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Review

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

- Contribution to the Development and Validation of the Therapeutics for Hypercholesterolemia and Atherosclerosis -

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A number of effective drugs have been developed through animal experiments, contributing to the health of many patients. In particular, the WHHL rabbit family (WHHL rabbits and its advanced strains (coronary atherosclerosis-prone WHHL-CA rabbits and myocardial infarction-prone WHHLMI rabbits) developed at Kobe University (Kobe, Japan) contributed greatly in the development of cholesterol-lowering agents. The WHHL rabbit family is animal models for human familial hypercholesterolemia, coronary atherosclerosis, and coronary heart disease. At the end of breeding of the WHHL rabbit family, this review summarizes the contribution of the WHHL rabbit family to the development of lipid-lowering agents and anti-atherosclerosis agents. Studies using the WHHL rabbit family demonstrated, for the first time in the world, that lowering serum cholesterol levels or preventing LDL oxidation can suppress the progression and destabilization of coronary lesions. In addition, the WHHL rabbit family contributed to the development of various compounds that exhibit lipid-lowering and anti-atherosclerotic effects and has also been used in studies of gene therapeutics. Furthermore, this review also discusses the causes of the increased discrepancy in drug development between the results of animal experiments and clinical studies, which became a problem in recent years, and addresses the importance of the selection of appropriate animal models used in studies in addition to an appropriate study design.

Key words: **Anti-atherosclerotic agents, Cholesterol-lowering agents, Development of therapeutics, Species differences, WHHL rabbit**

Introduction

According to a report by WHO (https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death), out of 56.9 million deaths worldwide in 2016, more than 9 million were due to ischemic heart disease and increased by approximately 2 million since 2000. It has long been pointed out that hypercholesterolemia is associated with the onset of ischemic heart disease. Animal models for human disease greatly contributed to the development or validation of therapeutic agents or devices and diagnostic equipment or technologies. In particular, the Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model of familial hypercholesterolemia[1-3], and its advanced strains developed by selective breeding, the WHHL-CA rabbits (provisional name)[4, 5] showing spontaneous coronary atherosclerosis, and the WHHLMI rabbit showing spontaneous coronary atherosclerosis and myocardial infarction[6] played an important role in the development and validation of therapeutic agents for hypercholesterolemia or atherosclerosis. 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. However, breeding of the WHHL rabbit family at Kobe University ended in June 2018. Currently, a few institutes are trying to breed WHHLMI rabbits. On this occasion, the contribution of the WHHL rabbit fam-

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Table 1. Studies using the WHHL rabbit family to develop compounds with lipid-lowering effects and anti-atherosclerotic effects

	Lipid-lowering effects		Anti-atherosclerosis	
	Cholesterol	Triglyceride	Aorta	Coronary
Statin	○	○×	○×	○
Anion resin	○	×	○	n.e.
Statin + resin	○	×	○	○
Squalene synthase inhibitor	○	○	○	○
MTP inhibitor	○	○	n.e.	n.e.
ACAT inhibitor	○×	×	○×	○×
Fish oil or ω3 fatty acids	○	○	○×	n.e.
ApoE	○	×	○	n.e.
Fibrate	×	×	○×	n.e.
D-47	○	○	n.e.	n.e.
Probucol	○	×	○	○
M-CSF and GM-CSF	○	○	○	n.e.
Thiazolidinedione	×	×	×	×
Thiazolidinedione + statin	○	×	○	○
Ca^{2+} antagonists	×	×	×	×
β-blockers	×	×	×	×
Angiotensin converting enzyme inhibitors	×	×	○	n.e.
Angiotensin-II type I receptor antagonists	×	×	○	n.e.
LDLR gene therapy	○	○×	○	n.e.
Apobec-1 expression in liver	○	○×	n.e.	n.e.
LPL expression in whole body cells	○	○	×	n.e.

○, effective; ×, no effect; n.e., not examined. References for studies about lipid-lowering or anti-atherosclerotic effects of each agent, compound or molecule are listed on the WHHL rabbit website (http://www.med.akita-u.ac.jp/~doubutu/WHHL/w-index.html).

ily in the development or validation of therapeutic agents and diagnostic equipment was summarized in this review. This review also addressed the issues of increasing discrepancies in drug development between the results of animal experiments and clinical studies. The history of the development of the WHHL rabbit family, the characteristics, and their contribution to studies on lipoprotein metabolism and atherosclerosis are described in the Part I[7]. In this review, terms "WHHL rabbit", "WHHL-CA rabbits", and "WHHLMI rabbit" indicate the homozygotes.

