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Production of human apolipoprotein(a) transgenic NIBS miniature pigs by somatic cell nuclear transfer

Yoshiki SHIMATSU[1)], Wataru HORII[1)], Tetsuo NUNOYA[2)], Akira IWATA[2)], Jianglin FAN[3)], and Masayuki OZAWA[4)]

[1)]*NIBS Laboratory Animal Research Station, Nippon Institute for Biological Science, 3331-114 Kamisasao, Kobuchisawa, Hokuto, Yamanashi 408-0041, Japan*
[2)]*Headquarters, Nippon Institute for Biological Science, 9-2221-1 Shinmachi, Ome, Tokyo 198-0024, Japan*
[3)]*Department of Molecular Pathology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan*
[4)]*Department of Biochemistry and Molecular Biology, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima-city, Kagoshima 890-8520, Japan*

Abstract: Most cases of ischemic heart disease and stroke occur as a result of atherosclerosis. The purpose of this study was to produce a new Nippon Institute for Biological Science (NIBS) miniature pig model by somatic cell nuclear transfer (SCNT) for studying atherosclerosis. The human apolipoprotein(a) (apo(a)) genes were transfected into kidney epithelial cells derived from a male and a female piglet. Male cells were used as donors initially, and 275 embryos were transferred to surrogates. Three offspring were delivered, and the production efficiency was 1.1% (3/275). Serial female cells were injected into 937 enucleated oocytes. Eight offspring were delivered (production efficiency: 0.9%) from surrogates. One male and 2 female transgenic miniature pigs matured well. Lipoprotein(a) was found in the male and one of the female transgenic animals. These results demonstrate successful production of human apo(a) transgenic NIBS miniature pigs by SCNT. Our goal is to establish a human apo(a) transgenic NIBS miniature pig colony for studying atherosclerosis.
Key words: apolipoprotein(a), atherosclerosis, somatic cell nuclear transfer, transgenic miniature pig

Introduction

In 2014, the World Health Organization announced the 10 leading causes of death in the world in 2000 and 2012, with ischemic heart disease in first place and cerebral stroke in second [36]. Most cases of ischemic heart disease and stroke occur as a result of atherosclerosis complicated by glucose intolerance, hyperlipidemia, and hypertension.

Lipoprotein(a) (Lp(a)) has gained increasing attention due to its role as a novel major risk factor for atherosclerosis and for the unusual nature of its distinguishing protein component, apolipoprotein(a) (apo(a)) [2]. Lp(a) is distinguished from low-density lipoprotein (LDL) by the presence of an additional protein component designated as apo(a). Apo(a) is complexed to apolipoprotein B-100 (apoB-100) by disulfide linkage [8, 9]. Although the physiological functions of Lp(a) remain unclear [28], Lp(a) was recently demonstrated to be selectively enriched in oxidized phospholipids, as detected by murine monoclonal antibody E06, in humans and in Lp(a) transgenic mice expressing a mini-apo(a) construct containing eight kringle IV repeats [1, 21, 29, 33]. Lp(a) is selectively trapped in atherosclerotic lesions through a lysine

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Address corresponding: Y. Shimatsu, NIBS Laboratory Animal Research Station, Nippon Institute for Biological Science, 3331-114 Kamisasao, Kobuchisawa, Hokuto, Yamanashi 408-0041, Japan. M. Ozawa, Department of Biochemistry and Molecular Biology, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima City, Kagoshima 890-8520, Japan

binding site on kringle IV-10 [13, 23, 24] and may deliver apo(a) with its prothrombotic potential, cholesterol, lipids and pro-inflammatory oxidized phospholipids into atherosclerotic lesions. Apo(a) is naturally present in Old World monkeys and humans, while one non-primate species, the hedgehog, has independently evolved a particular apo(a) protein [18, 19]. Human apo(a) transgenic rabbits have been produced successfully and have been used in studies of atherosclerosis [5–7, 14].

