

High Pathological Reproducibility of Diet-induced Atherosclerosis in Microminipigs *via* Cloning Technology

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Abstract. *Background/Aim:* The reproducibility of atherosclerotic lesions was evaluated after the production of cloned-microminipigs and their offspring. *Materials and Methods:* Cloned-microminipig-parents were produced by microminipig-somatic cell nuclei. These parents were crossbred and delivered males (F1-offspring) were divided into two groups: normal chow diet (NcD)-fed and high-fat/high-cholesterol diet (HcD)-fed groups. One of the F1-offspring was subjected to cloning, and delivered males (F1-clones) were fed with HcD. After 8 weeks, all animals were necropsied for pathophysiological studies compared to non-cloned-microminipigs. *Results:* HcD-fed F1-offspring and F1-clones, but not NcD-fed F1-offspring, exhibited increased serum lipid levels and

systemic atherosclerosis, which were comparable to those of HcD-fed non-cloned-microminipigs. Homogeneity of variance analysis demonstrated that standard deviation values of serum lipoprotein and aortic atherosclerosis area from HcD-fed animals decreased in F1-offspring and F1-clones. *Conclusion:* HcD-induced atherogenesis was highly reproducible in F1-offspring and F1-clones, indicating that the atherosclerosis-prone genomic background was preserved in the cloned-microminipigs, which can be used for studies on human atherosclerosis and related diseases.

To study the pathogenesis of atherosclerosis, many animals, including mice, rats, rabbits, and swine are widely used worldwide (1). In contrast to human lipid metabolism, mice and rats are high-density lipoprotein (HDL)-cholesterol dominant in their serum lipid profiles and are essentially resistant to the high-fat/high-cholesterol diet (HcD) used to develop atherosclerosis. This is partially explained by the absence of expression of several genes that regulate lipid metabolism and atherogenesis (1-3). Therefore, gene modification, for example gene knockout of apolipoprotein E or low-density lipoprotein receptor (LDLr), is necessary to efficiently produce atherosclerosis in mice (1, 4). However, since the pathogenesis of atherosclerosis includes both genetic and environmental factors, animal models should ideally have anatomy, physiology, and life habits similar to humans (5). In this sense, swine are a potentially useful animal model for atherosclerosis research because their anatomy, physiology, lipid metabolism, and life habits are very similar to those of humans (6, 7).

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Recently, the microminipig™ (μ MP, Fuji Micra Inc., Shizuoka, Japan) has been established in Japan and maintained in closed-colony breeding as an experimental animal for atherosclerosis research (8). μ MPs are the world's smallest minipigs with body weights less than 10 kg, even at 7 months old. μ MPs are very sensitive to HcD, in which only 8 weeks of HcD feeding is sufficient to produce hyperlipidemia-induced atherosclerosis. This is partly achieved by the similarity to human gene expression profiles related to lipid metabolism, such as hydroxymethylglutaryl-CoA reductase, apoB mRNA editing enzyme catalytic polypeptide 1, Niemann-Pick C1-like 1 protein, cholesteryl ester transfer protein, and hepatic lipase. Therefore, μ MPs are a suitable animal models for research on atherosclerosis and related diseases (6-8). However, for newly established animal models, especially large animals, such as swine, the genomic background required for experimental animals is not homogenous enough, because the inbreeding coefficient is still very low (approximately 15%-20%) compared to that of mice (more than 99%). On the contrary, the low inbreeding coefficient, which gradually increases during closed-colony breeding, guarantees the heterogeneous genomic background for reproducing human polygenic or lifestyle-related diseases, such as atherosclerosis. Thus, the homogeneous phenotype harboring the diverse genomic background that is necessary for the reproducibility of human atherosclerosis would be maintained by cloned swine production (9).

The specific aims of the present study were to provide evidence that cloned- μ MPs exhibit good reproducibility regarding atherosclerosis lesion formation compared to that of non-cloned- μ MPs, and that the cloned- μ MP are a very useful animal model for research on atherosclerosis and related diseases.

Materials and Methods

Animals and maintenance. The μ MPs (inbreeding coefficient less than 15%) were obtained from the Swine and Poultry Department, Shizuoka Prefectural Research Institute of Animal Industry, Swine and Poultry Research Center (Shizuoka, Japan) and maintained under filtered air laminar flow conditions in a dedicated room. The room was maintained at a temperature of $24\pm 3^{\circ}\text{C}$ and a relative humidity of $50\pm 20\%$, with a 12-h light/dark cycle. Tap water was available *ad libitum*, and μ MPs were provided with normal or special diets. Body weight was measured once per week. All experimental protocols were approved by the Ethics Committee of Animal Care and Experimentation, Kagoshima University (VM15027, MD16009), and the research was performed in accordance with the Institutional Guidelines for Animal Experiments and in compliance with the Japanese Law Concerning the Protection and Control of Animals (Law No. 105 and Notification No. 6).

