbed through the intestine was ~60% in low and high responding opossums. On the HCHF diet, low responders reduced the percentage of absorbed cholesterol by 50%, whereas high responders did not [63]. Several genes that play a role in cholesterol absorption were analyzed to determine whether their expression differed between low and high responders on the HCHF diet. Dietary cholesterol increased expression of *ABCG5* and *ABCG8* in the small intestine of low and high responders to limit absorption of cholesterol by transporting cholesterol from enterocytes to the intestinal lumen; the extent of increase was similar in both strains of opossums. The *NPC1L1* gene transports cholesterol from the lumen into enterocytes, and the *ACAT2* and *MTP* genes facilitate chylomicron formation and secretion in the small intestine. These genes were expressed at similar levels in low and high responders. Therefore, the difference in cholesterol absorption between low and high responders is not due to differences in expression of genes that regulate influx and efflux of cholesterol in the small intestine [64].

Bile acid synthesis. Liver is the other major site that controls cholesterol homeostasis. In the liver, cholesterol is converted to bile acids, and bile acids and free cholesterol are secreted into bile for disposal via fecal excretion. Bile acids are synthesized by two pathways, the classic pathway and the alternate pathway. The rate-limiting enzyme in the classic pathway is 7α-hydroxylase. The enzyme sterol 27-hydroxylase initiates bile acid synthesis in the alternate pathway and catalyzes several oxidation reactions in the classic and alternate bile acid synthesis pathways [65]. Low and high responders on the HCHF diet had similar 7α-hydroxylase activities but differed in sterol 27-hydroxylase activities. Low responders had higher activity of hepatic sterol 27-hydroxylase (14.1 ± 0.8 pmol/mg protein/min) compared with high responders (10.1 ± 0.8 pmol/mg protein/min; $P<0.01$) [66]. Sterol 27-hydroxylase is encoded by the *CYP27A1* gene. Expression of the *CYP27A1* gene was also higher (2-fold) in low responders fed a high cholesterol diet [64]. Given that sterol 27-hydroxylase catalyzes several reactions in the classic and alternate pathways of bile acid synthesis, a reduction in enzyme activity was consistent with lower concentrations of total bile acids in gall bladder bile of high responders (194 ± 19 mol/ml for high responders versus 244 ± 15 mol/ml for low responders; $P=0.05$) [66].

Biliary cholesterol and biliary phospholipids. Bile samples collected from gall bladders were analyzed to determine whether there are any differences in the secretion of cholesterol into bile by low and high responders fed the HCHF diet. The results revealed differences in biliary cholesterol concentration and biliary phospholipid concentration in gall bladder bile. Choles-

terol concentration in the bile of low responders (5.7 ± 1.3 mg/ml) was higher than that of high responders (1.3 ± 0.7 mg/ml; $P<0.05$). Similarly, phospholipid concentration in the bile of low responders (29.7 ± 3.7 mg/ml) was also higher than that of high responders (6.9 ± 5.5 mg/ml for high responders; $P<0.05$) [24]. Biliary cholesterol secretion is mediated by ABCG5, ABCG8 and NPC1L1. ABCG5 and ABCG8 transport cholesterol from the liver into bile [45] whereas NPC1L1 transports cholesterol from the bile back into the liver [67]. Biliary phospholipid secretion is mediated by ABCB4 [41,42]. The difference in biliary cholesterol and biliary phospholipid secretion prompted an investigation of the expression of these genes in response to dietary challenge in low and high responders. *ABCG5* and *ABCG8* mRNA levels did not differ between low and high responding opossums, whereas *NPC1L1* mRNA levels were down-regulated in high responders [68]. There was no significant difference in *ABCB4* mRNA levels between low and high responders on the two diets [69]. Therefore, expression of cholesterol and phospholipid transporter genes cannot explain differences in biliary lipid concentrations.