Pigs have been used as an animal model for human cholesterol-related disease states for many decades. Many aspects of cholesterol and lipoprotein metabolism in pigs and their responses to dietary cholesterol and fat resemble those of humans. One of the most favorable characteristics of pigs is that they are omnivorous animals [27]. The purpose of this study was to produce a human apo(a) transgenic Nippon Institute for Biological Science (NIBS) miniature pig that could be used to study atherosclerosis by somatic cell nuclear transfer (SCNT). We previously established the NIBS miniature pig cloning technology [32].

Materials and Methods

Animals (surrogates)

A total of 9 NIBS miniature gilts and sows, aged 1–4 years, were used as surrogates. They were individually housed in stainless steel cages (700 × 1,200 × 700 mm) or cement pens (1,600 × 1,400 × 800 mm) and were given *ad libitum* access to fresh water in addition to 800–1,200 g/day of a commercial diet (Nexcelbreed, Nosan Corporation, Yokohama, Japan). Room temperature was maintained at 20–25°C, and the relative humidity was 40–70%. The animals were cared for and treated in accordance with the Regulations for Animal Experimentation of NIBS.

Vector construction

The plasmid vector used in this study is shown in Fig. 1. To facilitate the detection of apo(a), apo(a) was tagged with hemagglutinin (HA) at the *C*-terminus by using a PCR with the primers ATCCCTCTCTGTGCATCCTCT and CGAATTATTTCTCATCATTCCCTCAA and apo(a) cDNA as a template. After generating blunt ends using T4 DNA polymerase, the PCR product was cloned into the *Eco*RV site of the pC-SnailHA vector [26]. The *Eco*RI-*Kpn*I fragment of apo(a) was then cloned into the vector, yielding pC-apo(a) HA.

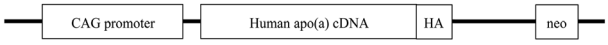

Fig. 1. The structure of the pC-apo(a) HA construct. The human apo(a) cDNA was tagged with an HA sequence for identification by immunohistochemistry and immunoblot. The CAG promoter comprises a cytomegalovirus (CMV) enhancer and a β-actin promoter. The neo gene encodes the resistance gene for G418.

Donor cells (nuclear transfer cells) and transfection

A 1-day-old male NIBS miniature piglet and a 10-day-old female piglet were anesthetized with inhaled isoflurane (Escain®, Mylan Seiyaku Ltd., Tokyo, Japan) and oxygen. After they were bled to death by exsanguination, the right kidneys were harvested. Both minced tissues were dissociated in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 1 mg/ml Collagenase P (Roche Diagnostics GmbH, Mannheim, Germany) for 1 h at 37°C. The cultured media were strained by stainless steel meshes and seeded onto 100 mm dishes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) at 37°C in a humidified atmosphere of 5% CO_2. After reaching confluence, the cells were trypsinized by trypsin solution (Trypsin-EDTA, Invitrogen Corp.) and centrifuged at 300 g for 3 min. Suspended cells in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Corp.) supplemented with 10% (v/v) fetal bovine serum (FBS) were passaged once.

Transfection of kidney epithelial cells with the pC-apo(a) HA plasmid was performed with an Amaxa Nucleofector System (Amaxa GmbH, Cologne, Germany) as previously described [22]. Briefly, 10 μl of a solution containing 10 μg plasmid DNA was mixed with 90 μl of Nucleofector Solution (Amaxa GmbH), and then the combined solution was mixed with 1×10^6 kidney epithelial cells. After transfection, the cells were split into two 100 mm dishes and incubated in DMEM supplemented with 10% (v/v) FBS at 37°C in a humidified atmosphere of 10% CO_2. Five days later, the cells were incubated with a culture medium containing 200 μg/ml of G418 (Geneticin®, Life Technologies Japan Ltd., Tokyo, Japan) for an additional 10–15 days to isolate drug-resistant colonies. Colonies (comprising 300–700 cells) were picked using Pipetman tips and directly transferred to individual wells in 24-well plates (Becton, Dickinson