Cloned animal production. As shown in Figure 1, male and female μ MP clones (F0) have been produced from a stock of cryopreserved somatic cells, as previously described (9). The F0- μ MP clones were

mated and 11 males (F1-offspring) were obtained by natural mating of two paired cloned parents. Four male clones (F1-clones) were produced from a stock of cryopreserved somatic cells from one of the F1-offsprings (9).

Study design for diet-induced atherosclerosis. A total of 24 male μ MPs (3- to 4-month-old, weighing 5-7 kg) were used for the study. Eleven F1-offsprings were divided into two groups: Group I (n=5) were fed a normal chow diet (NcD) and Group II (n=6) were fed a HcD. Group III (n=4) were F1-clones that were fed a HcD. The HcD was composed of fat (10% w/w, refined lard; Miyoshi Oil & Fat Co., Ltd., Tokyo, Japan) and cholesterol (0.5% w/w, Wako Pure Chemical Industries, Ltd., Osaka, Japan) mixed with a NcD (Kodakara 73; Marubeni Nisshin Feed Inc., Tokyo, Japan), as previously reported (6, 7). After 8 weeks, all μ MPs were anesthetized and sacrificed by bilateral axillary artery exsanguination, and necropsied. For comparison with non-cloned animals, data from a previous study (7) have been used as a NcD-fed Group IV (n=4) and HcD-fed Group V (n=5).

Biochemical and lipoprotein analysis. Peripheral blood samples were collected from the cranial vena cava at weeks 0, 4, and 8 to examine general hematology, biochemistry, and lipoprotein profiles. Measured biochemical parameters included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), γ -glutamyl transpeptidase (γ -GTP), total bilirubin, and glucose. The levels of total cholesterol (TC), very low-density lipoprotein-cholesterol (VLDL-C), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), and triglycerides (TG) were analyzed using an automated agarose gel electrophoresis apparatus (Epalyzer 2, Helena Laboratories, Saitama, Japan).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). Hepatic tissues were stored at -80°C in RNeasy Lysis Buffer (Takara Bio., Shiga, Japan) immediately following necropsy until use. Total RNA was extracted using the mirVana miRNA isolation kit (Invitrogen, Carlsbad, CA, USA), and mRNA expression of LDLR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), and sterol regulatory element binding protein 2 (SREBP2) were quantified using qRT-PCR, according to a previous report (7).

Pathological examination. At necropsy, the aorta and branching arteries, heart, liver, kidneys, spleen, and intestine were resected for pathological examination. The heart, liver, kidneys, spleen, and visceral adipose tissue (omental and mesenteric adipose tissues) were weighed. All organs were fixed in 10% phosphate-buffered formalin for 24 h, and routinely processed into paraffin-embedded tissue sections of 5 μm thickness. Sections were stained with hematoxylin and eosin (H&E). The aortas were cut open longitudinally, fixed with 10% phosphate-buffered formalin, and stained with Oil Red O stain. *En face* images of the thoracic and abdominal aorta were captured using a digital camera. The Oil Red O-positive area relative to the whole surface area was calculated using the Image J software program, according to a previous report (7).

Statistical analysis. All results are expressed as the mean \pm standard deviation (SD). Statistical analysis of the differences between groups was assessed using one-way analysis of variance followed by the Tukey-Kramer multiple comparison test and Mann-Whitney *U*-test.

