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Original Article

Phenotypic features of dystrophin gene knockout pigs harboring a human artificial chromosome containing the entire dystrophin gene

Masahito Watanabe,[1,8] Hitomaru Miyamoto,[2] Kazutoshi Okamoto,[3] Kazuaki Nakano,[1,8] Hitomi Matsunari,[1,8] Kanako Kazuki,[4] Koki Hasegawa,[3] Ayuko Uchikura,[1] Shuko Takayanagi,[1,8] Kazuhiro Umeyama,[1,8] Yosuke Hiramuki,[4] Elisabeth Kemter,[5,6] Nikolai Klymuik,[5,6] Mayuko Kurome,[1,5,6] Barbara Kessler,[5,6] Eckhard Wolf,[1,5,6] Yasuhiro Kazuki,[2,4,7] and Hiroshi Nagashima[1,3]

[1]Meiji University International Institute for Bio-Resource Research, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan; [2]Department of Chromosome Biomedical Engineering, School of Life Science, Faculty of Medicine, Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan; [3]Laboratory of Medical Bioengineering, Department of Life Sciences, School of Agriculture, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan; [4]Chromosome Engineering Research Center (CERC), Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan; [5]Chair for Molecular Animal Breeding and Biotechnology, Gene Center and Department of Veterinary Sciences, LMU Munich, 81377 Munich, Germany; [6]Center for Innovative Medical Models (CiMM), LMU Munich, 85764 Oberschleissheim, Germany; [7]Chromosome Engineering Research Group, The Exploratory Research Center on Life and Living Systems (ExCELLS), National Institutes of Natural Sciences, 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan

Mammalian artificial chromosomes have enabled the introduction of extremely large amounts of genetic information into animal cells in an autonomously replicating, nonintegrating format. However, the evaluation of human artificial chromosomes (HACs) as novel tools for curing intractable hereditary disorders has been hindered by the limited efficacy of the delivery system. We generated dystrophin gene knockout (*DMD*-KO) pigs harboring the HAC bearing the entire human *DMD* via a somatic cell cloning procedure (DYS-HAC-cloned pig). Restored human dystrophin expression was confirmed by immunofluorescence staining in the skeletal muscle of the DYS-HAC-cloned pigs. Viability at the first month postpartum of the DYS-HAC-cloned pigs, including motor function in the hind leg and serum creatinine kinase level, was improved significantly when compared with that in the original *DMD*-KO pigs. However, decrease in systemic retention of the DYS-HAC vector and limited production of the DMD protein might have caused severe respiratory impairment with general prostration by 3 months postpartum. The results demonstrate that the use of transchromosomic cloned pigs permitted a straightforward estimation of the efficacy of the DYS-HAC carried in affected tissues/organs in a large-animal disease model, providing novel insights into the therapeutic application of exogenous mammalian artificial chromosomes.

INTRODUCTION

The development of transgenic animals has facilitated the understanding of the function of specific genes in complex living body systems. During transgenic animal generation, the limitations of plasmid-derived vectors in terms of stability and tissue specificity of transgene expression have been overcome via artificial chromosome technologies.[1–5] Bacterial and yeast artificial chromosome (BACs and YACs) characteristics have been improved further by the development of mammalian ACs,[6–9] including human ACs (HACs) and mouse ACs (MACs), which provide means of introducing larger amounts of genetic information into cells in an autonomously replicating, nonintegrating format.[10,11] HACs have recently been utilized as novel tools for biopharmaceutical production, transgenesis, and gene-based cell therapy.[12–14] The target diseases of gene-based therapy using HACs include intractable hereditary disorders, such as hemophilia A,[15,16] amyotrophic lateral sclerosis (ALS),[17–19] and Duchenne muscular dystrophy (DMD).[20,21]

DMD is caused by mutations in the dystrophin gene (*DMD*).[22,23] HACs are considered suitable for the delivery of intact *DMD*, which is the largest known gene in humans. In previous studies, HACs harboring intact *DMD* have been delivered to the affected muscle tissues of DMD model animals using carrier cells.[20,21] However, limited engraftment of the carrier cells is thought to hinder the evaluation of the effect of exogenous *DMD* expression.[24–26] Evaluation of the effect of DMD gene therapy with HACs has also been limited

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[8]Present address: PorMedTec Co., Ltd., 2-3227 Mita, Tama-ku, Kawasaki, Kanagawa 214-0034, Japan

Correspondence: Yasuhiro Kazuki, Department of Chromosome Biomedical Engineering, School of Life Science, Faculty of Medicine, Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan.
E-mail: kazuki@tottori-u.ac.jp
Correspondence: Hiroshi Nagashima, Meiji University International Institute for Bio-Resource Research, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan.
E-mail: hnagas@meiji.ac.jp

