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Review

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The History of the WHHL Rabbit, an Animal Model of Familial Hypercholesterolemia (I)

- Contribution to the Elucidation of the Pathophysiology of Human Hypercholesterolemia and Coronary Heart Disease -

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Animal models that closely resemble both human disease findings and their onset mechanism have contributed to the advancement of biomedical science. The Watanabe heritable hyperlipidemic (WHHL) rabbit and its advanced strains (the coronary atherosclerosis-prone and the myocardial infarction-prone WHHL rabbits) developed at Kobe University (Kobe, Japan), an animal model of human familial hypercholesterolemia, have greatly contributed to the elucidation of the pathophysiology of human lipoprotein metabolism, hypercholesterolemia, atherosclerosis, and coronary heart disease, as described below. 1) The main part of human lipoprotein metabolism has been elucidated, and the low-density lipoprotein (LDL) receptor pathway hypothesis derived from studies using fibroblasts was proven *in vivo*. 2) Oxidized LDL accumulates in the arterial wall, monocyte adhesion molecules are expressed on arterial endothelial cells, and monocyte-derived macrophages infiltrate the arterial intima, resulting in the formation and progression of atherosclerosis. 3) Coronary lesions differ from aortic lesions in lesion composition. 4) Factors involved in the development of atherosclerosis differ between the coronary arteries and aorta. 5) The rupture of coronary lesions requires secondary mechanical forces, such as spasm, in addition to vulnerable plaques. 6) Specific lipid molecules in the blood have been identified as markers of the progression of coronary lesions. At the end of the breeding of the WHHL rabbit family at Kobe University, this review summarizes the history of the development of the WHHL rabbit family and their contribution to biomedical science.

Key words: **Atherosclerosis, Coronary heart disease, Lipoprotein metabolism, Serum markers for coronary lesions, WHHL rabbit**

Introduction

Animal models for human disease have been greatly contributed to the elucidation of the pathophysiology of human lipoprotein metabolism and atherosclerosis. In particular, the Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model of familial hypercholesterolemia[1], and its advanced strains developed by selective breeding, the coronary atherosclerosis-prone WHHL rabbits (WHHL-CA; provisional name)[2, 3] showing spontaneous coronary atherosclerosis, and the myocardial infarction-prone WHHL rabbits (WHHLMI) showing spontaneous coronary atherosclerosis and myocardial infarction[4], have played an important role in advancing these research areas. These rabbit strains were developed at Kobe University (Kobe, Japan). In this review, WHHL rabbits, WHHL-CA rabbits, and WHHLMI rabbits are referred to as the WHHL rabbit family. Unlike mice and rats, rabbits are similar to humans in lipoprotein metabolism, atherosclerosis, and myocardial function (see the latter part of this review[5] for details). Compared with human hypercholesterolemia and atherosclerosis, cholesterol-fed rabbits also have several difficulties. 1) The cause of hypercholesterolemia is due to excess fat-supplemented feed, and the major

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lipoproteins are not low-density lipoprotein (LDL) but remnant lipoproteins (β- very low-density lipoprotein [VLDL])[6]. 2) There are large individual differences in serum lipid levels due to individual differences in the response to cholesterol feeding. 3) Arterial lesions are characterized by excessive macrophage accumulation[7], and there are large individual differences in the development of coronary lesions. 4) It is difficult to conduct long-term experiments due to fatty liver caused by excessive lipid loading[8]. However, the WHHL rabbit family resembles humans for the pathophysiology of lipoprotein metabolism and atherosclerotic lesions, and relatively small individual differences in serum lipid levels and the degree of atherosclerosis. Consequently, the WHHL rabbit family contributed remarkably to the elucidation of the pathophysiology of human lipoprotein metabolism, atherosclerosis, coronary heart disease, and the development of statins, inhibitors of cholesterol biosynthesis. In Kobe University, breeding of each WHHL rabbit strain was terminated each time after an advanced rabbit strain was developed, and only WHHLMI rabbits had been bred since 1999. However, breeding of the WHHLMI rabbit at Kobe University ended in June 2018. Currently, a small number of WHHLMI rabbits are bred at Saga University (Saga, Japan) for cryopreservation of WHHLMI rabbit embryos, and a few other institutions are trying to breed WHHLMI rabbits. Until the end of breeding of the WHHL family, 4,639 rabbits had been provided by Kobe University to many researchers around the world (**Supplementary Table 1**). At the end of the breeding of WHHLMI rabbits at Kobe University, the contribution of the WHHL rabbit family to these studies are summarized here. With regard to the history of the WHHL rabbit family, the first part summarizes the history of the development of the WHHL rabbit family and their contribution to the pathophysiology of human lipoprotein metabolism, atherosclerosis, and coronary heart disease, and the second part summarizes their contribution to the development of agents for improving lipoprotein metabolism and atherosclerosis[5]. In this review, the terms “WHHL rabbit”, “WHHL-CA rabbit”, and “WHHLMI rabbit” indicate the homozygotes, and the heterozygote is described as “heterozygous WHHL rabbit”, “heterozygous WHHL-CA rabbit”, or “heterozygous WHHLMI rabbit”.