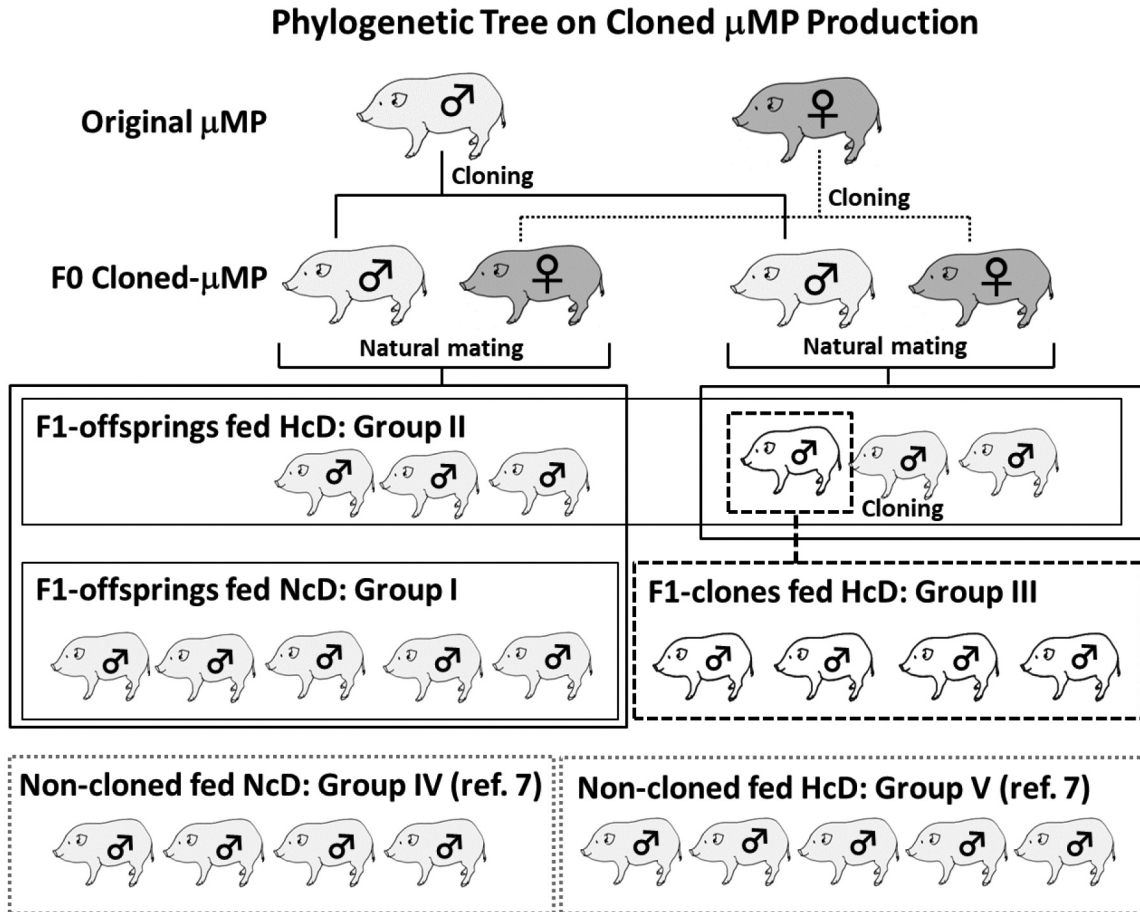


Figure 1. Schematic presentation of the strategy for the production of F1-offspring and F1-clones. Two pairs of male and female cloned- μ MPs (F0) were mated, and the offspring (F1-offspring) were divided into a NcD-fed Group I ($n=5$) and a HcD-fed Group II ($n=6$). One male F1-offspring was subjected to cloning and delivered male μ MPs (F1-clones) that were fed a HcD (Group III, $n=4$). For comparison, the data from non-cloned- μ MPs fed both NcD and HcD were used as Groups VI ($n=4$) and V ($n=5$).

SD values were assessed using the homogeneity of variances (HOV) test for comparison with the cloned- and non-cloned- μ MPs. $p<0.05$ was considered significant. Statistical analyses were performed using IBM SPSS Statistics 25 software (IBM, Tokyo, Japan).

Results

Body weight, visceral adiposity, and blood pressure. The body weights of Groups I, II, and III increased during the 8-week experimental period. HcD-fed Groups II and III μ MPs showed a rapid increase in body weight compared to those of the NcD-fed Group I (Figure 2A), and the growth curve was nearly similar to that of non-cloned- μ MPs fed a HcD, as previously reported (7). The relative weights of the omental and mesenteric adipose tissues also increased in HcD-fed Groups II and III compared to that in the NcD-fed Group I (Figure 2B). No animals were excluded during the 8-week study period for unexpected diseases or death. Data

for the cloned- μ MPs were comparable to those of non-cloned- μ MPs (Groups VI and V).

Hematology and blood biochemistry. Compared to μ MP normal reference data (10, 11), no anemia, leukopenia, or leukocytosis was observed in all groups after they were fed the NcD or HcD for 8 weeks. Blood biochemistry demonstrated no increased levels of AST, ALT, ALP, LDH, γ -GTP, and total bilirubin in either the NcD- or HcD-fed μ MP groups. No increase in blood glucose was noted in any of HcD-fed μ MPs as compared to that of NcD-fed μ MPs (data not shown).