Association of ABCB4 with hypercholesterolemia. Development of a genetic linkage map of *Monodelphis* [70], coupled with the *Monodelphis* genome sequence [62], facilitated the identification of genes that predispose high responders to develop hypercholesterolemia on the HCHF diet. Genome-wide linkage analyses on data from pedigreed opossums located two quantitative trait loci (QTL) influencing V+LDL cholesterol levels. The QTL on chromosome 1 influences V+LDL cholesterol on the basal diet, and the QTL on chromosome 8 influences V+LDL cholesterol on the HCHF diet [69]. One gene in the chromosome 8 QTL is *ABCB4*. Since ABCB4 plays a role in biliary secretion of phospholipids and cholesterol, lower levels of biliary lipids in high responding opossums could be due to an impairment of ABCB4 function. We tested this hypothesis by sequencing the *ABCB4* gene to identify mutations and found two single nucleotide polymorphisms (SNPs) that cause missense mutations in exons 2 and 7 of the *ABCB4* gene from high responders. In exon 2, Gly at amino acid 29 in allele 1 is substituted by Arg in allele 2. In exon 7, Leu at amino acid 235 in allele 1 is substituted by Ile in allele 2. Allele 1 is predominant in the high responding strain, whereas allele 2 is predominant in the two low responding strains [24,69].

Using a pedigree-based genetic association approach, matings of high responders with low responders were carried out to produce F2 progeny in two different crosses (designated JCX and KUSH6) to determine whether ABCB4 has an effect on response to dietary cholesterol. Animals from both crosses were genotyped for the ABCB4 Ile235Leu polymorphism and subjected to measured genotype analysis using plasma cholesterol data from a basal diet and from a 4-week HCHF diet. The average concentration of plasma total cholesterol and V+LDL cholesterol on the HCHF diet was highest in JCX animals homozygous for the *ABCB4* '1' allele, intermediate in animals with the *ABCB4* '1/2' genotype, and lowest in animals homozygous for the *ABCB4* '2' allele. A similar pattern was observed in animals from the KUSH6 cross. The results showed that genetic variation in *ABCB4* had a significant effect on variation in V+LDL cholesterol levels in response to the HCHF diet, and implicated defects in biliary phospholipid and biliary cholesterol secretion in causing diet-induced hypercholesterolemia in the opossum model. However, it was apparent from the analysis that there is at least one additional gene

that influences diet-induced hypercholesterolemia because some opossums that are homozygous for the missense mutations are not high responders [24,69].

Variations in the *ABCB4* gene have not been shown previously to be associated with variations in plasma LDL cholesterol in response to diet in other experimental animals or humans. ABCB4 mutations affect secretion of phospholipids, and clinical symptoms are due to production of bile with a low phospholipid content which cannot prevent bile salts from damaging the membranes of cells lining the bile ducts. Moreover, the phospholipid deficient bile has a high cholesterol saturation index. In humans, *ABCB4* variants that have a severe effect are associated with progressive familial intrahepatic cholestasis type 3, a liver disease that often develops in the first year of life. *ABCB4* variants that have a moderate effect are associated with a gallstone disease in adults known as low-phospholipid associated cholelithiasis, and a reversible form of cholestasis known as intrahepatic cholestasis of pregnancy that develops in women during the third trimester of pregnancy and resolves after delivery of their babies [71]. *ABCB4* knockout mice lacking phospholipid transport function develop sclerosing cholangitis, which progresses to metastatic liver cancer [72,73]. Mutations in the opossum A*BCB4* gene do not have a severe effect as high responders exhibit no adverse symptoms when the animals are fed the basal diet. The reduction in biliary cholesterol and biliary phospholipids associated with *ABCB4* mutations leads to an increase in plasma V+LDL cholesterol when high responders are challenged with the HCHF diet. However, a gene whose identity is still unknown seems to be able to compensate for the reduction in biliary cholesterol secretion and rescues high responders that are homozygous for the *ABCB4* mutations from developing diet-induced hypercholesterolemia. Identification of this gene will lead to a better understanding of the process involving *ABCB*4 in controlling plasma LDL cholesterol concentration in response to dietary cholesterol.