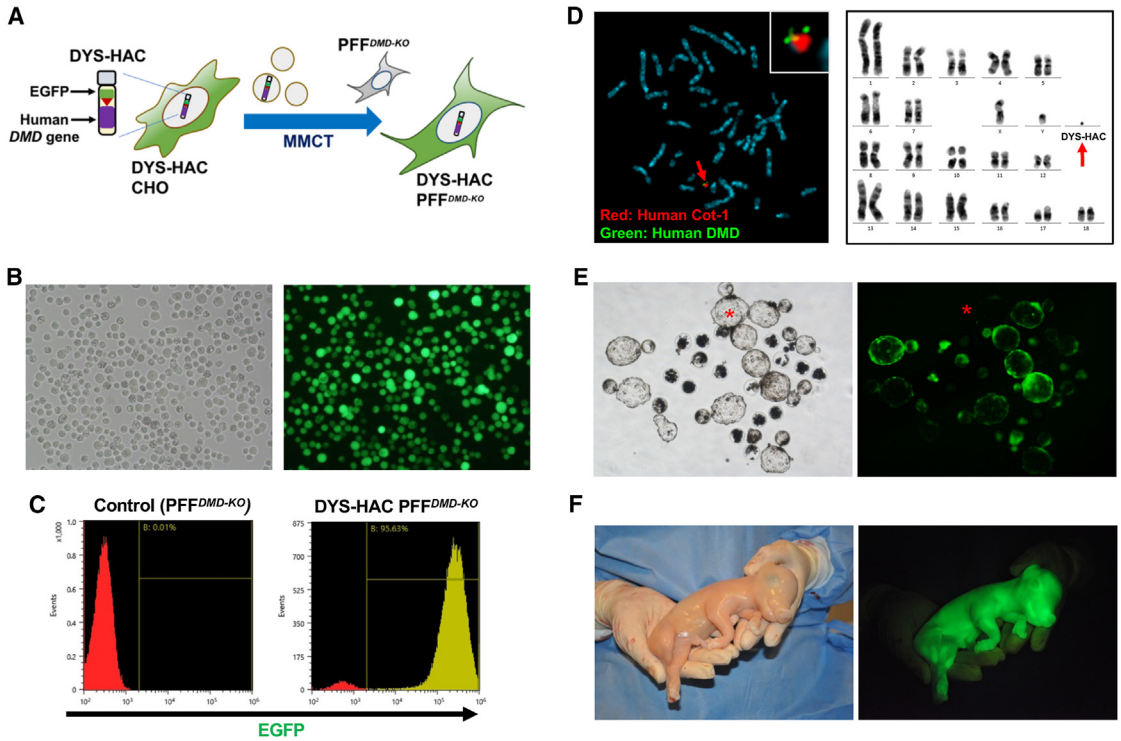


Figure 1. DYS-HAC-carrying nuclear donor cells for SCNT and the cloned fetuses

(A) Schematic representation of the isolation of nuclear donor cells carrying the DYS-HAC vector. (B) Phase contrast (left) and EGFP fluorescence (right) images of the DYS-HAC PFF$^{DMD\text{-}KO}$ established as nuclear donor cells. (C) Flow cytometry analysis for the expression of EGFP on the DYS-HAC vector in the nuclear donor cells (left; PFF$^{DMD\text{-}KO}$, right; DYS-HAC PFF$^{DMD\text{-}KO}$). (D) Chromosomal analysis of nuclear donor DYS-HAC PFF$^{DMD\text{-}KO}$. An arrow indicates the episomal DYS-HAC vector. The digoxigenin-labeled human Cot-1 DNA and the biotin-labeled DMD-BAC (RP11-954B16) detected the human chromosome (red) and the human dystrophin genome (green), respectively. (E) Phase-contrast (left) and fluorescence (right) images of DYS-HAC-cloned embryos at the blastocyst stage. The asterisk indicates a cloned blastocyst with no EGFP expression. (F) Macroscopic view of a DYS-HAC-cloned fetus (#1920-2) at day 91 of gestation.

due to the use of rodent models that exhibit relatively mild DMD symptoms.

We have previously generated DMD model pigs carrying loss-of-function mutations in *DMD* by means of somatic cell cloning techniques.[27,28] The cloned *DMD* X^{KO}Y (knockout [KO]) embryos were produced by transferring donor nuclei derived from gene KO cells into the cytoplasm of enucleated porcine oocytes. Therefore, the somatic cell nuclear transfer (SCNT) procedure allowed the introduction of the HAC into porcine oocytes with the donor nucleus, which would make it possible to generate porcine embryos carrying the HAC throughout development from the beginning of embryogenesis. We hypothesized that the analysis of animals carrying the HAC harboring functional *DMD* from the early developmental stage could provide novel insights into the therapeutic effect of exogenous mammalian ACs. Therefore, in the present study, we attempted to generate *DMD* X^{KO}Y-cloned pigs that systemically carry human *DMD*-bearing HAC (DYS-HAC). Various biological phenomena, including mitotic stability, gene expression, and phenotypic effects of the HAC introduced *de novo* in the cloned embryos and offspring, were investigated.