Serum lipoprotein profile. As shown in Figure 3A, HcD-fed Groups II (closed circle) and III (open square) μ MPs become hypercholesterolemic, but the NcD-fed Group I μ MPs do not (open circle). Levels of VLDL-C, LDL-C, and HDL-C also increased in HcD-fed μ MPs during the 8-week experimental

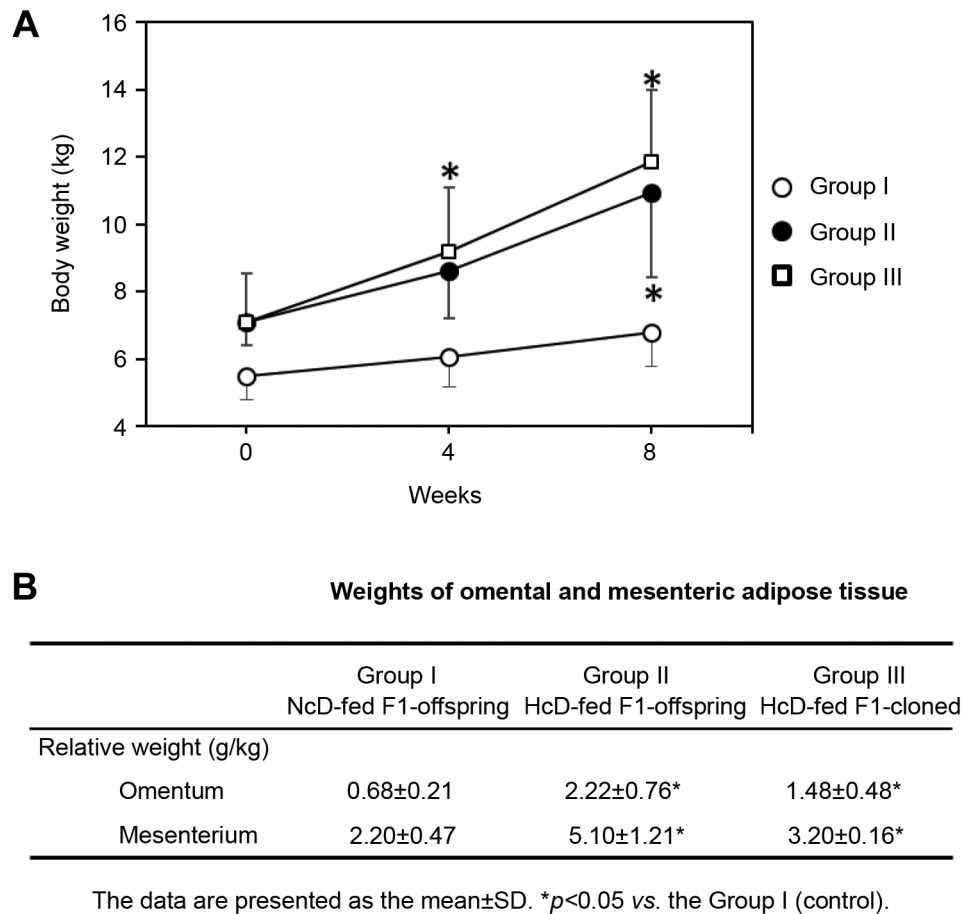


Figure 2. Growth curve and visceral adiposity in F1-offsprings and F1-clones. A) Compared to NcD-fed animals (Group I: open circle), HcD-fed μ MPs (Group II: closed circle and Group III: open square) show a significant increase in the body weights. B) Visceral adiposity represented by the omental and mesenteric adipose tissue weights increased in HcD-fed Groups II and III compared to that in the NcD-fed Group I. * $p < 0.05$ vs. Group I.

period (Figure 3B, C, and D). Percent distributions of cholesterol increased in the LDL fractions and decreased in the HDL fractions in HcD-fed Groups II and III compared to that of the NcD-fed Group I (Figure 3E and F). Serum TG levels in Group III (71.2 ± 30.0 mg/dl) increased as compared to that of the NcD-fed Group I (32.4 ± 6.8 mg/dl) at week 4 of the experiment, while the TG levels at other experimental times were not different among the groups (data not shown). All serum lipoprotein profiles were very comparable to those obtained from the studies using non-cloned- μ MPs (Groups VI and V), as shown in a previous report (7).

Expression of LDLr, HMGCR, and SREBP2. Hepatic expression of LDLr and HMGCR was significantly down-regulated by HcD feeding in cloned- μ MPs (Groups II and III), and the mRNA expression levels of these genes corresponded well with that of SREBP2 (Figure 4). These results are very comparable to those obtained from non-cloned- μ MPs (Groups

VI and V) indicating that the gene regulation in μ MPs fed a high-fat diet is very similar to that in humans (12).