3.4. Pathologic features of high responders

Fatty livers. Dysregulated cholesterol homeostasis causes high responders to develop fatty livers and atherosclerotic lesions. Cholesterol accumulates in the livers of high responders as a result of impaired biliary cholesterol secretion. After 4 weeks of HCHF diet, serum levels of liver enzymes (alanine aminotransferase, aspartate aminotransferase, and γ–glutamyltransferase) and bilirubin were significantly elevated, indicating high responders had liver injury. Histology revealed steatosis, inflammation and ballooned hepatocytes in their livers after 8 weeks of HCHF diet. The pathologic condition in the liver worsened as high responders continued to consume the HCHF diet. In one study in which high and low responders were fed the HCHF diet for 24 weeks, livers of high responders were markedly enlarged compared to those of low responders. The enlarged livers had an increase in free cholesterol (2-fold), esterified cholesterol (11-fold) and triglycerides (2-fold), but no significant increase in free fatty acids. Low responders did not display any significant morphological changes in the liver after 24 weeks on the HCHF diet. Prolonged HCHF dietary challenge caused high responders to develop fibrosis in addition to steatosis, inflammation and ballooned cells. Liver fibrosis is a characteristic feature of the severe form of nonalcoholic fatty liver disease known as nonalcoholic steatohepatitis.

Expression of a set of hepatic genes associated with inflammation, oxidative stress and fibrogenesis was up-regulated in high responders, and the gene expression pattern was consistent with the histopathological features in the livers of high responders [74].

Atherosclerotic lesions. Similar to humans, hypercholesterolemia leads to development of atherosclerotic lesions in the arteries of high responding opossums. Low responding opossums whose V+LDL cholesterol was below 75 mg/dl did not develop gross or histologically detectable lesions after consuming the HCHF diet for one year. In contrast, high responding opossums whose V+LDL cholesterol was over 500 mg/dl developed gross and histologically detectable lesions after 40 weeks of HCHF diet. The opossum lesions were similar in histologic characteristics to those observed in cholesterol-fed mouse models of atherosclerosis [23].

4. Non-human primate models

Non-human primate models stand out as the most biologically similar to humans in physiologic and genetic characteristics of hypercholesterolemia [25]. This is because nonhuman primates and humans share similar biochemical, anatomical and physiological characteristics, including lipid synthesis and metabolism. Both humans and primates exhibit spontaneous and diet-induced hypercholesterolemia [15] and develop atherosclerosis [11,75]. Commonly used nonhuman primates include African green monkey (green monkey), rhesus monkey, cynomolgus monkey and baboon. These species not only have a high degree of physiological similarity with humans, but also have many of the same genes underlying relevant phenotypes. The size of nonhuman primates by comparison to mice enables the collection of tissue and organ samples of equivalent sizes to humans, including arteries and hearts. It is important to mention that great apes share greater similarities to humans than other nonhuman primates. However, cost and ethical considerations prohibit use of great apes for most human disease studies.

4.1. Nonhuman primate responses to HCHF diet

Nonhuman primates respond to HCHF diet as do humans. Most nonhuman primates respond to HCHF diet by an increase in average total plasma cholesterol concentration ranging from 200-800 mg/dl with no change in weight [11,15]. Total plasma cholesterol levels positively correlate with LDL cholesterol, VLDL cholesterol and triglyceride levels with no change in HDL cholesterol levels. In addition, triglyceride concentration is positively correlated with VLDL cholesterol and LDL cholesterol concentrations [15,25]. However, there are differences among species and among individuals in response to HCHF diet in nonhuman primates.

Variation among species. Nonhuman primate species differ in their response to HCHF diet challenges by exhibiting variation in plasma cholesterol [11,76]. Baboons and green monkeys display moderate response with an average plasma cholesterol concentration of 204 and 275 mg/dl, respectively, while cynomolgus monkeys and rhesus macaque have the highest response, 307 and 467 mg/dl, respectively [11,15,76]. The response of baboons is similar to that of humans [11].