Contribution of the WHHL Rabbit Family to the Development of Therapeutic Agents and Imaging Technology for Atherosclerosis

Studies using the WHHL rabbit family that examined lipid-lowering effects and/or anti-atherosclerotic effects of compounds, foods, proteins, and gene therapy are summarized in **Table 1**.

Contribution of the WHHL Family to the Establishment of Statins' Lipid-Lowering Effects and Anti-Atherosclerosis Effects

Nowadays, the first-choice medicine for hypercholesterolemia is statins, which are competitive inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, a rate limiting enzyme in the early stage of cholesterol biosynthesis. The first statin, compactin, was developed by Akira Endo (Sankyo Co., Ltd., Tokyo, Japan) in 1973[8, 9]. Since compactin results in no lipid-lowering effect in mice and rats, the research group considered discontinuing the development[9]. However, compactin continued to be developed because it showed a potent cholesterol-lowering effect in other animals[9]. The continued development of compactin led to the development of seven statins, which are currently available. The compactin research group considered that it is important that cholesterol-lowering agents should be effective in animal models with spontaneous hypercholesterolemia. In 1980, cholesterol-lowering effects of compactin were examined in WHHL rabbits (homozygotes), and the serum cholesterol levels were decreased dose-dependently[10]. After cessation in compactin development, pravastatin, a compactin metabolite, was developed[11], and pravastatin also lowered serum cholesterol levels dose-dependently in WHHL rabbits[11]. In addition to compactin and pravastatin, various statins, such as lovastatin[12], simvastatin[13], fluvastatin[14], cerivastatin[15],

pitavastatin[16], and atorvastatin[17], also showed potent serum cholesterol-lowering effects in the WHHL rabbit family. The cholesterol-lowering effects of statins are considered to be due to an increase in the number of LDL receptors in the liver. The hepatic LDL receptor activity in WHHL rabbits (homozygote) was increased 11.2-fold with pravastatin treatment[18], and similar results were observed in fluvastatin[19]. Mutant LDL receptor proteins of WHHL fibroblasts were biosynthesized similar to a variant of human familial hypercholesterolemia[20], and serum LDL cholesterol levels decrease by approximately 80% at the end of pregnancy in WHHL rabbits[21], similar to another variant of human familial hypercholesterolemia[22]. In that paper, the patient was considered as a heterozygote but was diagnosed as a homozygote in the later examination (personal communication with Kajinami K). These studies suggest that mutant LDL receptors in the WHHL rabbit family may be able to increase, and increased LDL receptors may be able to bind LDL particles. In addition, cholesterol content in newly secreted very low-density lipoprotein (VLDL) was decreased by 18% in pravastatin treated WHHL-CA rabbits[23]. These studies suggest that statins' cholesterol-lowering effects were due to a dual mechanism, an increase in hepatic LDL receptors, and a decrease in VLDL-cholesterol secretion from liver.