and Co.) containing 1 ml culture medium with 200 μg/ml of G418. The cells were cultured for 6–10 more days, and then a portion of each colony was analyzed for apo(a) expression by immunofluorescence staining with anti-HA antibodies (Roche Diagnostics GmbH). Kidney epithelial cells at passages 7–10 were used for SCNT. A single-cell suspension in Dulbecco's Phosphate-Buffered Saline (DPBS, Invitrogen Corp.) supplemented with 0.5% (v/v) FBS was prepared by trypsinization immediately prior to nuclear transfer (NT).

Oocytes for SCNT

All oocytes were prepared using the regimen described by Shimatsu *et al.* [32]. Briefly, cumulus-oocyte complexes of commercial pigs were cultured in a medium based on Medium 199 (Invitrogen Corp.) at 39°C in a humidified atmosphere of 5% CO_2. After culturing for 22 h, cumulus-oocyte complexes were transferred to hormone-free medium and cultured for 15–17 h. Oocytes were freed from cumulus cells by repeated pipetting in 0.1% (w/v) hyaluronidase.

SCNT procedure

A micromanipulation system (Narishige Group, Tokyo, Japan) attached to an inverted microscope (TE2000-S, Nikon Corporation, Tokyo, Japan) was used. The SCNT procedure was carried out using the regimen described by Shimatsu *et al.* [32]. Donor cell–oocyte complexes were fused and simultaneously activated with a single 1.7 kV/cm DC pulse for 60 μs using a Super Electro Cell Fusion Generator (ECFG21, Nepa Gene Co., Ltd., Chiba, Japan) and then cultured in porcine zygote medium (PZM-5, Research Institute for the Functional Peptides Co., Ltd., Yamagata, Japan) supplemented with 2 mM 6-dimethylaminopurine for 1 h.

Embryo transfer and surrogate maintenance

Surrogates received 10 mg/day altrenogest (Regumate® Porcine, Intervet International B.V., Boxmeer, Netherlands) in their feed for 12 days. Seven days after treatment, they were anesthetized with an intramuscular injection of atropine sulfate (Atropine Sulfate Injection, Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) and midazolam (Dormicum®, Astellas Pharma Inc., Tokyo, Japan), followed by inhalation of isoflurane and oxygen. The cloned embryos directly transferred into the ampulla of the oviduct via exploratory laparotomy [31, 32, 34]. Clinical status was monitored daily after embryo transfer (ET). We judged the surrogates to be pregnant if they did not experience estrus at any time in the 42 days after ET.

Reagents

All reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless otherwise stated.

Identification of the transgene

Genomic DNA was isolated from either auricle or liver tissues by the method described by Herrmann and Frischauf [12], purified using a QIAquick DNA Purification Kit (Qiagen N.V., Venlo, Netherlands), and used as templates for PCR reactions using GoTaq DNA Polymerase (Promega Corp., Madison, WI, USA). The following oligonucleotides were used as primers for PCR amplification of a human apo(a) sequence of 158 bp: TCCCTCTCTGTGCATCCTCT and GCCTCCACAGAAGTGCTTTC. Sampling from offspring was carried out under anesthesia with isoflurane and oxygen.

Serum Lp(a) and lipid level analysis

Blood was collected from human apo(a) transgenic miniature pigs via a distal portion of the sinus venarum cavarum at 2, 5, and 7 months of age, and serum Lp(a) was analyzed by a latex agglutination method (SRL Inc., Tokyo, Japan). Serum total cholesterol and triglycerides were analyzed using a high-performance liquid chromatography (HPLC) method (Skylight Biotech Inc., Tokyo, Japan).

Semen quality analysis

Six whole ejaculates were collected from a human apo(a) transgenic miniature boar at around 10 months of age. Semen volume, sperm motility, sperm concentration and total number of sperm were analyzed [30].