In addition, green monkeys and baboons show unusual increases in HDL cholesterol levels in response to HCHF diet compared to most nonhuman primate species [11,76]. The mechanisms underlying the marked difference in response to HCHF diet for green monkeys and baboons are not well understood. Sorci-Thomas et al. [76] compared the responses of green and cynomolgus monkeys to HCHF diet. Because green monkeys develop modest hypercholesterolemia compared to cynomolgus monkeys when challenged with HCHF diet, green monkeys were fed diet with more cholesterol than cynomolgus monkeys to induce equivalent extent of hypercholesterolemia in both species. Surprisingly green monkeys still had 2-3-fold higher plasma HDL cholesterol and apoA-I concentrations than cynomolgus monkeys, indicating that higher plasma HDL cholesterol in green monkeys was due to factors independent of level of dietary cholesterol. Further investigation indicated that green monkey hepatic apoA-I and mRNA expression levels were respectively 2-fold and 3.7-fold higher, and intestinal *apoA-I* mRNA level was 3.7-folder higher than in cynomolgus monkeys. These observations indicate that factors that regulate mRNA transcription and post-transcription, including microRNA (miRNA) gene regulation, may be determinants of resistance to HCHF diet.

Variation among individuals. Similar to humans, nonhuman primates display variation among individual animals of the same species in response to HCHF diet [77-80]. This variation is one of the important features of nonhuman primate models that enables us to identify genetic variants that predispose individuals to develop hypercholesterolemia.

a. *Plasma lipoprotein cholesterol levels.* The response of plasma cholesterol level to HCHF diet differs among individuals. Baboons challenged with HCHF diet for 2 years exhibited an increase in plasma cholesterol levels from 5 to 197 mg/dl [81]. Based on these observations, McGill et al. selectively bred two lines of baboons with extreme plasma cholesterol levels; low responders and high responders to HCHF diet. Subsequent studies have shown that low and high responders differed in LDL cholesterol levels when challenged with HCHF diet. High responders had approximately 2-fold higher plasma cholesterol than low responders [11,80]. In addition, LDL *apoB* concentrations were 2-3-fold higher in high responders compared with low responders [11]. This difference was due to higher production of apoB in high responders. However, apoB mRNA levels did not differ between low and high responders on HCHF diet, suggesting that apoB production is regulated at the post-transcriptional level and is influenced by plasma cholesterol levels, which differ between low and high responders.

   McGill et al. [82] examined the effect of cholesterol or saturated fat on plasma cholesterol response in low and high responders. The study revealed that high responders challenged with diet containing high cholesterol (1.7 mg/kcal) displayed a higher percent increase of LDL and VLDL cholesterol levels than low responders, and that there was no difference in HDL cholesterol levels between high and low responders. The type of saturated fat, corn or coconut oil in the diet did not influence plasma cholesterol variation between the two lines of baboons. Genetic analysis revealed that genetic factors explained 57% of the response to dietary cholesterol. These findings indicated that dietary cholesterol, and not saturated fat, drives variation in plasma cholesterol in baboons.

b. *Expression of 27-hydroxylase.* Nonhuman primates exhibit individual variation in the synthesis of bile acids from cholesterol. Sterol 27-hydroxylase is an important enzyme for bile acid synthesis in both the classic and alternate pathways. Kushwaha et al. [83] measured plasma and hepatic 27-hydroxycholesterol levels, hepatic 27-hydroxylase activity and mRNA levels in 12 low and 12 high responding baboons. Low responders displayed higher 27-hydroxycholesterol levels, 27-hydroxylase activities and mRNA levels than high responders when fed the HCHF diet but not when fed the chow diet. These parameters were negatively correlated with LDL and VLDL cholesterol concentrations in low responders. These findings indicate that sterol 27-hydroxylase is induced by HCHF diet and that the induction is higher in low responding baboons, resulting in higher bile acid synthesis. Thus, the ability to induce sterol 27-hydroxylase influences LDL cholesterol variation in baboons.