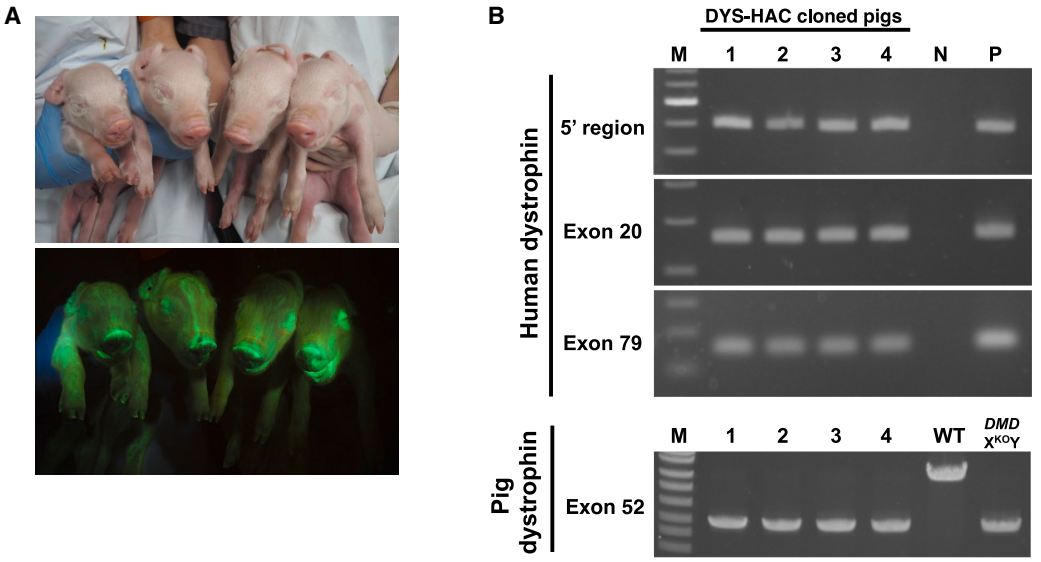
RESULTS

Isolation of nuclear donor cells harboring DYS-HAC

The DYS-HAC vectors were transferred to *DMD* X^{KO}Y porcine fetal fibroblast cells (PFF$^{DMD\text{-}KO}$) via microcell-mediated chromosome transfer (MMCT) (Figure 1A).[28,29] Almost all the cells isolated with blasticidin S selection were confirmed to be EGFP positive according to flow cytometry analysis results (Figures 1B and 1C). PCR analyses confirmed that DYS-HAC was successfully transferred to PFF$^{DMD\text{-}KO}$ (Figure S1). Fluorescence *in situ* hybridization (FISH) analyses demonstrated that DYS-HAC was present as an individual chromosome in PFF$^{DMD\text{-}KO}$ (Figure 1D). The isolated PFF$^{DMD\text{-}KO}$ harboring DYS-HAC, designated as DYS-HAC PFF$^{DMD\text{-}KO}$, were used as nuclear donors for SCNT.

Generation of cloned embryos and fetuses harboring DYS-HAC

The *in vitro* developmental competency of SCNT embryos reconstructed with DYS-HAC PFF$^{DMD\text{-}KO}$ as nuclear donor cells was comparable to that of cloned embryos lacking HAC generated in our previous studies.[30–32] The cleavage and blastocyst formation rates of the reconstructed DYS-HAC-cloned embryos were 79% (79/100) and 60% (60/100), respectively.



C

	Donor cell	No. of embryos transferred	Recipients No.	Fetuses or piglets obtained	EGFP expression	Production efficiency of cloned pigs (%)
Fetuses (E91)	DYS-HAC PFF$^{DMD\text{-}KO}$	176	#1920	2	+	1.13
Piglets		86	#2022	4	+	4.65
		106	K338	1	−	0.94

Figure 2. Generation of DYS-HAC-cloned pigs

(A) Bright-field (top panel) and fluorescence (bottom panel) images of the four newborn DYS-HAC-cloned pigs (K358, K359, K360, and K361) from a recipient gilt (#2022). (B) Genotyping for four DYS-HAC-cloned pigs. The top panel indicates the detection of three regions of the human dystrophin gene (5′ region, exon 20, and exon 79) on the DYS-HAC vector in the cloned piglets. The bottom panel indicates the detection of the *DMD*-KO mutation in the genomic DNA of DYS-HAC-cloned pigs. M, DNA size marker; 1–4, DYS-HAC-cloned pigs (K358, K359, K360, and K361); N, negative control (PFF$^{DMD\text{-}KO}$); P, positive control (nuclear donor DYS-HAC PFF$^{DMD\text{-}KO}$); WT, wild type; *DMD* X^{KO}Y, *DMD*-KO pig. (C) Summary of production of DYS-HAC-cloned fetuses and piglets in this study.

EGFP fluorescence in DYS-HAC-cloned embryos was first confirmed at the eight-cell stage. The level of expression increased as development progressed (Figure S2A). The EGFP expression pattern was similar to that of cloned porcine embryos harboring a fluorescent protein (monomeric Plum) gene regulated by the CAG promoter, as previously reported.[33] EGFP fluorescence was observed at the blastocyst stage in almost all DYS-HAC-cloned embryos produced (Figure 1E).

The developmental competence of DYS-HAC-cloned embryos was further confirmed by embryo transfer experiments to recipient females. Transfer of 176 DYS-HAC-cloned embryos to one recipient gilt (#1920) gave rise to two live fetuses (#1920-1 and #1920-2) via cesarean section at day 91 of gestation. No external abnormalities were observed in either of the cloned fetuses. Systemic green fluorescence derived from the EGFP gene on the DYS-HAC vector was observed in both cases (Figures 1F and S2B). The genotypes of the cloned fetuses were confirmed to be identical to those of the nuclear donor, DYS-HAC PFF$^{DMD\text{-}KO}$ (Figure S2C).