In the original WHHL rabbit strain, atherosclerotic lesions were observed in the aorta but were rare in the coronary arteries[1, 4]. WHHL-CA rabbits demonstrating coronary atherosclerosis were developed by selective breeding at Kobe University[4, 5], and the WHHL-CA rabbit made the possibility to investigate the suppression effect of drugs on coronary lesions exist. Thereafter, anti-atherosclerotic effects of pravastatin were examined using young WHHL-CA rabbits[24]. Twenty-four weeks of the statin treatment reduced serum cholesterol levels by 28%, and the atherosclerotic lesions were suppressed in the aorta and coronary arteries. This was the first study that statin can suppress coronary atherosclerosis. Since the serum cholesterol levels treated with pravastatin were still high, cholestyramine, a bile acid sequstrant, and pravastatin were co-administered to mature WHHL-CA rabbits aged 10 month for one year to lower serum cholesterol levels to almost normal. These WHHL-CA rabbits had established atherosclerotic lesions. The purpose of this study was to investigate whether atherosclerotic lesions could regress or not[25]. In the combination treatment, the serum cholesterol levels decreased to 229 ± 23 mg/dl. The degree of atherosclerotic lesions decreased markedly in both coronary arteries and the aorta. However, the degree of atherosclerosis in the combination treatment group (22 months old) was comparable to control rabbits examined at the start of the treatment (10 months old). In the histopathological examination, arterial lesions of the combination treatment group were fibrous lesions rich in collagen fibers and scarce in macrophages and extracellular lipid deposits, whereas arterial lesions in control and placebo groups were atheromatous lesions enriched in macrophages or necrotic cores. These results suggested that cholesterol-lowering therapy can regress atherosclerotic lesions if the lesions are not fibrotic. In the REGRESS study[26], one of clinical trials of statins, coronary events were suppressed by pravastatin treatment despite almost no changes in the degree of coronary stenosis evaluated with quantitative coronary angiography. This study indicated that statins prevent coronary events by some function other than the suppression of the degree of coronary stenosis. At that time, acute coronary syndromes are thought to occur with sudden coronary occlusion after rupture of coronary vulnerable lesions[27-29]. Therefore, statins' protective effects on coronary events were considered to be statin's atheroma stabilizing effects that prevent rupture of coronary lesions. Using mature WHHL-CA rabbits or WHHLMI rabbits that established coronary lesions, atheroma stabilizing effects of statins were examined (**Fig. 1**)[30-33]. In a quantitative analysis of lesion components[34], compared to the placebo group, statin treatment increased collagen fibers and suppressed a decrease in smooth muscle cells during lesion progression, but treatment decreased macrophage derived foam cells and extracellular lipid accumulation. Consequently, an increase in lesion vulnerability score calculated by dividing the sum of the areas of macrophages and extracellular lipid by the sum of the areas of smooth muscle cells and collagen fibers was decreased. These observations about stabilization of coronary lesions were associated with a decrease in the expression of matrix metalloproteinases in atheromatous lesions[31-33]. In addition, studies using WHHL rabbits revealed that statin preserved the endothelium-dependent relaxation of the coronary arteries[35] and improved disturbed endothelial barrier function[36]. Similar findings were observed in normal rabbits fed with a mild fat chow[37-39]. Recently, researchers reported that administration of statins after radiation therapy for cancer suppressed the onset of cerebrovascular and cardiovascular diseases[40]. These results in statin treatments may be related to statins' vascular endothelial cell protective effects and anti-inflammatory effects. Therefore, statins' anti-atherosclerotic effects are considered to be due mainly to lowering of plasma LDL cholesterol and protection of arterial endothelial cells. As described above, the WHHL rab-