c. *ApoE levels and LDL receptor expression.* The liver clears excess plasma lipoprotein cholesterol through the LDL receptor and the LDL receptor-related protein. ApoE is a component of chylomicrons and most of the lipoproteins, and aids in receptor mediated-clearance of plasma lipoprotein cholesterol [31]. A study in cynomolgus and green monkeys fed an HCHF diet demonstrated that apoE concentrations were positively correlated with total plasma cholesterol concentrations, plasma LDL cholesterol concentrations and LDL particle size [12]. Since apoE is a high-affinity ligand for the LDL receptor and the LDL receptor-related protein, plasma cholesterol clearance by the liver is expected to increase when apoE levels are elevated, but this is not the case. A possible explanation is that LDL receptors are down-regulated in hypercholesterolemic monkeys, and clearance of VLDL and LDL particles is impeded. ApoE-enriched LDL particles accumulate in the plasma of hypercholesterolemic animals because VLDL particles are metabolized to LDL particles rather than being removed from the circulation by the LDL receptor as in monkeys with normal plasma cholesterol concentrations [12].

Together, these findings suggest that plasma cholesterol variation in nonhuman primates may be influenced by the level of sterol 27-hydroxylase activity in the liver. A decrease in conversion of cholesterol to bile acids may lead to an increase in plasma cholesterol, which in turn decreases LDL receptor expression. As a consequence, the rate of clearance of plasma VLDL and LDL cholesterol is reduced and circulating levels of LDL cholesterol are elevated.

4.2. Genetic mechanisms that influence individual variation in plasma cholesterol levels

Baboon genetic resources to study lipid metabolism. The baboon is the most commonly used primate model for genetic studies of complex traits and susceptibility to complex diseases [14]. The Southwest National Primate Research Center (SNPRC) at Texas Biomedical Research Institute maintains approximately 1,500 living baboons for biological research. These baboons have been used to develop nonhuman primate genomic resources to study responses to environmental factors, such as diet, and how these factors interact with genomic factors in causing complex diseases or disorders. In addition, SNPRC maintains an extensive pedigree database consisting of 16,000 baboons across seven generations. This is the largest nonhuman primate pedigree in the world. The pedigreed population includes approximately 384 founders of olive

(*P. h. anubis*) and yellow (*P. h. cynocephalus)* baboons, and their hybrid descendants. These resources provided a unique opportunity to map baboon genes, resulting in the first ever nonhuman primate linkage map [84,85]. In addition, tissues and blood samples have been collected from 8,000 baboons, and DNA, serum and buffy coats from 4,000 animals [14].

Genetic factors for hypercholesterolemia in baboons. Baboon response to HCHF diet is modest, similar to the response of humans. Because of this similarity as well as other baboon characteristics that mimic human characteristics, numerous studies have utilized baboon resources available at SNPRC to understand how genetic variation influences lipoprotein cholesterol in response to diet. This initiative started more than three decades ago when scientists at the Texas Biomedical Research Institute observed differential response of baboons to HCHF diet [81]. These observations led to selective breeding and characterization of distinct phenotypes of baboons and revealed that differential response to HCHF diet is heritable [86,87].

Attempts to find the major genes influencing plasma cholesterol in response to dietary challenge revealed that polymorphisms in the LDL receptor gene contributed only 6% of the variation [88], suggesting that lipid response to HCHF diet is multigenic. Hypercholesterolemia is a complex disorder plausibly influenced by complex genetic networks. Therefore, to elucidate the mechanisms that underlie cholesterol variation, a system biological approach is most appropriate. Using available pedigree and genotypic information for more than 2,400 baboons, important lipid/lipoprotein-related QTL have been identified [27,89,90]. In addition, improved Next Gen Sequencing techniques for RNA and DNA sequencing and genetic network analyses have enabled understanding of genes encoding these QTL.