Flow cytometry analysis of umbilical cord blood-derived leukocytes revealed that EGFP was expressed by 57% and 79.1% of the cells in fetuses #1920-1 and #1920-2, respectively (Figure S2D). EGFP expression was also confirmed in 17 types of tissues and organs, including skeletal muscle (biceps femoris and diaphragm), heart, brain, and digestive organs, observed after autopsy (Figure S3). The findings indicate that the DYS-HAC vectors were retained systemically in the cloned fetuses. The ubiquitous expression of EGFP was similar to that in transchromosomic mice harboring the same DYS-HAC vector used in the present study.[29]

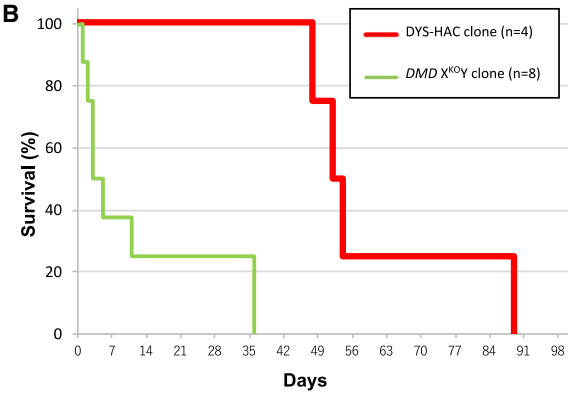
To confirm whether the human *DMD* on the DYS-HAC vector was properly expressed, tissues from six organs were subjected to RT-PCR using three pairs of specific primers for human *DMD*. Dp427l, an isoform of human dystrophin, was expressed mainly in the brain, heart, and skeletal muscle (biceps femoris); Dp427m was mainly expressed in the heart and skeletal muscle tissues; and Dp140 was specifically expressed in brain tissue (Figure S4).

Generation of live DYS-HAC-cloned pigs

The postnatal viability of the DYS-HAC-harboring cloned piglets was demonstrated. After transferring 86 cloned embryos to a recipient gilt (#2022), four live piglets (K358, K359, K360, and K361) were obtained by natural birth. EGFP fluorescence derived from the DYS-HAC vector was confirmed in all the cloned individuals obtained (Figure 2A).

A

B



C

Parameter [U/L]	DYS-HAC clone (n=4)					WT	*DMD* $X^{KO}Y$ clone
	K358	K359	K360	K361	Mean ± SEM	(n=19)	(n=2)
CK	30,730	46,490	33,165	16,687	31,768 ± 6105[a]	913 ± 128[b]	194,260 ± 61,340[c]
AST	1,076	1,473	959	322	958 ± 239[a]	37 ± 3[b]	1,048 ± 93[a]
ALT	2,059	1,197	1,101	717	1,269 ± 283[a]	38 ± 1[b]	959 ± 39[a]
LDH	8,696	7,923	6,264	4,016	6,725 ± 1036[a]	940 ± 44[b]	12,807 ± 5,014[a]

Figure 3. Phenotypic features of DYS-HAC-cloned pigs

(A) Photographs of four DYS-HAC-cloned pigs (K358, K359, K360, and K361) at 3 weeks of age. (B) Survival rates of the DYS-HAC-cloned pigs and the *DMD* $X^{KO}Y$-cloned pigs. The survival data of the *DMD* $X^{KO}Y$-cloned pigs were collected in our previous study.[28] (C) Comparison of serum biochemical parameters of the DYS-HAC-cloned pigs at 1 month of age with age-matched WT and *DMD* $X^{KO}Y$-cloned pigs. Parameters: CK, creatinine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase. Values represent the mean ± SEM. Different letters (a, b, c) within rows denote significant differences ($p < 0.05$).

PCR analyses revealed that all four piglets possessed the full-length human dystrophin gene and that the endogenous *DMD* gene was knocked out as in *DMD* $X^{KO}Y$ pigs (Figure 2B). The average birth weight of the four piglets was 1,195 ± 98 g (n = 4), which was slightly higher than that of the *DMD* $X^{KO}Y$-cloned piglets produced in our previous experiment using the same $PFF^{DMD\text{-}KO}$ nuclear donor cells (946 ± 144 g, n = 8).[28] The production efficiency of the DYS-HAC-cloned pigs (4.65%; Figure 2C) was comparable to that of genetically modified cloned pigs produced in our previous studies using non-HAC-carrying nuclear donor cells.[30–32]

In our previous studies, *DMD* $X^{KO}Y$-cloned pigs generated using $PFF^{DMD\text{-}KO}$ nuclear donor cells developed gait and respiratory abnormalities at infancy, and many (approximately 75%) died within the first month.[27,28] In contrast, all four DYS-HAC-cloned piglets grew well and survived beyond 1 month of age (Figures 3A and 3B). The surviving animals preserved motor function until 7 weeks of age (Figure S5). However, the symptoms observed in the *DMD* $X^{KO}Y$ pigs, such as difficulty ambulating and abnormal breathing, began to appear at approximately 7 weeks of age, and all animals were eventually euthanized before reaching 3 months of age (Figure 3B).

Blood biochemical analyses revealed that the level of serum creatine kinase (CK), a marker of muscle damage, was significantly lower in the four cloned pigs than in *DMD* $X^{KO}Y$ pigs (31,768 ± 6,105 vs. 194,260 ± 61,340 U/L) at 1 month of age. However, the CK level in the cloned pigs was significantly higher than that in wild-type (WT) animals (913 ± 128 U/L; Figure 3C). The CK levels in the DYS-HAC-cloned pigs had significantly increased by the time of euthanasia (325,098 ± 68,555 U/L). Thus, the improved CK level was inconsistent. No significant differences in aspartate aminotransferase (AST), alanine aminotransferase (ALT), or lactate dehydrogenase (LDH) values were observed between the DYS-HAC-cloned pigs and the *DMD* $X^{KO}Y$-cloned pigs. The values were significantly higher than those in the WT animals (Figure 3C).