The Outline of the Characteristics of the WHHL Rabbit Family

Fig. 1 illustrates the characteristics of the WHHL rabbit family. The proband of WHHL rabbit strain, a mutant rabbit with hypercholesterolemia, showed hyperlipidemia, aortic and coronary atherosclerosis, and xanthoma on the digital joints[9, 10]. The original WHHL rabbit showed the following features: hypercholesterolemia due to LDL receptor deficiency[11, 12], low high-density lipoprotein cholesterol, atherosclerotic lesions on the aorta (**Fig. 2**) and various arteries except coronary lesions[13-15], and other related diseases, such as xanthoma on tendons of the limb[1, 16] osteonecrosis due to the abnormal accumulation of fat in osteocytes and marrow cells[17], corneal opacities due to the infiltration of macrophages and epithelial cells[18], sensorineural hearing loss due to inner ear cell damage[19], and metabolic syndrome-like findings[20, 21]. In addition to the characteristics of WHHL rabbits, coronary atherosclerosis occurred in WHHL-CA rabbits[2, 3], and in WHHLMI rabbits, unstable coronary lesions (**Fig. 3**)[4, 22, 23], myocardial infarction (**Fig. 3**)[4], acute coronary syndromes-like findings[22, 24], calcific aortic valve sclerosis with decreased aortic valve area[25], cutaneous xanthoma[26], and overactive bladder[27] were developed. However, the mean systolic blood pressure of WHHLMI rabbits measured at the auricular artery was approximately 100 mmHg[28] and had similar levels with normal Japanese white rabbits. Coronary lesions of WHHLMI rabbits are initiated from fatty streaks, and then progress to fatty patches, atheroma, fibroatheroma, thin-capped fibroatheroma, and advanced complicated lesions with reduced cellular components, calcification, vasa vasorum, etc.[4, 22, 29]. These features of coronary lesions of WHHLMI rabbits resemble human atherosclerosis[30]. Regarding thrombus formation, the activities of vitamin K-dependent clotting factors and levels of the clotting factor VIII and fibrinogen were significantly higher in WHHL rabbits than in normal rabbits[31]. However, platelet aggregation induced by collagen, platelet-activating factor, or adenosine diphosphate was reduced in WHHL rabbits[32]. Consequently, thrombin and prothrombin times of WHHL rabbits were similar to normal rabbits[33]. In the WHHL rabbit family, despite the development of hypercholesterolemia and atherosclerosis, thrombus formation is not always enhanced. Thrombus was formed when coronary spasm disrupted coronary plaques or injured the endothelial cell layer[22, 24]. These studies suggest that scattering of tissue factor into the extracellular matrix due to the collapse of macrophage-derived foam cells rather than intracellular tissue factor may be involved in thrombus formation.

The History of the WHHL Rabbit Family

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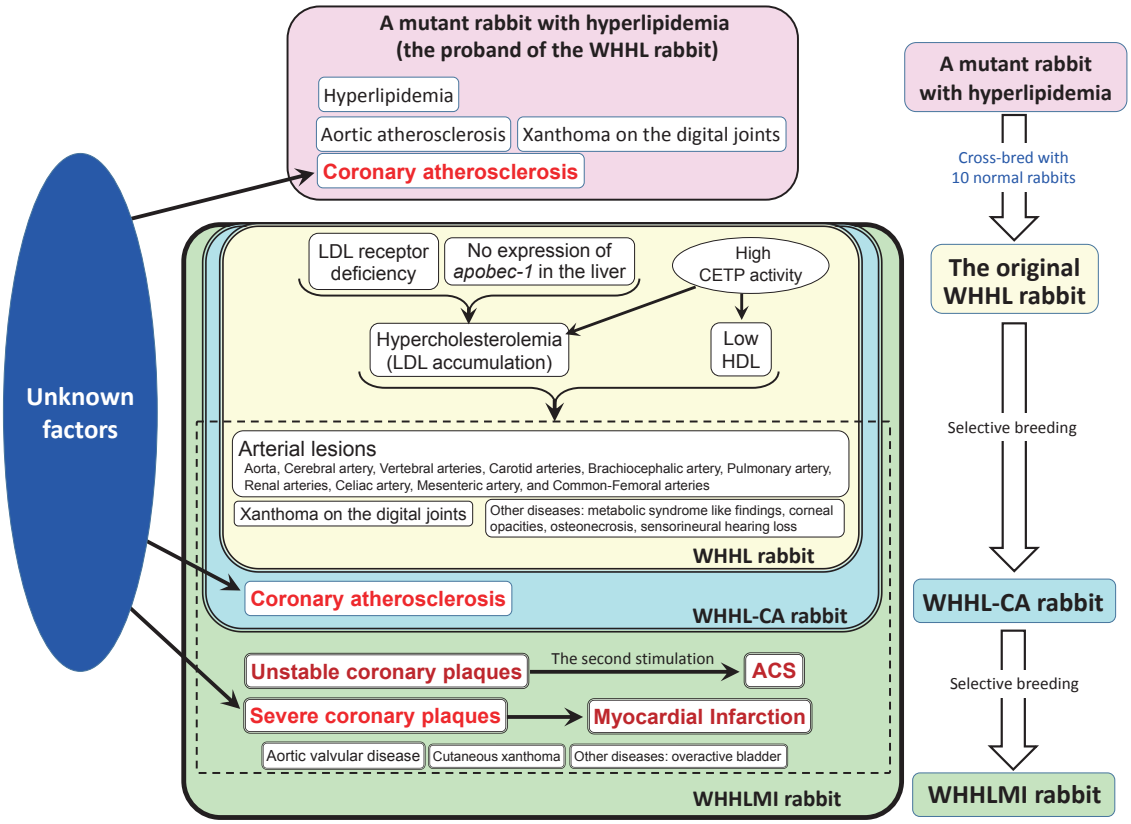

Fig. 1. The characteristics of the WHHL rabbit family and the proband rabbit

The WHHL rabbit family is a collected term for WHHL rabbits, coronary atherosclerosis-prone WHHL rabbits (WHHL-CA rabbits), and myocardial infarction-prone WHHL rabbits (WHHLMI rabbits). The characteristics shown in the yellow square are specific to WHHL rabbits. The characteristics shown in the yellow and blue squares are specific to WHHL-CA rabbits. The characteristics shown in the yellow and green squares are those of WHHLMI rabbits. Black arrows indicate causes and the results.