Studies were undertaken to interrogate the QTL to discover gene, genetic variants and functional mechanisms that influence variation in response to diet in baboons. In one study, four novel candidate genes (*TENC1, ACVR1B, ERBB3, DGKA*) were identified that encode a QTL for LDL cholesterol concentration variation. This QTL overlaps multiple other QTL for LDL related traits, including particle size, suggesting that these genes have pleiotropic effects [13]. *TENC1* was downregulated while *ACVR1B, ERBB3* and *DGKA* were upregulated in response to HCHF diet. The protein products of all four genes are central molecules for a single pathway, affirming that multiple genes influence LDL cholesterol variation. Interestingly these genes are associated with cancer in the *AKT/GSK3B* signaling pathway [91]. Several studies have alluded a link between cancer, hypercholesterolemia and atherosclerosis [92-95], but the link is not well understood. One aspect that is clear is that cholesterol is intertwined in the etiology of cancer and atherosclerosis. In addition, tumorigenesis thrives by the ability to alter important biological processes, including regulation of cholesterol levels. For cancer cells to proliferate uncontrollably, essential cell components, such as cholesterol must be available for plasma membrane synthesis. In order to meet the demand for cholesterol, pathways regulating cellular cholesterol homeostasis are altered in cancer cells.

Other studies have investigated the role of miRNA in LDL cholesterol variation in baboons [13,80]. miRNAs were hypothesized to regulate genes encoding variation in LDL cholesterol in response to HCHF diet. Hepatic miRNA expression profiling in low and high LDL cholesterol half–sibling baboons by RNA sequencing revealed 226 miRNAs were differentially expressed (160 downregulated and 66 upregulated) between low and high responders in

response to HCHF diet. In order to identify molecular mechanisms that may regulate LDL cholesterol variation, these miRNAs were overlaid onto gene networks that differ between low and high baboon responders. Seven miRNAs were inversely expressed with respect to the four candidate genes. Together, these findings demonstrate that hepatic miRNAs are responsive to diet, and that response differs among baboons with different plasma LDL cholesterol levels.

4.3. Nonhuman primates and atherosclerosis, the clinical endpoint of hypercholesterolemia

Atherosclerosis, a complex progressive disease, is the leading cause of mortality and morbidity in developed countries [96,97]. The clinical end-point of atherosclerosis is cardiovascular disease primarily caused by thickening and/or occlusion of coronary arteries. Atherosclerotic heart disease is the leading cause of death in the world and is projected to remain the single leading cause of death by 2030 [98].

Atherogenesis is similar in nonhuman primates and humans. Atherogenesis is a multifactorial process. Initial events during atherogenesis include deposition of modified or oxidized cholesterol (ox-cholesterol) in the artery wall, resulting in endothelial dysfunction; adhesion of circulating monocytes onto the endothelium; entry of ox-cholesterol and monocytes into the intima layer of the artery; engulfment of ox-cholesterol by monocytes and transformation into macrophages and foam cells; production of pro-inflammatory cytokines and connective matrix; conversion and proliferation of smooth muscle cells; cell apoptosis; and intima thickening. During these processes, atherosclerotic lesions, which are grossly and microscopically heterogeneous, develop on the intimal arterial surface. In nonhuman primates, as in humans, the initial lesions are flat fatty streaks, which are not elevated on the intimal surface, containing predominately foam cells derived from monocytes and smooth muscle cells filled with minimal lipid. These lesions advance to raised fatty streaks that are characterized by lipid-filled foam cells. Raised lesions may progress to advanced fibrous plaques with lipid cores and accumulated connective matrix [26,75,99].