Another group of 106 cloned embryos generated from the same DYS-HAC $PFF^{DMD\text{-}KO}$ nuclear donor cells were transferred to a recipient (K338) and resulted in a live piglet that did not exhibit EGFP fluorescence (Figure 2C). This cloned piglet was likely to have developed from a nuclear donor cell that did not carry the DYS-HAC vector (Figures 1C and 1E).

Phenotypic characteristics of DYS-HAC-cloned pigs

Histological analysis of the skeletal muscle (biceps femoris) of the DYS-HAC-cloned pigs indicated a mosaic EGFP fluorescence pattern in the muscle fibers of all four individuals (Figure 4, top panels). Immunofluorescence staining also revealed a mosaic staining

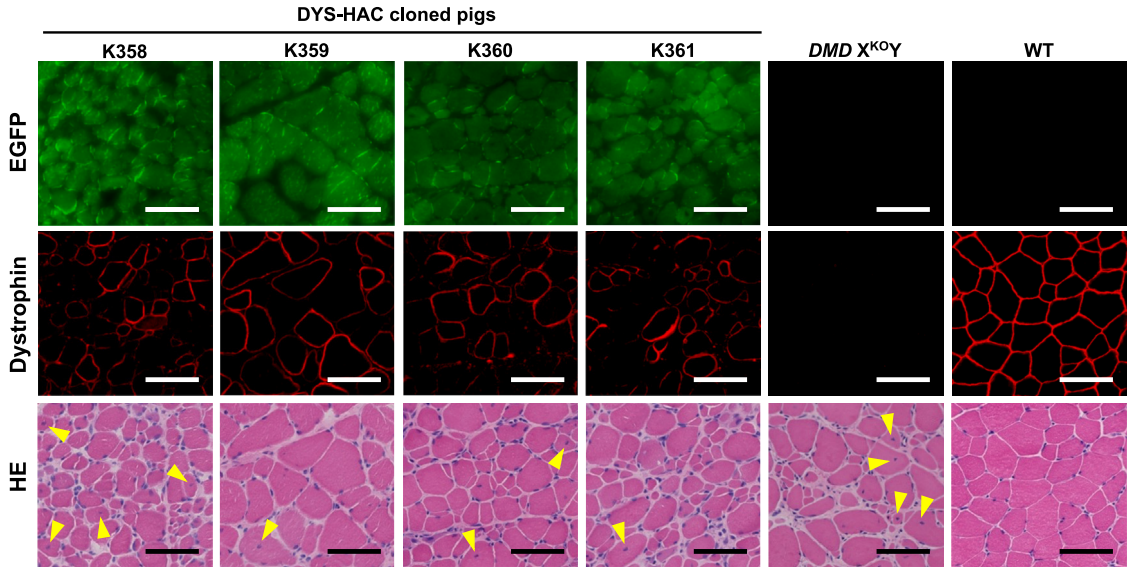


Figure 4. Histological examination of skeletal muscle from the DYS-HAC-cloned pigs

Mosaic expression pattern of EGFP (top panels) and human dystrophin (middle panels) in the muscle fibers (biceps femoris) of the four cloned pigs. Yellow arrowheads in the bottom panels indicate central nuclei. Samples of the DYS-HAC-cloned pigs were collected at days 48 (K358), 89 (K359), 52 (K360), and 54 (K361). Skeletal muscle samples from the *DMD* $X^{KO}Y$-cloned pig and WT pigs at 1 month of age were used as controls. Scale bars: 100 μm.

pattern of human dystrophin derived from the DYS-HAC vector in the muscle fibers (Figure 4, middle panels). Hematoxylin and eosin (H&E) staining of the muscle tissue showed some pathological features that were also observed in the *DMD* $X^{KO}Y$-cloned pigs, including nonuniform muscle fiber size, increased interstitial tissue volume, and central nuclei, as a reparative response to muscle necrosis (Figure 4, bottom panels). The dystrophin protein level of the skeletal muscle in the DYS-HAC-cloned pigs, as measured by western blot, was estimated to be <1% of the WT level (Figure S6).

Tissue-specific isoforms of human dystrophin were detected by RT-PCR using three specific pairs of primers for human dystrophin tissue-specific transcripts (Figure 5). Isoforms Dp427l and Dp427m were mainly expressed in the heart and skeletal muscles of the DYS-HAC-cloned pigs. The Dp140 isoform was detected in the brain. This pattern was comparable to the *DMD* expression profile in humans.

The retention rate of the DYS-HAC vector was examined in peripheral leukocytes and eight organs (brain, pancreas, heart, lung, liver, kidney, spleen, and skeletal muscle) of the four DYS-HAC-cloned pigs. Flow cytometry analysis of peripheral leukocytes showed that the proportion of EGFP-expressing cells ranged from 29.3% to 54.6% at the age of 1 month, with a decrease to approximately 20% (20.1%–23.9%) at the time of euthanasia (Figure S7). Quantitative PCR (qPCR) analyses showed that the retention rates of the DYS-HAC vector in solid organs at the time of sacrifice were different among the organs of individual animals, and the retention rate in each organ also differed among animals (Figure 6).