Results

The production efficiency of human apo(a) transgenic NIBS miniature pigs is shown in Table 1. A total of 275 embryos constructed using male donor cells were transferred to 2 surrogates. Only one surrogate successfully delivered offspring (3 offspring). The production rate was 1.1% (offspring/embryos). Two (Transgenic (Tg) #6 and #7) of the 3 male offspring died at 1 and 2 days of age, respectively. Bruises on them suggested that they might have been attacked by the surrogate.

Table 1. Production efficiency of human apo(a) transgenic NIBS miniature pigs by SCNT

No. of embryos	Average no. of embryos transferred	No. (%) of surrogates		No. of offspring	No. (%) of offspring/ embryos
		Total	Delivered offspring		
275[a)]	136, 139	2	1 (50)	3[d)]	3/275 (1.1)
937[b)]	133.9 ± 9.5[c)]	7	4 (57)	8[e)]	8/937 (0.9)

[a)]Donor cells were male. [b)]Donor cells were female. [c)]Mean ± SD. [d)]The birth weights of the offspring were 300, 480, and 480 g. [e)]The average birth weight of the offspring, including 4 stillbirths and one case of fetal mummification, was 309 ± 82.0 g.

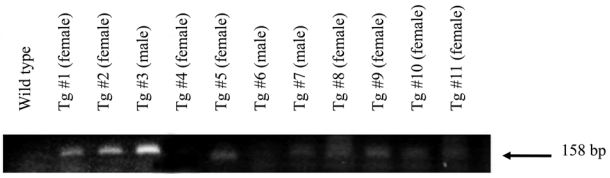

Fig. 2. PCR analysis of genomic DNA isolated from wild-type and human apo(a) transgenic NIBS miniature pigs. A PCR product of approximately 158 bp was amplified from the targeted human apo(a) gene, except in the case of the wild-type pig and Tg #4.

Fig. 3. Picture of 3 human apo(a) transgenic NIBS miniature pigs. The body weights were 6.3 kg for the pig on the far left (male Tg #3, 2 months of age), 9.8 kg for the pig in the middle (female Tg #2, 3 months of age), and 12.7 kg for the pig on the far right (female Tg #1, 4 months of age).

The female donor cells were injected into 937 enucleated oocytes, which were transferred to 7 surrogates (Table 1). Four of the 7 surrogates successfully delivered offspring (8 offspring; production efficiency: 0.9%). Successful induction of pregnancy was unrelated to parity. Three female offspring were born alive; there were also 4 stillbirths (Tg #5, #8, #9, and #10) and one case of fetal mummification (Tg #4). A PCR product of approximately 158 bp was not amplified from the targeted human apo(a) gene in the genomic DNA of Tg #4 (Fig. 2), and it was thought that the DNA might not have been extracted completely. One (Tg #11) of the 3 living offspring was crushed to death by the surrogate shortly after birth.

In the present study, a male (Tg #3) and 2 female (Tg #1 and #2) transgenic NIBS miniature pigs were observed to have matured well (Fig. 3) by the time they were one year of age. The serum Lp(a) levels of the male (Tg #3) and one (Tg #2) of the female transgenic miniature pigs were clearly high, and the values for the male animal increased with growth. However, the levels of the other female (Tg #1) transgenic pig were ≤1 mg/dl at all points (Fig. 4). Pigs normally do not have endogenous Lp(a), and it is known that the serum level of wild-type pigs is 0. The serum total cholesterol levels of all of the transgenic miniature pigs were similar to those of wild-type pigs. The serum triglyceride levels of one (Tg #1) of the female transgenic pigs were slightly higher than those of the other transgenic and wild-type pigs at 5 and 7 months old (Fig. 5).