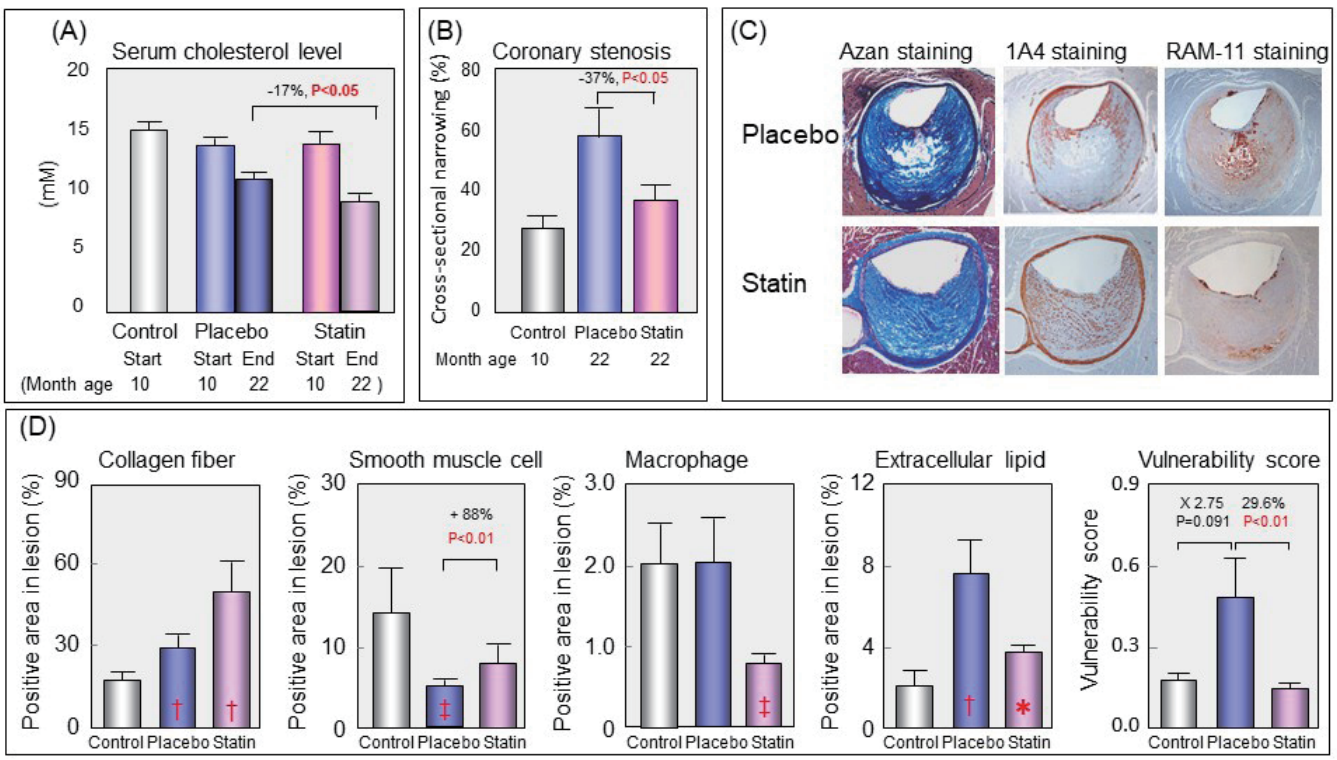


Fig. 1. Suppressive effect of pravastatin on destabilization of coronary lesions in WHHL-CA rabbits

Coronary lesions were examined at the start of treatment (10 months old) in control group and at the end of treatment (22 months old) in placebo and statin groups. (A) Plasma cholesterol levels of each group at the start (10 months old) and the end of the treatment (22 months old), (B) The degree of coronary lesions of each group, (C) Photomicrographs of coronary lesions of placebo and pravastatin treated rabbits, and (D) Lesional composition of coronary lesions of each group. Vulnerability score was calculated by dividing sum of macrophage area and extracellular lipid area by sum of collagen area and smooth muscle cell area. Panels A – D are modified from Shiomi M, *et al.*[30], and panel C is modified from Shiomi M, *et al.*[31] *, $P<0.05$; ‡, $P<0.01$; †, $P<0.001$ (vs. 10 months old control)

bit family contributed significantly to the development and efficacy evaluation of statins.

Development or Validation of the Lipid-Lowering Effects of Various Non-Statin Compounds Using the WHHL Rabbit Family

Lipid-lowering effects of various non-statin agents or molecules were examined in the WHHL rabbit family (**Table 1**). Similar to statins, various compounds and molecules showed lipid-lowering effects in the WHHL rabbit family. Since the microsomal triglyceride transfer protein (MTP) plays an important role in the assembly of VLDL particles in the liver and of chylomicron particles in the intestine[41], MTP inhibitors lower plasma cholesterol levels by a mechanism different from statins. Lipid-lowering effects of MTP inhibitors were potent in WHHL-CA rabbits[42], but a concern existed about lipid accumulation in the liver. Since a compound D-47, which exhibits unique lipid metabolism improving effects, decreased not only serum lipid levels but lipid accumulation in the liver and adipose tissues in WHHLMI rabbits[43], D-47 may be effective to suppress lipid accumulation in the liver of animals treated with an MTP inhibitor. In addition, D-47 increased in the expression of *CYP7A1*, mRNA of cholesterol-7α-hydroxylase, and *CPT-1*, mRNA of carnitine palmitoyltransferase-1, but suppressed the expression of *MTP* and *FAS*, mRNA of fatty acid synthase, in the liver, and an increase in the expression of *CPT-1* and *LPL*, mRNA of lipoprotein lipase, in the mesentery[43]. Therefore, D-47 may exhibit functions to prevent non-alcoholic fatty liver disease and metabolic syndrome. Further studies on D-47 are required. Triglyceride lowering effects in the WHHL rabbit family were observed with squalene synthase inhibitors[44], MTP inhibitors[42], fish oil[45] or omega-3 fatty acids[46], monocyte-specific colony-stimulating factor (M-CSF)[47], granulocyte-macrophage colony-stimulating factor (GM-CSF)[48], compound D-47[43] but not with fibrates[43, 49]. Omega-3-fatty acids inhibit the assembly and secretion of VLDL particles[50] and enhanced fatty acid oxidation in the liver[51]. Conversely, fibrates function through activation of the per-

oxisome proliferator-activated receptor alpha[52]. Since fenofibrate decreased plasma triglyceride levels in normal New Zealand white rabbits fed normal chow[53], fibrates may not work well in hypercholesterolemia due to LDL receptor deficiency. Although the mechanism of the cholesterol-lowering effects of colony stimulating factors was not elucidated sufficiently, an increased clearance of apoB-100 containing lipoproteins through both LDL receptor-dependent and -independent pathways[47] and an enhancement of VLDL receptor-mediated uptake of plasma VLDL[48] may be involved. As described above, the WHHL rabbit family have been used to examine the lipid-lowering effects of various compounds, foods, and proteins.