DISCUSSION

Various next-generation therapies, including cell-based therapy, gene therapy, and exon skipping, have been used to treat patients with DMD. However, a clinically curative treatment has not yet been developed.[34,35] The extremely large *DMD* gene (2.4 Mb) produces a range of different transcripts encoding various dystrophin isoforms via at least seven promoters within the gene.[36,37] The expression of all these isoforms under physiological conditions requires the introduction of the entire dystrophin gene. This is currently thought to be possible only with mammalian AC vectors. The presently observed 100% survival rate of the DYS-HAC-cloned pigs at 1 month of age, the significant improvement in serum CK levels, and the recovery of motor function in the hind leg indicated that the introduction of DYS-HAC was effective in alleviating muscle damage caused by *DMD* KO. Our results also suggest the presence of a critical threshold below which the carriage of the DYS-HAC vector in the cells of affected tissue could not prevent the development of DMD symptoms. The data obtained from the DYS-HAC-cloned pigs may suggest the requirement of a minimum amount of the vector in cells to cure the affected organ or tissue. Nevertheless, our data demonstrated that the episomal DYS-HAC, even with a minimum retention level, preserved therapeutic effect over a considerable period, thereby documenting the great potential of the novel therapy based on mammalian ACs. The stability of mammalian ACs in transchromosomic animals may be affected by the compatibility between the AC donor species and the host animal species.[11] Indeed, MAC is reportedly more suitable than HAC for generating transchromosomic mice.[38,39] Therefore, it will be worth developing a porcine AC suitable for pigs.

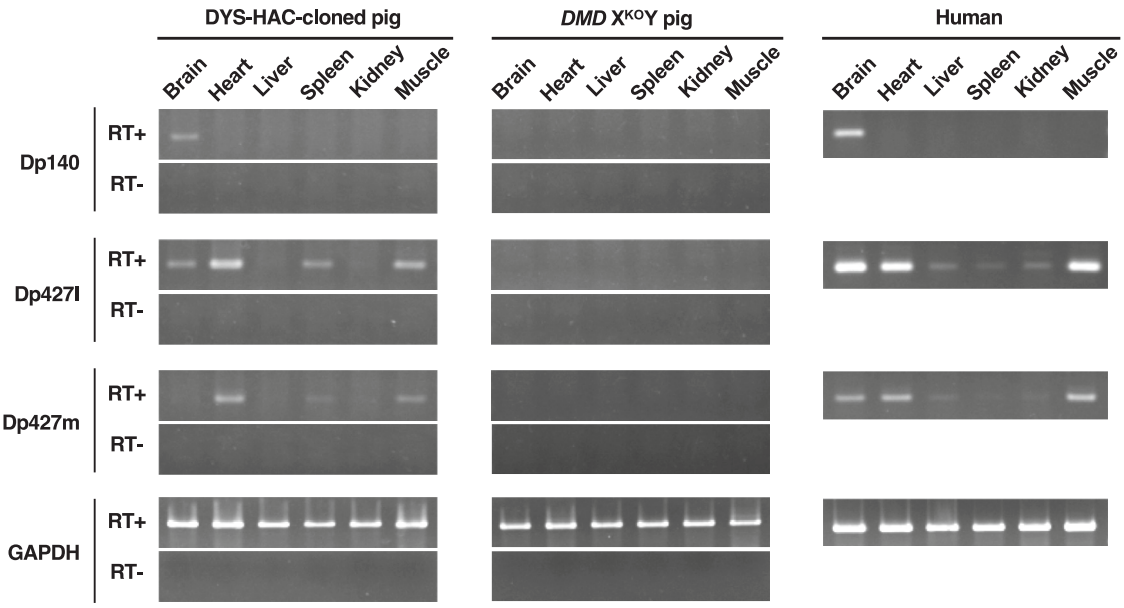


Figure 5. Expression of human dystrophin in various tissues of DYS-HAC-cloned pigs

Detection for the expression of the human dystrophin gene derived from the DYS-HAC vector in each tissue of the DYS-HAC-cloned pig (K359) by RT-PCR using three different sets of human dystrophin-specific primers.[29] *GAPDH* was used as an internal control. cDNA from *DMD* $X^{KO}Y$ pig tissues and human cDNA were used as negative and positive controls, respectively. RT, reverse transcriptase; RT−, negative control reaction.

A recent report described that adeno-associated virus (AAV)-based exon skipping restored dystrophin expression and prolonged survival in dystrophin-deficient *DMD* $X^{KO}Y$ pigs.[40] The *DMD* $X^{KO}Y$ pigs systematically administered with AAV displayed mosaic-like expression of endogenous truncated dystrophin (ΔExons 51–52) in the quadriceps and diaphragm. The amounts of restored DMD protein in quadriceps and diaphragm tissue were estimated to be 54% and 34%, respectively, of those in the WT control. In the present study, dystrophin protein in the skeletal muscle of the DYS-HAC cloned pigs was detected by western blot analysis; however, the amount of dystrophin protein restored in the DYS-HAC-cloned pigs was very low (<1% of the WT level). The limited improvement in DMD symptoms is likely ascribed to the low dystrophin production in the muscular tissues that possess the DYS-HAC vector in a mosaic manner (18.2%–23.2%). It has been estimated that even 15% of normal levels of dystrophin would provide substantial therapeutic benefits for patients with DMD.[41–43] Therefore, the reason why the rescue of the phenotype was insufficient was not only due to the loss of the DYS-HAC vector but also because the dystrophin levels expressed from the DYS-HAC vector were lower than expected. The results of the present study indicate the importance of stable dystrophin expression in a substantial proportion of cells in the affected tissue for the achievement of clinically satisfactory resolution of DMD symptoms. The use of large-animal models to determine the dystrophin expression level required for therapeutic efficacy may contribute to DMD treatment strategies.[44]

Despite the development of various genetic modification techniques, the creation of genetically modified pigs with extremely large genes has remained a challenge. Compared with plasmids and BACs, which have been used to generate many transgenic animals, mammalian ACs, such as HACs or MACs, have the advantage of no restrictions on the size of genes that can be introduced. Mammalian ACs have been used for the transduction of foreign genes into mice,[29,45] rats,[46] cattle,[47] and goats.[48] However, to the best of our knowledge, mammalian ACs have not been used in pigs. In the present study, we generated the first transchromosomic pig via SCNT with somatic cells harboring HAC.