Hyperlipidemia-induced atherosclerosis and histopathological examination. An *en face* analysis of the aortas demonstrated that atherosclerotic lesion areas significantly increased in the HcD-fed Groups II and III (Figure 5A and B). Atherosclerotic lesions developed at the aortic arch, entry of the spinal arteries, and abdominal aorta. Histological examination of H&E sections showed typical atherosclerosis in the aortas, coronary, carotid, and basilar arteries that were infiltrated by foam cells and showed proliferation of smooth muscle cells (Figure 5C). Even severe atherosclerotic lesions were induced by HcD, no thrombosis, myocardial, or cerebral infarctions were encountered during the 8-week experiment. The macroscopic and microscopic features of HcD-induced atherosclerosis in cloned- μ MPs were identical to those of non-cloned animals in Groups VI and V (7). No significant histological changes were

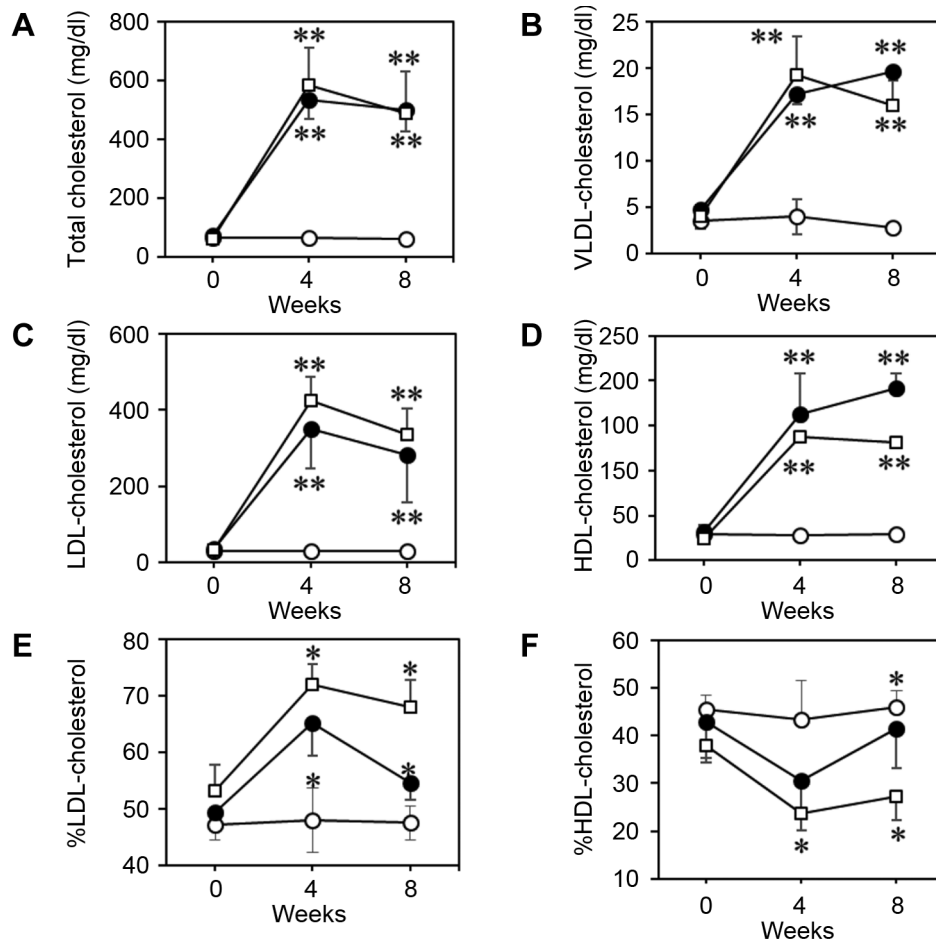


Figure 3. Serum lipoprotein profiles in F1-offsprings and F1-clones. A) HcD-fed Groups II (closed circle) and III (open square) μ MPs exhibit marked hypercholesterolemia compared to that of Group I, fed the NcD (open circle). B-D) The VLDL-C (B), LDL-C (C), and HDL-C (D) also significantly increased in HcD-fed μ MPs compared to that in NcD-fed animals. E-F) The cholesterol fractions markedly increased in LDL (E) and decreased in HDL (F) in Groups II (closed circle) and III (open square) fed a HcD compared to that of Group I fed the NcD (open circle). * $p < 0.05$, ** $p < 0.01$ vs. Group I.

observed in the lungs, kidneys, spleen, and intestine (data not shown). Moderate fatty degeneration is observed in the liver, as previously reported (13, 14).

Homogeneity of variances test for lipoprotein profile and atherosclerotic lesion area. To demonstrate the reduction of variance of the SD in atherosclerotic matrices, including lipoprotein profiles and aortic atherosclerotic lesion areas in HcD-fed cloned- μ MPs (Groups II and III) compared to that in non-cloned- μ MPs (Group V), SD values for these data were analyzed using the HOV test for a 3-group comparison. The SD values of serum lipoprotein data showed a tendency to gradually decrease in cloned- μ MPs (Groups II and III) compared to that of non-cloned- μ MPs (Group V), but the difference was not significant, whereas that of the lesion area of aortic atherosclerosis exhibited a significant reduction ($p = 0.04$) in cloned- μ MPs (Figure 5D).