Lipoprotein cholesterol and atherosclerosis. Nonhuman primates provide a unique opportunity not only to understand the factors that underlie differential response to HCHF diet but the link between diet response and development of atherosclerosis in humans. In both nonhuman primates and humans, dyslipidemia is associated with atherosclerosis. High levels of non-HDL cholesterol, including LDL cholesterol, VLDL cholesterol and triglycerides, induced by HCHF diet are positively correlated with the extent and severity of atherosclerosis while HDL cholesterol is negatively correlated [75]. This implies that HCHF diets indirectly influence atherogenesis through induction of hypercholesterolemia. Another study with baboons revealed that plasma HDL1 levels are negatively correlated with extent and severity of atherosclerosis [11]. These results are consistent with results from human studies that indicate plasma lipoprotein cholesterol levels and lipoprotein subclasses are indicators of the extent of atherosclerosis [100]. Stevenson et al. [12] observed in cynomolgus monkeys that an increase in apoE correlates with extent of atherosclerosis, suggesting that apoE may represent an atherogenic feature of diet-induced hypercholesterolemia.

Arterial distribution of atherosclerosis. Atherosclerosis in nonhuman primates and humans displays a distinctive topographical distribution in the arterial system. The extent and severity of the disease is greater in the abdominal and common iliac arteries than in the thoracic and

aortic arch, whereas flat lesions are more abundant in thoracic and aortic arch in nonhuman primates and humans [11,26]. It is hypothesized that the distinct localization of atherosclerotic lesions is a consequence of hemodynamic stress induced by blood flow; and anatomic, cellular, or biochemical variations in the arterial wall, particularly in the endothelium. These hypotheses are consistent with observations of more abundant lesions in branches and bifurcations of medium-sized arteries, including abdominal and common iliac arteries in nonhuman primates [101]. However, the mechanisms underlying the varied distribution of atherosclerosis in both humans and nonhuman primates are not well understood.

Variation among species and individuals in development of atherosclerosis. Nonhuman primate species display variation in susceptibility to developing atherosclerosis. Paralleling the different responses to HCHF diet, rhesus and cynomolgus monkeys are more susceptible to atherosclerosis [102] than green monkeys and baboons, which develop moderate atherosclerosis [103] as do humans [11]. Individuals within any one species also display differential susceptibility to atherosclerosis. High responders to HCHF diet develop more severe atherosclerosis than low responders, consistent with differential levels of plasma non-HDL cholesterol [26]. It is this variation that is critical for identification of genetic factors underlying variation in atherosclerosis development. This variation is heritable [104] and may correspond to genetic variation that underlies observed plasma cholesterol variation in response to HCHF diet.

5. Conclusion

ApoE deficient mice, generated by gene targeting, have a lipoprotein profile similar to humans in that most of the plasma cholesterol is carried on VLDL and IDL particles rather than HDL particles as in non-genetically modified mice. *ApoE-/-* mice have elevated levels of plasma cholesterol even on a chow diet and develop atherosclerotic lesions spontaneously. Advanced lesions with plaque rupture that resemble those in humans are frequently observed in *apoE-/-* mice fed an HCHF diet, and these mice are used for developing drugs to reduce atherosclerosis.

A nonbiliary pathway for cholesterol excretion in humans was suggested more than five decades ago based on measurement of intestinal cholesterol secretion from patients with bile duct obstruction. This finding was largely ignored because hepatobiliary secretion is believed to be the only route to dispose of cholesterol in feces. Observations from studies using several genetically modified mice (*G5G8-/-*, *Abcb4-/-* and *Npc1l1*$^{\text{-LiverTg}}$) that have severe defects in biliary cholesterol secretion, but normal or even increased fecal neutral sterol excretion, prompted several groups of researchers to investigate the TICE pathway. They reported that TICE accounts for 20%-30% of fecal neutral sterol excretion. However, mechanistic details of TICE still remain unknown. Because cholesterol excretion is an important process to eliminate cholesterol from the body, stimulation of TICE by pharmacological agents may be a novel therapeutic strategy to limit atherogenesis.