Fig. 2. Aortic atherosclerosis of WHHLMI rabbits

Aortas were stained with Sudan staining. Lesion area was evaluated as the ratio of the lesion area to the surface lumen area.

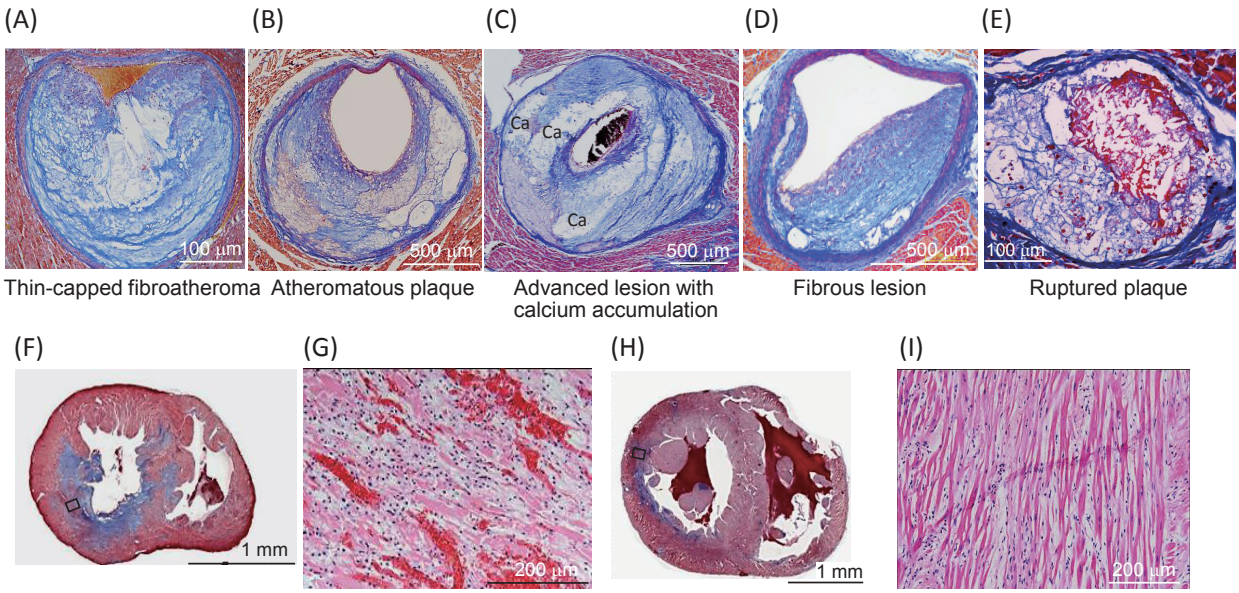

Fig. 3. Photomicrographs of coronary lesions (A–E) and myocardial lesions (F–I) of WHHLMI rabbits

Panels A–D demonstrate spontaneously developed coronary lesions, and panel E demonstrated a ruptured coronary lesion after spasm provocation. Panels G and I are magnified photomicrographs of square in panel F and panel H, respectively. Panels A-F, and H show Azan staining, and panels G and H show HE staining. Panels A and E are modified from Shiomi M, *et al.*[22], panels B–D are modified from Shiomi M, *et al.*[23], and panels F-I are modified from Shiomi M, *et al.*[4].

shown in **Fig. 4**.

Discovery of a Mutant Rabbit Showing Hyperlipidemia and Establishment of the Original WHHL Rabbit Strain

In 1973, Yoshio Watanabe (1927–2008) at Kobe University accidentally found a male rabbit with hyperlipidemia. In normal chow feeding, the serum cholesterol level was 447 mg/dl at 8 months old without any abnormality in other serum biochemical parameters[9]. In this rabbit, atherosclerotic lesions were observed in the aorta and coronary arteries. He cross-bred the mutant rabbit with 10 female Japanese white rabbits to establish a new rabbit strain for spontaneous hyperlipidemia, and the offspring were bred with the mutant. Thereafter, he crossed between rabbits with hyperlipidemia and tried to establish a rabbit strain. However, reproductive ability is drastically depressed after three or more inbreeding generations in rabbits. He attempted to establish a rabbit strain with hyperlipidemia by crossing between above 10 lines to avoid inbreeding depression. After overcoming the crisis of inbreeding depression, he reported this strain as the hyperlipidemic rabbit (HLR) to a Japanese journal for laboratory animals[10]. From these breeding experiments, hyperlipidemia of the HLR was inherited in accordance with Mendel's laws. In 1973, the LDL receptor pathway[34] and the first statin, compactin[35], were also discovered. After seven years of cross-breeding, Watanabe established a rabbit strain of hyperlipidemia and submitted a paper to Atherosclerosis in 1979[1]. In the review process, this rabbit strain was renamed the Watanabe heritable hyperlipidemic (WHHL) rabbit by the advice of the chief editor, Adams CWM. After publication, many researchers around the world requested him to provide the WHHL rabbit for their study. In response to their request, 4,639 WHHL rabbit families have been provided to 126 research institutes in 19 countries (1980–2018) (**Supplementary Table 1**), and more than 700 papers written in English have been published by July 2019. Research papers using the WHHL rabbit family are listed on the WHHL website (http://www.med.akita-u.ac.jp/~doubutu/WHHL/w-index.html) with subject search function.