To evaluate the fertility potential of the transgenic NIBS miniature boar, the semen quality profile was examined (Table 2). Each value was recorded as the mean ± SD. The semen volume was 57.5 ± 8.29 ml. Sperm motility (13.0 ± 0.2%) and the sperm concentration (0.66 ± 0.18 × 10^8/ml) were comparatively low.

Discussion

The results of this study indicate that human apo(a) transgenic NIBS miniature pigs can be produced through SCNT for studying atherosclerosis. Miniature pigs have an advantage over commercial pigs in terms of their size and subsequent ease of handling [4]. The NIBS miniature pig breed, which was originally derived from Pitman-Moore miniature pigs, Taiwanese small-ear pigs, and Göttingen miniature pigs, was established in 1993 [25].

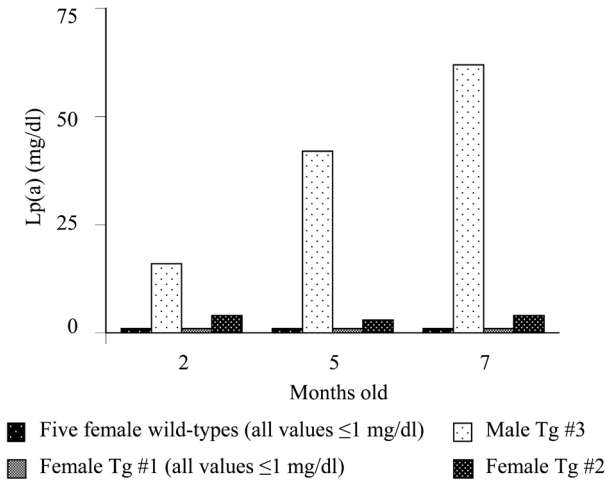

Fig. 4. Serum Lp(a) levels of wild-type and human apo(a) transgenic NIBS miniature pigs.

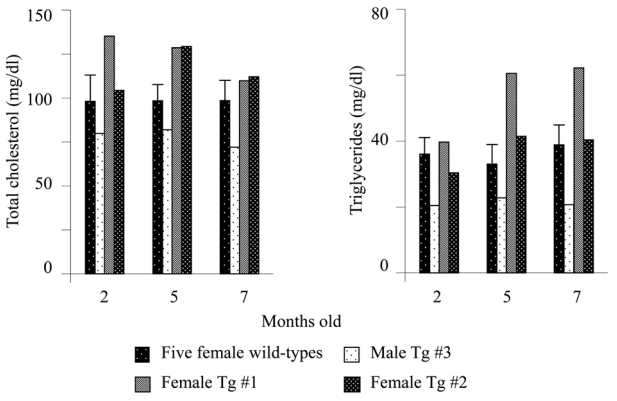

Fig. 5. Serum total cholesterol and triglyceride levels of wild-type and human apo(a) transgenic NIBS miniature pigs.

Table 2. Characterization of human apo(a) transgenic NIBS miniature boar semen

Volume (ml)	Sperm motility (%)	Sperm concentration ($\times 10^8$/ml)	Total sperm ($\times 10^8$)
57.5 ± 8.29	13.0 ± 0.2	0.66 ± 0.18	30.2 ± 2.3

Six whole ejaculates were collected from a transgenic miniature boar. Each value is shown as the mean ± SD.

The body weights of NIBS miniature pigs are around 30 kg at 1 year of age, and they are very friendly to humans.

We demonstrated that the human apo(a) transgenic production efficiency of 0.9 or 1.1% was comparable to the production efficiency of cloned NIBS miniature pigs (1.0%) [32] and cloned commercial pigs (1–2%) [17]. In addition, the birth weights of human apo(a) transgenic miniature pigs (Males, 300 or 480 g: Females, 309 ± 82.0 g) were comparable to those of normal sex-matched NIBS piglets. The average birth weight of the male NIBS piglets was 452 ± 95.5 g, and that of the female NIBS piglets was 403 ± 75.3 g (unpublished data).