Studies using genetically modified mice have shown that they are indispensable for advancing our knowledge of the genes and pathways that govern cholesterol homeostasis, as well as for

developing pharmacological agents to treat atherosclerosis. Traditional gene targeting using embryonic stem cells is a complex and time-consuming procedure to produce mutant mice and is limited to targeting one gene at a time. Mice carrying mutations in multiple genes are produced either by sequential gene targeting or intercrossing mice with a single mutation. The CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 (CRISPR-associated nuclease 9) system is a new and more efficient genome engineering technology [105]. It allows targeting multiple genes at the same time by direct co-injection of RNA encoding the Cas9 nuclease and several gene-specific guide RNA into a one-cell embryo to generate mice with multiple modified genes [106]. Application of the CRISPR/Cas system will accelerate the production of mouse models carrying multiple modified genes to study complex disease such as hypercholesterolemia.

High responding opossums have naturally occurring genetic variants that predispose them to develop hypercholesterolemia when challenged with an HCHF diet. At least two major genes control the plasma cholesterol response to dietary challenge in high responding opossums. One has been identified as the *ABCB4* gene. Mutations in *ABCB*4 impair the ability of high responding opossums to secrete phospholipids into the bile. Because secretion of cholesterol into bile requires phospholipids, biliary cholesterol secretion is also impaired in high responding opossums. As a consequence, plasma V+LDL cholesterol becomes elevated in high responders, and free and esterified cholesterol accumulates in their livers. However, some opossums that are homozygous for the *ABCB4* mutations are resistant to diet-induced hypercholesterolemia. The compensatory mechanism that allows these opossums to overcome the defect in biliary cholesterol secretion is not known. In light of the finding of a nonbiliary route of cholesterol excretion that functions in normal mice as well as in mice that have very low biliary cholesterol secretion, cholesterol excretion by the nonbiliary route may compensate for the defect in biliary excretion in opossums that are homozygous for the *ABCB4* mutations.

Phylogenetic similarities between humans and nonhuman primate models are core aspects for consideration in investigations of environmental and genetic factors that contribute to complex diseases/disorders, including hypercholesterolemia and atherosclerosis. Moreover, like humans, nonhuman primates exhibit diet-induced hypercholesterolemia and naturally develop atherosclerosis, making it possible to identify phenotypic variations without altering genetic background as is required for this line of research with mice. In addition, environmental factors, including diet, can be controlled for a prolonged period of time, invasive and terminal experiments can be conducted, and tissues and organs can be easily collected. These research activities are not attainable when working with human subjects.

Recent scientific advances have led to discovery of therapeutic regimes, including statins for lowering LDL cholesterol and retarding the development of atherosclerosis [107]. However, these therapies are limited by side effects and ineffectiveness in some individuals [108,109]. Thus, there is a need for continued searching for novel therapeutic agents. Diet-induced hypercholesterolemia in nonhuman primates provides an opportunity 1) to identify lipid profiles important for the development of atherosclerosis in primates in a controlled environment, 2) to identify variation in responses to diet, and 3) to assess progression of and variation in development of atherosclerosis in response to dietary cholesterol and saturated fat. Identification of these variations is essential for genetic analysis to develop novel therapeutic agents

for lowering plasma cholesterol and biomarkers for detection of early atherosclerosis, a precursor for cardiovascular disease.

Nonhuman primate genetic resources for studying complex diseases are becoming increasingly sophisticated and available at SNPRC and Texas Biomedical Research Institute. These resources enable scientific collaborations to study human diseases using multidisciplinary approaches. Significant steps have been achieved in the identification of genetic causes of hypercholesterolemia and atherosclerosis, including discovery of QTL and genes and gene variants that influence plasma LDL and HDL cholesterol levels, and triglyceride levels.

Further improvement and enhancement of unique genetic resources for research with mice, laboratory opossums, and nonhuman primates will be critical for future research aimed at understanding genetic and epigenetic factors influencing human health and disease.

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