Contribution of WHHL Rabbits to Studies of Lipoprotein Metabolism

Table 1 summarizes lipoprotein metabolism elucidated in studies using WHHL rabbits. At the establishment of the WHHL rabbit strain, only hyperlipidemia, aortic atherosclerosis, and xanthomas on the digital joints were reported as the characteristics of the original WHHL rabbit strain (**Fig. 1**)[1]. To elucidate the cause of the hyperlipidemia, plasma lipoprotein profile and LDL receptor function were examined in collaboration with Sankyo Co. Ltd. (Tokyo, Japan). WHHL rabbits showed LDL accumulation in the

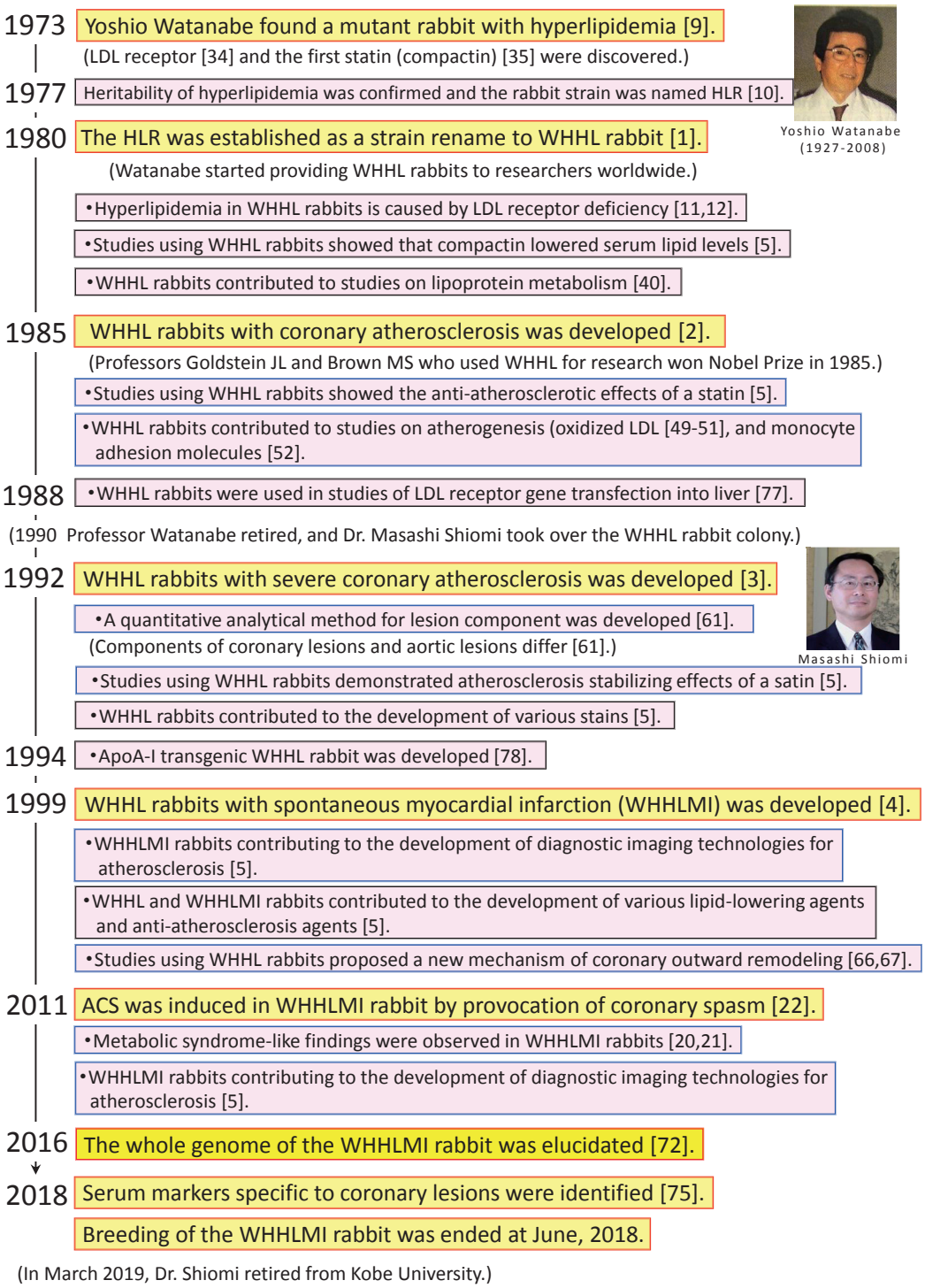

Fig. 4. The history of the WHHL rabbit family

plasma, delayed clearance of radiolabeled LDL from the plasma, and almost no LDL binding activity in fibroblasts[11], similar to human familial hypercholesterolemia[34]. However, skin fibroblasts do not play a central role in lipoprotein metabolism *in vivo*. Goldstein JL and Brown MS, who proposed the LDL receptor pathway hypothesis from *in vitro* studies, also requested Watanabe to provide WHHL rabbits for *in vivo* studies of the LDL receptor pathway. Toru Kita from Goldstein's group demonstrated that the LDL

Table 1. Lipoprotein metabolism elucidated in studies using WHHL rabbits

- Role of hepatic LDL receptors as a key player in the regulation of plasma cholesterol levels[12, 36, 37, 40]
- Secretion of VLDL particles from liver and subsequent transformation of VLDL particles into IDL particles[36]
- Rapid uptake of IDL through hepatic LDL receptors[40], and transformation of remaining IDL particles into LDL particles[36, 40]
- The relevance of non-expression of apoB-editing enzyme in liver in the accumulation of LDL in the circulation[41]
- Delay of the maturation of LDL receptor protein[39] by small deletions of DNA bases in the LDL binding site of the LDL receptor gene[38, 72]