Discussion

Cloned animals have been successfully produced in various mammals (15-19); however, abnormalities, such as abnormal placentation and extremely high birth weights (large-offspring syndrome) have been reported in calves, cattle, and sheep (20). The cloned- μ MPs and their offspring present no apparent external abnormalities compared to non-cloned μ MPs, as reported in a previous study (9). The F1-offspring and F1-clones also showed neither external and internal malformations nor abnormalities in growth and blood biochemical parameters. In the present study, it was clearly demonstrated that the F1-offspring and F1-clones were sensitive to a HcD, subsequently developing hyperlipidemia and atherosclerosis. The serum lipoprotein profiles, and atherosclerosis histology are very identical to those of non-cloned- μ MPs, as previously described (7). Furthermore, the

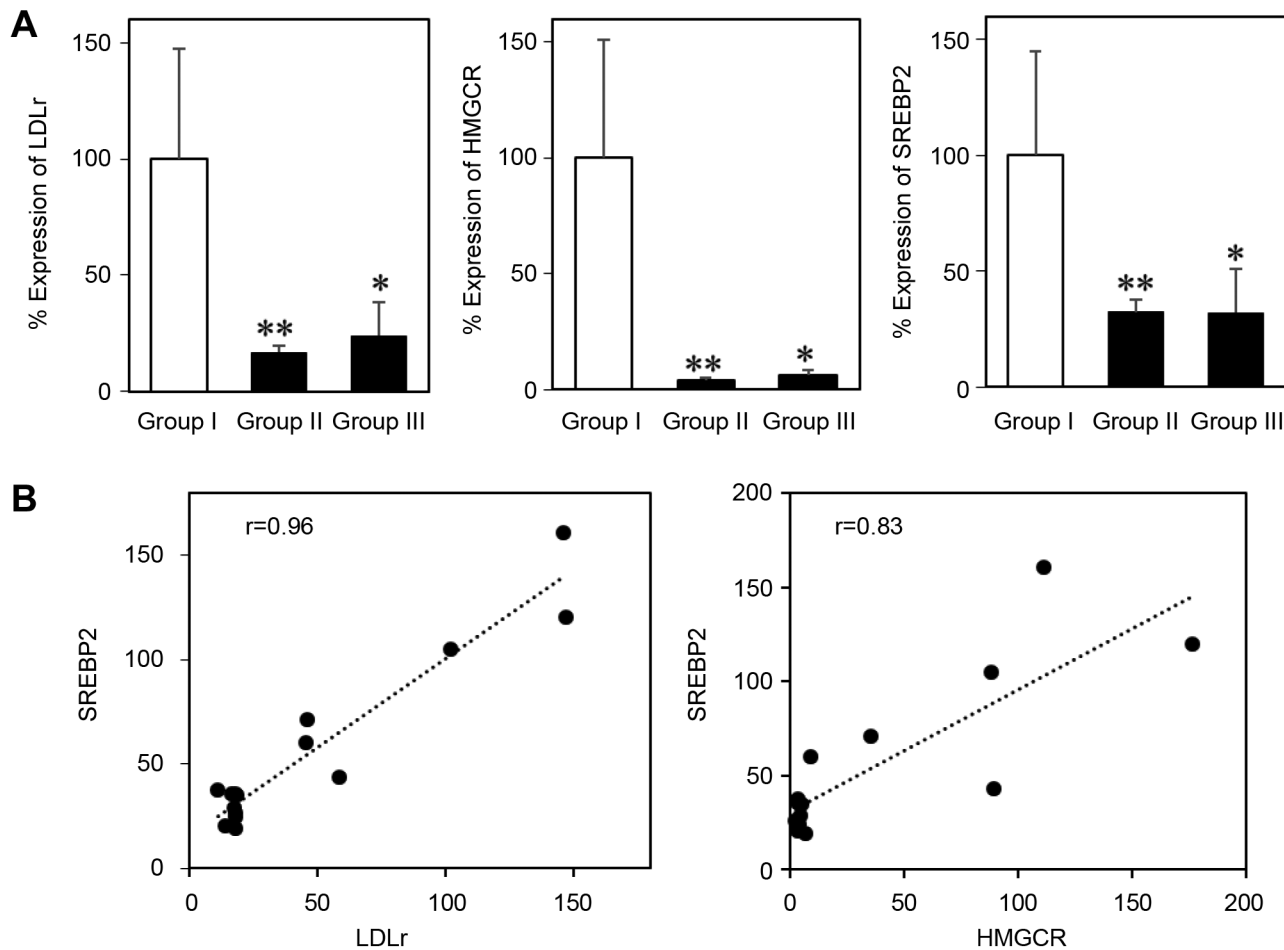


Figure 4. Expression of LDLr, HMGCR, and SREBP2 in F1-offspring and F1-clones. A) The hepatic mRNA expression of LDLr, HMGCR, and SREBP2 was down-regulated by HcD feeding in Groups II and III. B) The mRNA expression levels of these genes corresponded well with that of SREBP2. * $p < 0.05$, ** $p < 0.01$ vs. Group I.