The production efficiency of the DYS-HAC-cloned pigs was comparable to that of various other genetically modified pigs generated via SCNT using cells lacking HACs.[30–32] The finding indicated that the carriage of HAC in the donor nuclei does not interfere with the development of the cloned embryos. During the early development of SCNT embryos, nuclear reprogramming events occur. These include chromatin remodeling of the somatic state nucleus to the embryonic state accompanying histone replacement and modification.[49] The remodeling events that occur on the mammalian AC following transfer into the ooplasm remain unclear. The possible influence of post-SCNT modification on exogenous HAC needs to be further investigated.

In conclusion, the efficacy of mammalian ACs in the treatment of genetic diseases in a large-animal model was demonstrated for the first time. Transchromosomic cloned pigs produced via SCNT technology permitted a straightforward estimation of the therapeutic efficacy of the mammalian ACs carried in cells of the affected tissues/organs, and stable or controlled retention of the ACs needs to be achieved. The fact that the human *DMD* gene on the DYS-HAC vector has demonstrated therapeutic efficacy in *DMD* $X^{KO}Y$ pigs provides valuable information for developing next-generation therapies. The combination of

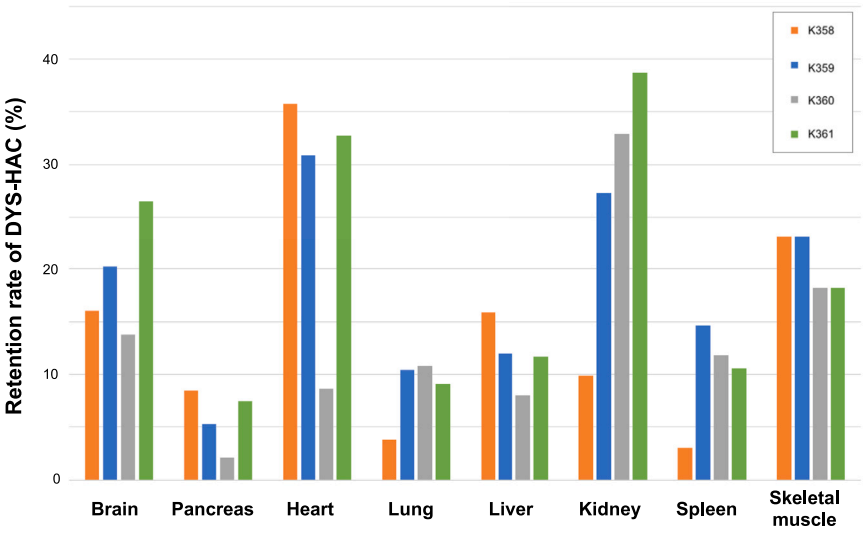


Figure 6. Retention rate of the DYS-HAC vector in various tissues of the DYS-HAC-cloned pigs

Vertical bars indicate the retention rates of the DYS-HAC vector in eight tissues of the four DYS-HAC-cloned pigs at days 48 (K358), 89 (K359), 52 (K360), and 54 (K361) after birth.

somatic cell cloning technology and mammalian AC technology may revolutionize the development of genetically modified pigs.

MATERIALS AND METHODS

Animal care

All animal experiments performed in the present study, including genetic modifications, were approved by the Institutional Animal Care and Use Committee of Meiji University (MUIACUC2020-111 and MUIACUC2020-125). All experiments were performed in accordance with the relevant guidelines and regulations.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Preparation of DYS-HAC donor cells and nuclear donor cells for SCNT

The construction of a HAC vector containing the entire human dystrophin genomic region was described previously.[29] Briefly, the DYS-HAC vector constructs, consisting of the centromere and telomere regions of human chromosome 21, the entire 2.4 Mb human dystrophin gene, and the EGFP gene driven by the CAG promoter flanked by HS4 insulators, were transferred into Chinese hamster ovary (CHO) cells to prepare donor cells.[29] A previously established primary culture of $PFF^{DMD\text{-}KO}$ was used to generate cloned *DMD* $X^{KO}Y$ pigs harboring DYS-HAC vectors.[28] The DYS-HAC vectors were introduced into $PFF^{DMD\text{-}KO}$ from donor CHO cells harboring DYS-HAC (DYS-HAC CHO) using MMCT.[29] DYS-HAC-transferred $PFF^{DMD\text{-}KO}$ cells were cultured in blasticidin S (3 μg/mL) selection medium, and drug-resistant and EGFP-positive clones were established as nuclear donor cells. For genotyping of the nuclear donor cells, PCR analyses were performed as described previously.[29,50] FISH analysis for the nuclear donor cells isolated was performed on fixed metaphase spreads of each cell bearing both $PFF^{DMD\text{-}KO}$ and the DYS-HAC vector using digoxigenin-labeled (Roche, Basel, Switzerland) human Cot-1 DNA (Invitrogen, Waltham, MA, USA) and biotin-labeled BAC DNA (RP11-954B16, located in the human dystrophin genomic region), as described previously.[29,50] Chromosomal DNA was counterstained with DAPI. Images were captured using an AxioImagerZ2 fluorescence microscope (Carl Zeiss, Jena, Germany).