Lp(a) has gained increasing attention due to its role as a novel major risk factor for atherosclerosis and for the unusual nature of its distinguishing protein component, apo(a) [2]. Lp(a) is distinguished from LDL by the presence of an additional protein component designated as apo(a) [8, 9]. However, apo(a) is naturally present in only Old World monkeys, humans, and one non-primate species (hedgehogs) [18, 19]. Against such a background, we planned to produce human apo(a) transgenic NIBS miniature pigs. Pigs have been used as an animal model for human cholesterol-related disease states for many decades [27], and it has been reported that the lipoprotein pattern of pig serum is similar in many ways to that of human serum [15].

In the present study, serum Lp(a) levels were analyzed as an index of apo(a) levels by a company specializing in human clinical laboratory testing. The levels of the male and one of the 2 female human apo(a) transgenic NIBS miniature pigs were clearly high, and the values for the male pig increased with growth. The other female transgenic pig showed levels of ≤1 mg/dl at all points. In these tests, levels of 0 to 1 mg/dl were not analyzed, and we concluded that the human apo(a) transgenic miniature pigs had Lp(a) in their sera and that the levels of one female were low.

Human apo(a) cDNA was provided by Dr. J. Fan; his group has succeeded in producing human apo(a) transgenic rabbits [5, 7]. Fan *et al.* [5, 7] analyzed plasma human apo(a) levels of human apo(a) transgenic rabbits by an enzyme-linked immunosorbent assay and found that the average levels were 11 nM, which was equivalent to 3 mg/dl of Lp(a). Finally, they concluded that the transgenic rabbits had the equivalent of a relatively low apo(a) level in humans [7].

On the other hand, hepatic apoB mRNA editing enzyme 1 (APOBEC-1) is not expressed in humans, rabbits, and pigs [10], and APOBEC-1 generates only apoB-100 to form very LDL, including both apoB-100 and apolipoprotein B-48 (apoB-48). In contrast, mice express hepatic APOBEC-1 to generate apoB-48 [10]. Human apo(a) was probably bound to apoB-100 successfully,

and Lp(a) was found in our transgenic miniature pigs.

The serum total cholesterol and triglyceride levels of the male human apo(a) transgenic miniature pig hardly changed in spite of the increase in Lp(a) levels depending on age. One female transgenic pig showed triglyceride levels that were slightly higher than the levels of the other transgenic pigs and the wild-type pigs at 5 and 7 months old. We are analyzing cholesterol and triglyceride levels of lipoprotein subclasses using the HPLC method now. The analysis results will clarify the lipoprotein profiles of human apo(a) transgenic miniature pigs.

Cholesteryl ester transfer protein (CETP) catalyzes the transfer of cholesteryl ester from high-density lipoprotein to apoB-containing lipoproteins and is considered to be a key protein for reverse cholesterol transport, which contributes to protection against atherosclerosis [37]. No CETP activity or very low levels of CETP activity are detected in pigs [11, 16]. It is also important to examine the CETP activity of our human apo(a) transgenic miniature pigs.

The semen quality profile of the human apo(a) transgenic NIBS miniature boar was within the normal range observed in some miniature pig breeds [3, 30]. However, the sperm motility and concentration of the transgenic boar were comparatively low, and the semen would not be suitable for use in artificial insemination. Deep intrauterine insemination technology [20, 35] may be indispensable for the production of human apo(a) transgenic piglets using the semen.

Based on the present findings, the relationship between the semen quality of the transgenic miniature boar and the human apo(a) transgene remains unclear. However, it is well known that there is great individual variation in semen quality in pigs, and it is possible that poor semen quality is a hereditary factor.

The results of the present study suggest that human apo(a) transgenic NIBS miniature pigs could be produced successfully by SCNT. Our goal is to establish a human apo(a) transgenic NIBS miniature pig colony for the study of atherosclerosis, and we are going to push forward with the breeding of transgenic miniature pigs.

Acknowledgments

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