SD values of serum lipoprotein profile data converged into narrow ranges, and those of the aortic atherosclerotic lesion area converged and significantly decreased in the F1-offspring and F1-clones compared to that of non-cloned- μ MPs. These results indicate that the atherosclerosis-prone genetic background was preserved in the cloned- μ MPs and were less variable in the matrices for the atherosclerosis phenotype than non-cloned- μ MPs.

The essential requirements for an ideal animal model to reproduce human pathogenesis are the similarities of the anatomy, histology, physiology, and genetic background to those of humans (5, 21). Since human atherosclerosis is regulated by complex mechanisms consisting of lipid metabolism, homeostatic inflammation, and environmental or social stress (22), an atherosclerosis-prone genetic background controlling these factors is required for an ideal animal model. Mice are relatively easy to introduce gene

modifications because of the presence of mouse embryonic stem cells, and in fact, many transgenic and knockout mouse models are used for atherosclerosis studies (1, 4, 23). Therefore, mouse models are quite useful for investigating a specific gene function related to atherogenesis; however, mouse models have certain limitations for the extrapolation to human diseases because of their differences in lipid metabolism compared to those in humans (5, 24). To overcome these disadvantages, all the atherogenesis-related genes must be humanized by gene modification techniques that would be nearly unrealistic.

Another potential animal model of atherosclerosis is rabbit, which also shows similar lipoprotein and genetic profiles to humans, as well as similar physiology and pathology (2, 24, 25). Many atherosclerosis studies have been performed using HcD-fed rabbits (23). Watanabe Heritable Hyperlipidemic (WHHL) rabbits that manifest spontaneous hyperlipidemia,