Table 2. Pathophysiology of atherosclerosis elucidated in studies using WHHL rabbit family

- Accumulation of lipid-laden foam cells derived from macrophages and smooth muscle cells in atherosclerotic lesions[44-48]
- Suppression of the development of atherosclerosis in hypercholesterolemia by inhibition of LDL oxidation[49, 50]
- Accumulation of peroxidized apoB-100 containing lipoproteins in atherosclerotic lesions[51]
- Expression of monocyte adhesion molecules on vascular endothelial cells, and infiltration of macrophages derived from monocyte adhered into subendothelial area[52]
- Expression of VLDL receptors on macrophages in atherosclerotic lesions[58]
- Accumulation of lipid vesicles and cholesterol esters containing peroxidized fatty acids in the extracellular matrix[48]
- Differences in the lesion components depending on the type of artery[15, 61]

binding activity of WHHL liver membrane fraction was about 13% of that of normal rabbits[12], and the transformation from VLDL to LDL was delayed in WHHL plasma[36]. In addition, cholesterol biosynthesis in the liver was not increased in WHHL rabbits[37]. These results indicate that hypercholesterolemia in the WHHL rabbit family is due to reduced LDL uptake by the liver. Thereafter, the group of Goldstein and Brown demonstrated that 12 base pairs were deleted in the LDL binding domain in the WHHL LDL receptor protein[38], and the maturation of the LDL receptor protein was delayed in WHHL fibroblasts[39]. In a study of LDL receptor protein maturation kinetics[39], the kinetics of LDL receptor protein maturation in fibroblasts of heterozygous WHHL rabbits showed an intermediate pattern between normal and homozygotes although serum cholesterol levels of heterozygous WHHL rabbits are almost normal. Since rabbits are herbivorous and the cholesterol pool in the liver depends on its synthesis of cholesterol[6], half the number of normal LDL receptors in the heterozygote may be sufficient to maintain the hepatic cholesterol pool. Based on these studies, Goldstein JL and Brown MS clarified lipoprotein metabolism *in vivo*[40] and won the Nobel Prize in 1985. Furthermore, as similar to humans, since apoB-editing enzyme, which induces a stop codon into apoB-100 mRNA, is not expressed in the liver of rabbits[41], lipoprotein particles secreted from the liver have apoB100, and the fractional catabolic rate of apoB-100-containing lipoproteins is very slow compared with apoB-48-containing lipoproteins because apoB-100-containing lipoproteins are not catabolized via remnant receptors. Therefore, the absence of apoB-editing enzyme expression in the rabbit liver is also associated with hypercholesterolemia in the WHHL rabbit family. While in mice and rats, since the apoB-editing enzyme is expressed in their liver[42], serum levels of apoB-containing lipoproteins are very low. Thus, WHHL rabbits contributed greatly to the elucidation of human lipoprotein metabolism.

Contribution of WHHL Rabbits to Studies of Atherogenesis

Table 2 summarizes the pathogenesis of atherosclerotic lesions elucidated in studies using WHHL rabbits. After the elucidation of the major parts of lipoprotein metabolism, studies on atherogenesis were started using the aorta of WHHL rabbits. In histopathological observations, the WHHL aortas showed progressive atherosclerotic lesions, including fatty streaks, raised foam cell lesions, atheromatous plaques, and advanced lesions with cholesterol crystals[43-46]. Electron microscopy observations showed lipid-laden foam cells in the intima[43-46]. These foam cells were derived from smooth muscle cells and macrophages[47]. In the extracellular matrix, lipid vesicles were markedly accumulated among collagen fibers[47], and cholesterol esters containing peroxidized fatty acids were detected in the atherosclerotic aorta[48]. *In vitro* studies demonstrated that macrophages can ingest chemically modified lipoproteins and transform to foam cells. Kita *et al.*[49] and Carew *et al.*[50] separately demonstrated that probucol, an antioxidant, suppresses atherosclerosis in WHHL aorta. The results indicate that oxidized LDL is a causative substance of atherosclerosis. Thereafter, an immunohistochemical study dem-

Table 3. Characteristics of coronary lesions elucidated in studies using WHHL rabbit family

- Differences between coronary artery lesions rich in fibers and aortic lesions rich in lipids[15, 61]
- The relevance of factors other than conventional risk factors (such as serum total cholesterol levels, HDL levels, oxidative stress, hyperglycemia, hypertension, and others) in the development and progression of coronary lesions[75]
- Involvement of genetic factors different from aortic lesions in the development of coronary artery lesions[75], and involvement of the coronary curvature in the progression and composition of lesions[68]
- The relevance of additional factor(s) in the rupture of unstable coronary lesions characterized by thin-capped fibroatheroma[4, 22, 65]
- Involvement of coronary spasm in rupture of coronary lesions and the occurrence of acute coronary syndromes[22, 65]
- The relevance of several serum lipid molecules identified with lipidome analyses in the development and progression of coronary lesions[75]
- Involvement of macrophages infiltrated into the deep area of intima through vasa vasorum[29] in coronary outward remodeling[67]
- Suppression and destabilization of atherosclerotic lesions by lowering serum lipid levels[5]