Validation of the Anti-Atherosclerotic Effects of Various Non-Statin Compounds Using the WHHL Rabbit Family

Anti-atherosclerotic effects of various non-statin agents or molecules were examined in the WHHL rabbit family (**Table 1**). Similar to statins, a squalene synthase inhibitor, which inhibits cholesterol synthesis at a late stage in cholesterol biosynthesis pathway, also exhibits cholesterol-lowering effects and suppressed the destabilization and progression of atherosclerosis in WHHLMI rabbits[44]. Since squalene synthase inhibitors function distal to farnesyl pyrophosphate associated with the pleiotropic effects of statins[54] and an increase in derivatives derived from farnesyl pyrophosphate is said to be associated with atherogenesis, the anti-atherosclerotic effects of squalene synthase inhibitors were considered to be due mainly to cholesterol-lowering effects. Other interesting molecules are M-CSF[55] and GM-CSF[56]. M-CSF and GM-CSF are released activated cells in the arterial wall and can activate monocytes and macrophages[57]. However, M-CSF and GM-CSF suppressed atherosclerosis in WHHL-CA rabbits. Yamada *et al.*[58] observed a decrease in the cellular cholesterol ester content in macrophages incubated with acetyl-LDL by the addition of M-CSF in the medium and an increase in plasma high-density lipoprotein (HDL)-cholesterol levels in WHHL-CA rabbits after M-CSF administration. These results suggest that atherosclerosis can be suppressed by normally activated macrophages or normalization of macrophage function. Regarding the anti-atherosclerotic effects of antihypertensive agents on normotensive WHHLMI rabbits, inhibitors of angiotensin converting enzyme[59] and angiotensin-II type I receptor antagonists[60] showed anti-atherosclerotic effects, but not calcium antagonists and beta-blockers. Angiotensin II promotes atherosclerosis through enhancement of cell proliferation, oxidative stress, suppression of arterial endothelial cell function, and inflammation through angiotensin II type I receptors in the arterial wall[61]. It is well known that an important risk factor for atherosclerosis is oxidized LDL. Anti-atherosclerotic effects of vitamins and compounds exhibiting anti-oxidative action have also been studied in WHHL-CA rabbits. Among various compounds and foods (vitamin E, alpha-tocopherol, herbal mixture, wine, and others) demonstrating anti-oxidative function, only probucol showed a clear anti-atherosclerosis effect in the WHHL rabbit family[62-64]. Although a case of QT-interval prolongation by probucol is a problem in humans with a missense mutation in the N-terminus of *HERG* gene[65], QT-interval prolongation has not been observed in rabbits. As described above, the WHHL rabbit family have been used to examine the anti-atherosclerotic effects of various compounds, foods, and proteins.

Contribution of the WHHL Rabbit Family to the Development of Imaging Technologies for Atherosclerosis

The development of evaluation technologies for atherosclerotic lesions is of great importance in the diagnosis of atherosclerotic lesions and the evaluation of the therapeutic effects. Studies on atherosclerosis imaging are actively conducted using the WHHL rabbit family, and 71 papers have been reported. For imaging of vulnerable atheromatous lesions, several apparatuses have been used, such as positron emission tomography (PET), optical coherence tomography, computed tomography (CT), single photon emission computed tomography, magnetic resonance, intravascular ultrasound (IVUS), and others. In fact, these imaging technologies (CT-PET[64], IVAS[66], and iMAP IVUS[67]) were effective in evaluating the therapeutic effects on atherosclerotic lesions. It is desirable that the animals used for imaging atherosclerosis demonstrate histopathologically similar arterial lesions as human lesions and are of a physical size that can be repeatedly imaged. The WHHL rabbit family was suitable for this condition.