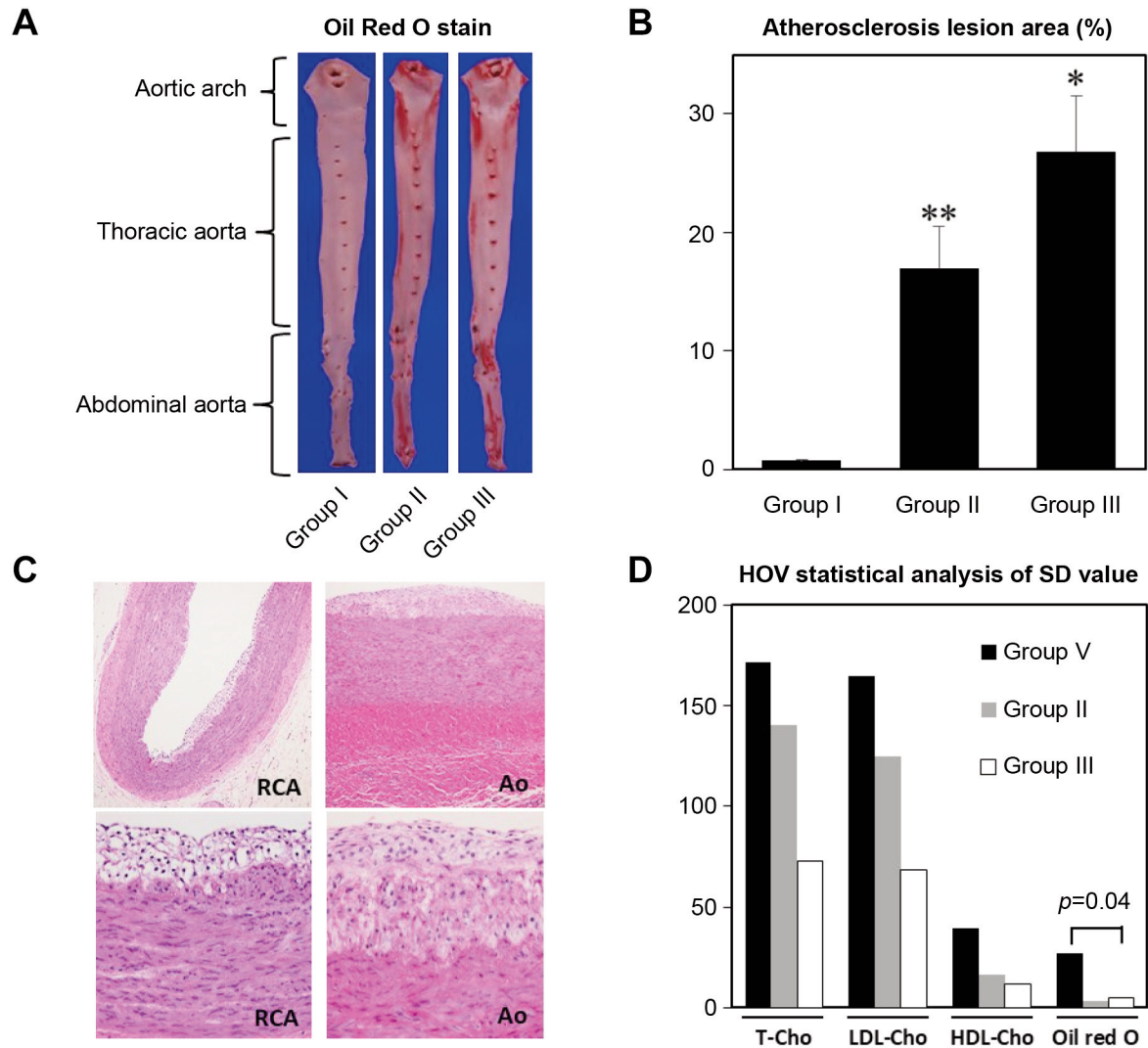


Figure 5. Pathology of atherosclerosis in F1-offsprings and F1-clones and HOV test for atherosclerosis parameters. A, B) The atherosclerotic lesion areas detected by En face Oil Red O stain significantly increased in HcD-fed Group II and III μ MPs. C) The H&E sections showed a typical atherosclerosis in the right coronary artery (RCA) abdominal aorta (Ao) that is characterized by infiltration of foam cells and proliferation of smooth muscle cells (representative field from a Group III μ MP). Upper panel, scanning view ($\times 100$); Lower panel, high power field ($\times 400$). D) The SD values of serum lipoprotein and aortic atherosclerosis lesion area from HcD-fed animals showed a gradual decrease in F1-offsprings (Group II) and F1-clones (Group III) compared to that of the noncloned (Group V) animals. * $p < 0.05$, ** $p < 0.01$ vs. Group I.

atherosclerosis, and xanthoma of the digital joints are naturally occurring LDLr-mutated rabbit strains and a model of human familial hypercholesterolemia (26, 27). WHHL rabbits are widely used for pharmacological and pathological research (27). Recently, transgenic rabbit models have been established using the somatic cell nuclear transfer technique and various transgenic rabbits have been used in atherosclerosis research (28). Even the production of gene-modified rabbits is more difficult than that of mice; transgenic rabbit models may be one of the strongest tools for atherosclerosis research at present.

Swine are available for life science research, including atherosclerosis and related diseases (21, 24, 29). Similar to rabbits, swine exhibit a resemblance to human physiology and pathology that is suitable for atherosclerosis studies as human disease model. One report indicates that the use of cloned swine (Duroc gilt), whose genome information is known, allows a more controlled and repeatable experiments (30). The genome of the μ MP has also been partially sequenced and the genomic information further facilitates atherosclerosis studies (31). In addition, knockout swine using domestic and miniature pigs are currently available for atherosclerosis research, including LDLr

and/or apolipoprotein E knockout swine reproducing human atherogenesis (32-36). The body size of these domestic and traditional miniature pigs, however, is still too big to be maintained and handled in an ordinary laboratory, especially for long-term experiments. In contrast, μ MPs are very small (approximately 9 kg in body weight at 7 months of age), less difficult to manage, and can breed in a cage designed for beagles (6, 7). Therefore, μ MPs compensate for the disadvantages of body size; cloned- μ MPs, as well as non-cloned- μ MP, would be a valuable tool for human atherosclerosis research and related diseases. As in the cases with other swine strains (32-36), transgenic and knockout μ MPs may be produced by gene modification techniques in somatic cell nuclear transfer or fertilized eggs. The development of swine embryonal stem cells would be another advancement for the production of gene-modified- μ MPs, possibly as a more efficient method than the somatic cell nuclear transfer.

In conclusion, production of cloned animals based on the somatic cell nuclear transfer technique resulted in the availability of μ MPs whenever needed, as long as frozen stocks of somatic cells are maintained. Therefore, cloned- μ MPs would be very useful atherosclerosis-prone animals, which would stably and uniformly exhibit the genomic diversity necessary for atherogenesis. Research would be further enhanced after the production of genetically modified- μ MPs. The utilization of cloned- μ MPs for atherosclerosis and life science research would also be beneficial for the reduction of animal sacrifice because of its higher reproducibility and lower data variation, in addition to its similarity to human pathogenesis.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

MO, HK, MS, and AT were involved in the design of this study and manuscript writing. MO, SE, AK and MS performed the cloned animal production. HK, KM and SY performed the animal experiments, and collected and analyzed the data. TK performed the statistical analysis. MMR and NM performed the qRT-PCR. MO, HK and AT proofread the manuscript. All Authors have read and approved the final version of the edited manuscript.

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