onstrated that peroxidized LDL accumulated in WHHL aorta[51]. In addition, monocyte adhesion molecules are expressed on the arterial endothelial cells of WHHL aorta[52]. So, macrophages in atherosclerotic lesions are derived from circulating monocytes. Ross's famous working hypothesis about the development of atherosclerosis, "Response-to-Injury Hypothesis", had been revised several times. In the first hypothesis, it was thought that smooth muscle cells migrated from the arterial tunica media and proliferated in intimal lesions by the stimulation of PDGF secreted by activated platelets, and atherosclerotic lesions were formed[53, 54]. However, after the observation of atherosclerotic lesions in WHHL rabbits by the Ross's group[44-47], this working hypothesis was extensively revised in 1993 by Ross himself. In his revised hypothesis, injury of arterial endothelial cells was expanded to endothelial dysfunction, and the role of macrophages and immune responses was added to the hypothesis[55]. This has led to the current "Inflammatory Theory" of atherosclerosis[56, 57]. Thus, WHHL rabbits have contributed to the elucidation of the pathogenesis of human atherosclerosis. However, the structure of the arteries is different in humans and many animal species. In human normal arteries, an intimal layer is observed inside the internal elastic lamina, whereas there is only an endothelium layer in animals. In the deep area of arterial intima in humans, lipid vesicles were observed in the extracellular matrix before the development of atherosclerosis[30]. In the early stages of atherosclerosis, macrophages infiltrate into the arterial intima from the surface of the intima and the vasa vasorum in the deep intima. In WHHLMI rabbits, the vasa vasorum was observed with extracellular lipid deposits and macrophage accumulation in the deep area of advanced coronary lesions, in addition to the intimal surface[29]. Therefore, it should be noted that the pathogenesis of atherosclerosis may be partially different between humans and animals. In addition, macrophages in atherosclerotic lesions express VLDL receptors in humans and WHHLMI rabbits but not in mice[58]. VLDL particles were also detected in human atherosclerotic lesions[59]. Therefore, VLDL is also directly related to atherogenesis in humans and rabbits.

Development of Coronary Atherosclerosis-prone WHHL Rabbits (the WHHL-CA Rabbit) and Myocardial Infarction-Prone WHHL Rabbits (the WHHLMI Rabbit)

Table 3 summarizes the characteristics of coronary lesions elucidated in studies using the WHHL rabbit family. In the proband mutant rabbit of WHHL rabbits, atherosclerosis was developed in the coronary arteries, as well as in the aorta[9]. However, in the original WHHL rabbit strain before 1980, the incidence of coronary atherosclerosis was very low[2]. The serum cholesterol levels were relatively lower in the proband mutant rabbit (216–450 mg/dl)[9] than in the original WHHL rabbit strain (518 ± 129 mg/dl)[1]. The decreased incidence of coronary lesions may be due to cross-breeding with normal rabbits during the development of the original WHHL rabbit strain, suggesting that factor(s) other than serum cholesterol levels are associated with the development of coronary lesions (**Fig. 1**). After the establishment of the original WHHL rabbit strain, Watanabe started a project to increase the incidence of coronary lesions. He performed selective breeding for 5 years using rabbits with coronary lesions. After selective breeding, the incidence of coronary lesions was markedly increased. However, the degree of coronary stenosis was still mild[3]. After 7 years of the second selective breeding for increasing the degree of coronary stenosis, the degree of cross-sectional narrowing was increased more than two-fold compared with the original

WHHL rabbits before the selective breeding[3]. This strain was provisionally named as the WHHL-CA rabbit. As a result, WHHL-CA rabbits can be used to study coronary atherosclerosis and the effects of statins on coronary atherosclerosis. However, the incidence of myocardial infarction was still very low. In human patients who died from myocardial infarction, many macrophages and/or T-cells were observed at the site of rupture or erosion of thrombosed coronary lesions[60]. In WHHL-CA rabbits, quantitative analyses of coronary lesions showed that coronary lesions were rich in collagen fibers, but aortic lesions were rich in macrophages and extracellular lipid deposits[61]. This analytical method was the first in the world and is widely used now. We added macrophage-rich coronary lesions to the selection criteria for the development of myocardial infarction and performed the third selective breeding. After 7 years of the third selective breeding, the cumulative incidence of fatal myocardial infarction increased to more than 90% at 30 months old[4]. Based on this result, the WHHL-CA rabbit that spontaneously develops myocardial infarction was renamed the WHHLMI rabbit. Myocardial lesions of WHHLMI rabbits were classified into subendocardial, intramural, and transmural infarctions (**Fig. 3**). Myocardial lesions of WHHLMI rabbits were myocardial fibrosis accompanied by eosinophilic myocardial cells, hyperemia, and infiltration of inflammatory cells. The electrocardiogram recorded just before sudden death showed the elevation of ST-segment and deep Q wave. The culprit coronary arteries exhibited more than 90% cross-sectional narrowing with vulnerable features (a necrotic core covered by a macrophage-infiltrated thin fibrous cap). These findings suggest that in WHHLMI rabbits, myocardial lesions progressed by repeated ischemic damage, probably due to tachyarrhythmia and died at the last ischemic attack. In young WHHLMI rabbits (10–15 months old) that suddenly died, no myocardial lesions were observed under a light microscope, although the coronary arteries were observed that were almost occluded by serial atherosclerotic lesions. In this case, fresh ischemic changes in the myocardial cells were detected using an electron microscope[62]. These young rabbits probably died by the first ischemic attack. There were no gender differences in the cumulative incidence of fatal myocardial infarction[63]. The incidence of myocardial infarction increased with the progression of coronary stenosis, although serum lipid levels were not associated with the incidence of myocardial infarction in WHHLMI rabbits. Therefore, the development of coronary lesions and/or myocardial infarction may be regulated by some genetic factor(s) other than serum cholesterol levels in WHHLMI rabbits. Similar to human coronary lesions[30], various types of coronary lesions were observed in WHHLMI rabbits (**Fig. 3**), such as fatty streak, fibrous lesion, fibroatheroma, thin-capped fibroatheroma, and advanced complicated lesions with reduced cellular components, calcification, vasa vasorum, etc.[22, 23, 29]. However, physiological intimal thickening in normal arteries observed in humans[30] was not observed in WHHLMI rabbits. WHHLMI rabbits promoted more studies of coronary lesions because severe coronary lesions always developed in WHHLMI rabbits compared with WHHL-CA rabbits. Quantitative analysis of the lesion components revealed differences between the aortic lesions and coronary lesions[61]. It has been clarified that the vasoconstrictor response was enhanced in the atherosclerotic coronary arteries[64]. This study suggests that the development of atherosclerotic lesions is likely to cause coronary artery spasm and has led to later studies on the provocation of acute coronary syndromes by spasm[22, 65]. It is well known that coronary arteries expand in response to the progression of atherosclerotic lesions, and this coronary artery enlargement is called compensatory remodeling. WHHLMI coronary arteries also expanded at the site of arterial lesions, but the coronary lumen area was nearly constant in the range of 10%–68% cross-sectional narrowing[66]. This coronary outward remodeling in WHHLMI rabbits was caused by attenuation of the tunica media by infiltration of macrophages into the tunica media, which expressed matrix metalloproteinase, and proliferation of smooth muscle cells on the adventitia side of the tunica media[67]. This coronary artery enlargement was not a mere physiological compensation. In addition, the degree of curvature of coronary arteries is related to the progression of lesions and the macrophage content in the lesions[68]. Thus, WHHL-CA rabbits and WHHLMI rabbits contribute to studies of coronary lesions that cannot be analyzed in studies of aortic lesions.