The WHHL Rabbit Family as a Model of Gene Therapy

WHHL rabbits were also used in studies of gene therapy. Target genes were LDL receptor[68, 69], apoB-editing enzyme[70], monocyte chemoattractant protein-1 (MCP-1)[71], vascular endothelial growth factor (VEGF)[72, 73], VEGF receptor[74], endothelial nitric oxide synthase[75], and β-galactosidase[76]. All of these gene therapies were effective in WHHL rabbits. Transfection of LDLR gene into WHHL liver using a lenti-

Table 2. Considerations in the designing animal experiments

1) Use of animal models that have similar pathophysiological mechanisms to the target disease in humans
2) Use of animals whose age corresponds to the age at which the target disease develops in humans
3) Use of animal species / animal models with moderate heterogeneity
4) Start of interventions such as drug administration after symptoms progress to some extent
5) The use of males and females both in experiments
6) Use of animal numbers calculated based on statistics, or more than one repetition of the same experiment using the same number of animals when the variance cannot be predicted
7) Use of doses resulting in blood levels corresponding to blood levels of human

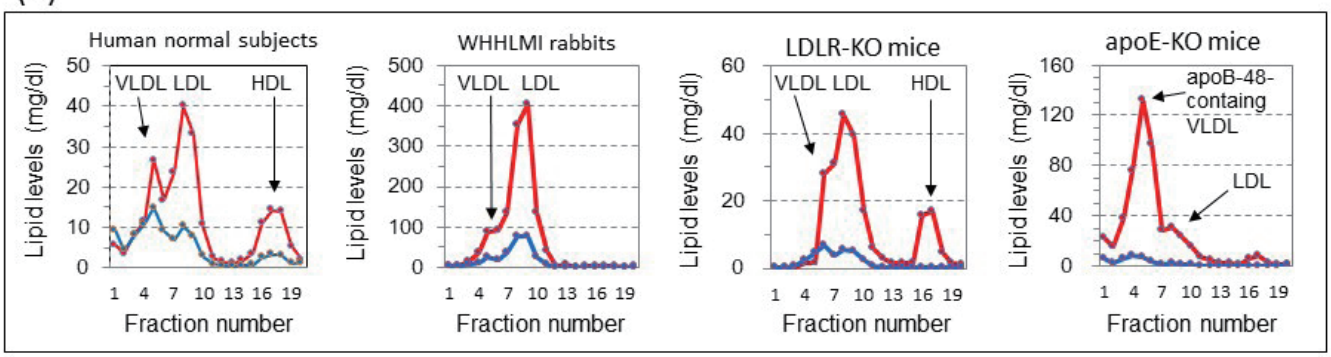
viral vector decreased serum cholesterol levels by 20 – 40% for one year without any side effects[69]. Transfection of a VEGF gene successfully mediated angiogenesis in skeletal muscle of WHHL rabbits[72, 73]. Thus, the WHHL rabbit family has also been used in studies of gene therapy, but further studies are needed to proceed gene therapy.

Translation of the Results of Animal Experiments into Humans

Although the WHHL rabbit family contributed to the development of therapeutics as described above, the Wall Street Journal issued February 24, 2004 said that the number of newly developed drugs decreased by approximately 60% in the United States from 1996 to 2003, despite the fact that the budget for new drug development increased almost doubled in this period. Since 2004, researchers frequently pointed out that an increasing discrepancy between the results of animal experiments and the results of clinical studies in the development of new drugs exists[77-82]. The possible causes are considered to be due to inadequate research target[79, 80], insufficient study design[77, 79-81], and species differences[77-80] (**Supplementary Table 1**). The differences between the disease state of human patients and therapeutic conditions of animal experiments are summarized in **Supplementary Table 2** [77, 79]. For reduction of the discrepancy between the results of animal experiments and human clinical studies, designing animal experiments that correspond to the pathophysiology of human patients is necessary, in addition to an adequate study target. **Table 2** proposes considerations in the design of animal experiments for translation of the results of studies using animals into humans. In studies using animals (especially mice and rats), considering the effects of age, circadian rhythm, genetic homogeneity, gender, cause of disease, time to begin treatment, and drug doses is better. These differences between human patients and animals used in studies can affect the translation of the results of animal experiments into humans. Van der Worp *et al.*[77] claimed that in animal studies the drug is administered at high doses that are toxic or intolerable to humans. Indeed, the dose of pravastatin to decrease serum cholesterol levels by approximately 30% without any side effects was 40 mg/body/day in humans[83], 50 mg/kg/day in cynomolgus monkeys[11], 3 mg/kg/day in beagle dogs[84], 12.5 mg/kg/day in normal rabbits[11], and 50 mg/kg/day in WHHL rabbits[11]. The causes of this species difference may be the difference in absorption of compounds in the intestine, degradation of the compounds in the liver, and the removal rate of the active form(s) of the compounds from the circulation. These results indicate that there are species differences in the effective dose of drugs, and the safety of the dose of drug that was effective in an efficacy test using animals should be examined in a safety study performed independently. The sample size in animal experiments is required to be as small as possible, from the viewpoint of animal welfare[77, 81]. However, studies with too few animals can lead to incorrect results. Calculating the number of animals needed for the experiment based on statistics is important.

Regarding species differences, lipoprotein metabolism, atherosclerosis, and myocardial function of mice and rats including genetically modified animals, these characteristics are very different from humans, but those of rabbits resemble human features (**Supplementary Table 3**)[85-88]. **Fig. 2** shows lipoprotein profiles and plasma lipid levels of healthy humans and several animal models. In human familial hypercholesterolemia homozygotes who are deficient in LDL receptors, the serum cholesterol levels are more than 500 mg/dl due to an elevation of LDL cholesterol levels. The WHHL family also showed a marked elevation of LDL fraction (**Fig. 2**), due to LDL receptor deficiency. However, in LDLR-KO mice fed standard chow, the serum cholesterol levels were 218 ± 17 mg/dl, and the elevation of LDL cholesterol levels was mild (**Fig. 2**)[89]. Similar findings were observed in LDLR-KO rats[90]. Since overexpression of apoB-100 in LDLR-KO mice liver markedly increased LDL-cholesterol levels[91] and LDL-cholesterol levels decreased markedly in WHHL rabbits expressed apoB mRNA editing enzyme (*APOBECK-1*) in the livers[70], these differences between human patients or the WHHL family and mice or rats may be due to expres-

(A)



(B)

Plasma lipid levels at normal chow feeding (mg/dl)				
	Healthy human	WHHLMI rabbit	LDLR-KO mouse	apoE-KO mouse
Total cholesterol	211±11	1487±68	218±17	513±33
Triglyceride	88±5	113±26	33.3±3.9	41.7±4.4

Fig. 2. Lipoprotein profiles (A) and plasma lipid levels (B) of healthy humans, WHHLMI rabbits, LDLR-KO mice, and apoE-KO mice

Lipoprotein profiles were analyzed with high performance liquid chromatography[87]. Animals were fed standard chow. Red lines indicate cholesterol and blue lines indicate triglyceride.

sion of *APOBECK-1* in the liver of mice and rats. Lipoprotein profiles of apoE-KO mice, another mice model for hypercholesterolemia, are also very different from human hypercholesterolemia (**Fig. 2**). In apoE-KO mice, the VLDL fraction is increased markedly, but the VLDL particles contain apoB-48 instead of apoB-100[92]. In addition, no cholesterol ester-transfer protein activity in the plasma[93], high activity of hepatic LDL receptor function[86], and high activity of hepatic lipase in pre-heparin plasma[94] in mice are also very different from humans. However, those of rabbits are close to humans[85-88]. In particular, the cholesterol-lowering effects of statins were hardly observed in mice[95] and rats[9, 11, 96] but were potent in rabbits and WHHL rabbit family[11-20, 23, 30-34]. In rats treated with statins, the activities of hepatic HMG-CoA reductase[96] and synthesis of hepatic fatty acids[96] were increased markedly. In mice, high activity of hepatic LDL receptor, high excretion of bile acid, and secretion of VLDL with apoB-48 from liver[88] may be associated with the lack of the effects of statins. Conversely, fibrates are effective in rats and normal rabbits but not in the WHHL rabbit family. Recently, monoclonal antibodies against proprotein convertase subtilisin/kexin type 9 (PCSK9) that inhibit lysosomal degradation of LDL receptor proteins have been developed, and mice have also been used for the development of PCSK9 antibodies[97]. Also, with respect to atherosclerosis and the characteristics of myocardium, mouse models are different from humans, but rabbits are similar to humans[85-88]. In humans and WHHLMI rabbits, macrophages in atherosclerotic lesions express VLDL receptors but not in LDLR-KO mice and apoE-KO mice[98]. These findings suggest that the mechanism of atherogenesis in mouse models may be different from those of humans and WHHLMI rabbits. Finally, Fan *et al.*[85] reported that distinctive phenotypes of lipoprotein metabolism and the onset of atherosclerosis differ between mice and rabbits that have been transfected the same gene. These observations about species differences give us the following two lessons: 1) In order to extrapolate the results of animal experiments to humans, using appropriate animal models in which pathogenesis and enzymes in the related metabolic pathway closely match humans is necessary, in addition to serological data such as total cholesterol levels; 2) Not all genetically modified animals correspond to human diseases. From these viewpoints, the WHHL rabbit family are suitable animal models for studies of lipid metabolism, atherosclerosis, and the development of the therapeutics. The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) proposed ARRIVE Guidelines to improve the discrepancy between the results of animal experiments and clinical research[99]. In order to translate the results of studies