Provocation of Acute Coronary Syndromes in WHHLMI Rabbits

On coronary angiography performed just before the onset of myocardial infarction, 67% of patients had coronary lesions with diameter stenosis less than 50%[69]. Falk reported that occlusive thrombi following the rupture of the coronary plaque were associated with coronary events[70]. These observations indicate that in human ischemic heart events, coronary lesions prone to rupture were more frequent than coronary lesions with higher stenosis. Although most WHHLMI rabbits had coronary lesions with more than 90% cross-sectional narrowing, several WHLMI rabbits died from myocardial infarction had coronary

lesions less than 70% cross-sectional narrowing[63]. Furthermore, no clear rupture of these lesions was observed in WHHLMI rabbits, although coronary lesions that appeared vulnerable were observed in WHHLMI rabbits[4]. These observations suggest that the second stimulation, such as mechanical force, plays an important role in the rupture of vulnerable lesions. We provoked coronary spasm by an intravenous injection of ergonovine during an intravenous infusion of norepinephrine[22, 65]. As a result, serum markers for myocardial injury (heart-type fatty acid-binding protein, cardiac troponin-I, and myoglobin) were markedly increased 4 h after spasm provocation, and fractional shortening of the left ventricle evaluated by echocardiogram was decreased at spasm provocation. In coronary lesions, a mild injury was observed in 83% of rabbits, and occlusive thrombus following lesion disruption (**Fig. 3E**) was observed in 9% of rabbits. In human acute coronary syndromes, no occlusive thrombus has been observed in the coronary arteries in about one-fifth of patients[71]. These results suggest that coronary spasm can be a cause of acute coronary syndromes. Infusion of angiotensin II using an osmotic pump caused coronary plaque erosion and rupture that were associated with thrombosis in WHHLMI rabbits[24]. In addition, a mild increase in blood pressure due to surgical treatment caused myocardial infarction along with myocardial hypertrophy[28]. Therefore, WHHLMI rabbits can be an animal model for acute coronary syndromes.

Arterial Lesions Developed in Other Arteries

In addition to coronary arteries and aortas, arterial lesions were observed in cerebral arteries, vertebral arteries, carotid arteries, brachiocephalic arteries, pulmonary arteries, celiac arteries, superior mesenteric arteries, renal arteries, common iliac arteries, and femoral arteries in the WHHL rabbit family fed with normal chow[13, 15]. However, lesion composition depends on the arteries. Fibrous lesions are frequently observed in cerebral arteries, vertebral arteries, renal arteries, and iliac-femoral arteries. Pulmonary lesions are rich in foam cells derived from macrophages. Various lesions, including atheroma, are observed in carotid arteries, brachiocephalic arteries, celiac arteries, and superior mesenteric arteries. However, arterial lesions were not observed in intracranial and intravisceral small arteries[13, 15]. Differences in lesion components in various arteries may depend on the differences in arterial structure, blood flow, blood pressure, and risk factors dependent on each arterial lesion.

Genome Analyses of WHHLMI Rabbits and Identification of Serum Markers Specific for Coronary Atherosclerosis

In 2016, the whole genome of WHHLMI rabbits was analyzed and compared with those of Japanese white rabbits and New Zealand white rabbits[72]. Although the authors described "genome information of WHHL rabbits" in the paper, they used "WHHLMI rabbits" in their study. In phylogenic tree analyses, three rabbit groups were different among each group. The genetic diversity of WHHLMI rabbits was low compared with other groups due to selective breeding for myocardial infarction. Deleterious mutation was observed in 25 genes on 15 chromosomes in WHHLMI rabbits, including genes that may be involved in inflammation or the development and progression of atherosclerosis, such as *ALDH2*, *VWF*, *DOCK4*, *OLR1*, *CRHR2*, and *HCK*. Analyzed WHHLMI rabbits had a large variation in the severity of coronary plaques (data not shown), although every WHHLMI rabbit has the same mutation at the same loci. Therefore, these mutations were not associated with the progression of coronary lesions and the development of myocardial infarction. In addition, the gene abnormality in LDLR was a 12 base pair deletion in a LDL binding domain in a previous study[38], but in this analysis it was an 11 base pair deletion. Rabbit genome information is now available from the National Center for Biotechnology Information database at http://www.ncbi.nlm.nih.gov/sra, and a comprehensive database containing both rabbit genome and transcriptome information has been comprehensively constructed by the Chinese Academy of Sciences[73] at http://www.picb.ac.cn/RabGTD/. Fan *et al.*[74] identified 29.8 million single nucleotide polymorphisms and 1.6 million small indels in the 30 genomes in WHHLMI rabbits in the above rabbit genome analyses, suggesting the difficulty in the detection of the gene(s) associated with the progression of coronary lesions and the development of myocardial infarction in WHHLMI rabbits.