Table 3. Conditions of animals used in studies to translate the results into humans

1) Animals with onset mechanism similar to human disease in addition to pathophysiology of human disease
2) Animals showing histopathological features similar to human lesions
3) Animals showing similar drug efficacy to human patients
4) Animals with a physical size that is compatible with repeated sampling and indwelling devices
5) Animals having appropriate heterogeneity.

using animals to humans, in addition to appropriate study designs and appropriate treatment targets, using animals corresponding to human conditions is important (**Table 3**). To perform translatable animal experiments, researchers should consider study design and animals used in studies more carefully.

Conclusion

With the end of breeding of the WHHL rabbit family at Kobe University, this review summarized the contribution of the WHHL rabbit family to the development of therapeutics for hypercholesterolemia and atherosclerosis. Studies using the WHHL rabbit family demonstrated that using animals suitable for study purpose is important for translating the results into humans. Information on the WHHL rabbit family and a list of studies using the WHHL rabbit family that can be used for subject searches can be found on the WHHL website (http://www.med.akita-u.ac.jp/~doubutu/WHHL/WHHL-home.html).

Author contribution

MS prepared this manuscript.

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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. Causes of poor reproducibility of the results of animal experiments in clinical trials

1) Species difference between humans and animals used in studies[77-80)]
2) Inadequate research targets[79, 80)]
3) Large differences between study design of animal experiments and the condition of human patients[77)]
4) Too small sample sizes in animal experiments[77, 80, 81)]
5) Insufficient study design[77, 79-81)]
6) No standard for protocols of animal experiments in drug development[79)]

Supplementary Table 2. Differences in condition between human patients and animals used in preclinical studies

	Humans	Animals
Age	Elder	Young
Presence of other diseases	Having other disease(s)	None
Circadian rhythm	Diurnality	Nocturnal
Genetic homogeneity	Individual differences	Homogenous strain
Gender	Both men and women	Males or females
Cause of disease	Spontaneous or disturbance of the immune or metabolism	Artificial induction or insufficient model
Time to begin treatment	After symptoms progress	At the time of induction or early stage
Drug dose		Extremely high dose

Summarized after van der Worp HB, *et al.*[77)] and Couzin-Frankel J.[79)].They discussed the difference between human treatment condition and experiments with primarily mice.

Supplementary Table 3. Species differences in lipoprotein metabolism, atherosclerosis, and myocardial characteristics

	Humans	WHHLMI rabbits	Mice
Lipoprotein metabolism			
Main lipoprotein	LDL	LDL	HDL, VLDL with apoB48
ApoB of VLDL	ApoB-100	ApoB-100	apoB-48
Expression of apoB editing enzyme	ilium	ilium	ilium and liver
CETP in plasma	Yes	Yes	none
Hepatic lipase activity in pre-heparin plasma	very low	very low	high
Response to dietary lipid	sensitive	sensitive	resistant
Effects of endothelial lipase on LDL	no effects	no effects	lowering of LDL
Cholesterol-lowering effects of statin	effective	effective	ineffective
Apolipoprotein(a)	Bound to LDL	Bound to LDL	Not bound to LDL
HDL	heterogeneous	heterogeneous	homogenous
Apolipoprotein AII	Dimmer	Absent	Monomer
Hepatic LDL receptor activity	Down regulated	Down regulated	Usually high
Main cholesterol pool	hepatic synthesis	hepatic synthesis	dietary origin
Excretion of bile acid	Low	Low	High
Atherosclerosis			
Atherogenesis	Sensitive	Spontaneous	Resistant
Coronary lesions	Frequent	Frequent	Rare
Feature of coronary lesion	Various types	Various types	Excessive lipid deposition
Expression of VLDL receptors in lesions	Macrophages	Macrophages	no expression
Destabilization of plaques by MMPs	Yes	Yes	Inconsistent results
Acute inflammation marker	CRP	CRP	SAP
Cardiac function			
Cardiac myosin			
Myosin heavy chain	β-type	β-type	α-type
Ion channel of myocardial myosin	I_{Kr} and I_{Ks}	I_{Kr} and I_{Ks}	I_{to} and $I_{k,\,slow}$
ECG	12-lead ECG	12-lead ECG	Single lead ECG
Wave form of ECG	T-wave	T-wave	J-wave

Summarized after Fan J, *et al.*[84, 85, 87)], and Shiomi M, *et al.*[86)]