We attempted to identify serum markers for the progression of coronary lesions and/or the development of myocardial infarction in WHHLMI rabbits[75]. Using WHHLMI rabbits has several advantages in studies of identification of serum markers for coronary lesions; 1) it is easy to keep constant several factors, such as dietary habits, environmental conditions, non-target diseases, and social stress; 2) there is no influence of artificial factors that occur when atherosclerotic lesions are induced artificially; 3) coronary lesions and/ or myocardial infarction are developed spontaneously. Our previous studies suggested that factors associated with coronary lesions differ from

those of aortic lesions[68, 75]. Because there were gender differences in the metabolome analysis of 59 metabolites and lipidome analysis of 313 lipid molecules, we analyzed data for females with large coronary artery disease deviations[75]. In female WHHLMI rabbits, molecules selected as serum markers for coronary lesions were lysophosphatidylcholine (LPC) 22:4 and diacylglycerol 18:0–18:0 at 4 months old, LPC 20:4 (sn-2), ceramide d18:1–18:2, citric acid plus isocitric acid, pyroglutamic acid at 8 months old, and phosphatidylethanolamine plasminogen 16:1p-22:2 at 16 months old. These markers were coronary lesion-specific markers independent of aortic lesions and conventional markers such as serum cholesterol levels. Although further studies will be required to extrapolate these data to humans, these serum markers may be useful to detect patients who will develop cardiovascular disease in the near future.

Genetically Modified WHHL Rabbits and Gene Therapy

Genetically modified animals are essential for elucidating the pathogenesis of diseases and for developing gene therapy technology. Improvement in gene-editing technology has made it possible to create genetically modified animals in various animal species. However, there is a difference in the characteristic phenotype of lipoproteins and atherosclerosis between mice and rabbits after gene transfection[76], and the characteristics of genetically modified mice do not always reflect human pathophysiological conditions[76]. The first gene transfer to WHHL rabbits was the transfer of the LDL receptor gene into the liver in 1988[77], and 32 studies were reported. The first transgenic WHHL rabbit was developed in 1993[78], and 9 studies were reported. Fan *et al.*[79] tabulated genetically modified rabbits (including knockout rabbits) related to lipid metabolism and atherosclerosis which were developed by 2018. Some of the gene-modified rabbits were cross-bred with the WHHL rabbit family to transfer a target gene to the WHHL rabbit family, and the influence of these genes on atherogenesis was examined[80, 81]. Studies using transgenic WHHL rabbits revealed that Lp(a) enhanced atherosclerosis[80] and overexpression of LPL improved hypercholesterolemia and obesity, but enhanced atherosclerosis[81], probably due to an increase in the expression of LPL on vascular macrophages. Gene transfer into WHHLMI rabbits and the development of genetically modified WHHLMI rabbits will be useful for identifying genes involved in the progression or suppression of atherosclerosis and elucidating the pathophysiological conditions. In 2014, Yang *et al.*[82] developed LDL receptor knockout rabbits using the gene-editing method, but they did not refer to coronary lesions. In our experience, LDL receptor deficiency in WHHL rabbits is responsible for hypercholesterolemia and aortic atherosclerosis, but the progression of coronary atherosclerosis is regulated by unknown factors[75]. There was a great variation in the severity of coronary lesions in the homozygous offspring obtained by cross-breeding the homozygous WHHLMI rabbits with the heterozygotes (unpublished results), despite similar levels of serum lipid levels and aortic lesions. Although the development of the LDLR-knockout rabbits is excellent, it is worrying whether severe coronary lesions always develop.

Conclusion

Since the breeding and providing of WHHL rabbit family have been completed, this review summarized the history of the development and research contribution of the WHHL rabbit family. The WHHL rabbit family has greatly contributed to the elucidation of lipoprotein metabolism and the pathogenesis of atherosclerosis and coronary heart disease. Studies using the WHHL rabbit family show that animal models for human disease corresponding well to human pathological conditions can greatly contribute to the progression of biomedical science. Finally, information on the WHHL rabbit family and a list of research articles using the WHHL rabbit family can be found on the WHHL website (http://www.med.akita-u.ac.jp/~doubutu/WHHL/WHHL-home.html).

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Conflicts of Interests

There is no conflict of interest associated with this manuscript within the past 36 months.

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Supplementary Table 1. The result about providing of WHHL rabbit family

Regions	Countries	Organizations	Provided rabbits
Asia	Hong Kong	1	12
	Japan	80	4,154
	Taiwan	1	8
	Singapore	1	4
	South Korea	1	34
Europe	Austria	1	6
	Belgium	2	32
	Finland	2	17
	France	1	6
	Germany	2	52
	Italy	1	unknown
	Spain	1	unknown
	Sweden	2	6
	Switzerland	1	4
	The Netherland	1	6
	United Kingdom	4	18
North America	Canada	2	80
	USA	21	184
Oceania	Australia	3	16
Total	19	128	4,639

The WHHL rabbit family is a collected term for WHHL rabbits, coronary atherosclerosis-prone WHHL rabbits (WHHL-CA rabbits), and myocardial infarction-prone WHHL rabbits (WHHLMI rabbits).