Generation of DYS-HAC-cloned pigs by SCNT

SCNT was performed as described previously with slight modifications.[51] Briefly, nuclear donor cells were used for SCNT following cell-cycle synchronization induced via serum starvation for 2 days. A single donor cell was electrically fused with each enucleated cytoplast prepared from an *in-vitro*-matured oocyte. The reconstructed embryos were electrically activated and cultured in porcine zygote medium-5 (PZM-5; Research Institute for Functional Peptides, Yamagata, Japan) for 3 h in the presence of 5 μg/mL cytochalasin B and 500 nM Scriptaid, and embryos were then cultured with 500 nM Scriptaid for another 12–14 h. The cloned embryos were cultured in PZM-5 under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C. Beyond the morula stage, the embryos were cultured in PZM-5 supplemented with 10% fetal bovine serum. At days 1–2, the cloned embryos were surgically transferred into the oviducts of estrus-synchronized recipients.

Genotyping of DYS-HAC-cloned pigs

Genomic DNA was extracted from the tissues and blood of the DYS-HAC-cloned pigs using a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. PCR analyses were carried out using standard techniques. The primer pairs for the detection of the human dystrophin region on the DYS-HAC vector were as follows: 5′-TCCTCCATAGCCTGAGGAGC and 5′-TCAACCACGGTAATGTATGC for the 5′ region; 5′-AACAACT GAACAGCCGGTGGA and 5′-GGGGTGGTGGGTTGGATTTT for exon 20; 5′- GCTGCTAGCAATGCCACGATT and 5′-GGATGGGC TGGGAATCCATAG for exon 79. For identification of *DMD* KO and sex, PCR was performed as described previously.[28]

Flow cytometry analysis

EGFP fluorescence of the nuclear donor cells and leukocytes was analyzed using an SH-800S flow cytometer/cell sorter (Sony, Tokyo, Japan). The leukocytes were harvested from the umbilical cord blood of the DYS-HAC-cloned fetuses and the peripheral blood of the cloned pigs using an erythrocyte lysis solution (PharmLyse; Becton Dickinson, Franklin Lakes, NJ, USA), according to the

manufacturer's instructions. The cell debris and aggregates were gated out of the analysis using bivariate forward/side scatter (FSC/SSC) parameters.

Analysis of DYS-HAC vector retention in various tissues by qPCR

The various tissues were collected from sacrificed DYS-HAC-cloned pigs to analyze the retention of the DYS-HAC vector. The genomic DNA was extracted from various tissues of the DYS-HAC-cloned pigs using the DNeasy Blood and Tissue Kit (QIAGEN), as described above. The retention rate of the DYS-HAC vector in various tissues was determined by a real-time PCR method (relative quantification) combined with a digital PCR method (absolute quantification). First, the retention rate of the DYS-HAC vector in skeletal muscle was evaluated by digital PCR analysis based on the copy number of the porcine *ACTB* gene (2 copies in diploid genome) as a reference. Subsequently, relative values for the retention of the DYS-HAC vector among various tissues, including skeletal muscle, were determined by real-time PCR. The ΔΔCT method was used to determine the relative values normalized to the *ACTB* gene. Finally, the retention rates were calculated by both digital PCR and real-time PCR. Digital PCR and real-time PCR were performed using the QuantStudio 3D Digital PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and the StepOne Plus Real-Time PCR System (Thermo Fisher Scientific), respectively, according to the manufacturer's instructions. Primers and probes for the assays were as follows: for DYS-HAC (human dystrophin gene) forward primer, 5′-AGCATCAAACAAGCCTCAGAAC, reverse primer, 5′-ATTGCTGTTGGCTCTGATGG, TaqMan probe, 5′-CTGGCTGGAGTATCAGAACAACATCATCGC; porcine *ACTB* (β-actin) forward primer, 5′-TGGATGACGATATTGCTGCGC, reverse primer, 5′-CCCACGATGGAGGGGAAGA, TaqMan probe, 5′-AGCATCGTCGCCCGCAAAGCCG.

RT-PCR

Total RNA from the DYS-HAC-carrying pig tissue specimens was prepared using ISOGEN (Nippon Gene, Tokyo, Japan), treated with deoxyribonuclease (Nippon Gene, Tokyo, Japan), and purified using RNeasy columns (QIAGEN), in accordance with the manufacturer's instructions. First-strand cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). RT-PCR analyses were performed as previously described.[29,50] In the case of fetal skeletal muscle (biceps femoris), an RNeasy Plus Micro Kit (QIAGEN) was used for total RNA extraction. Human tissue cDNA from Multiple Tissue cDNA Panels I and II (Takara Bio, Shiga, Japan) was used as a control.

Biochemical analysis of blood components

The pigs were anesthetized, and blood samples were obtained from the ear vein. The blood was allowed to clot completely, and the serum was collected. Serum biochemistry tests to determine the concentrations of CK, AST, ALT, and LDH were performed using a dry-chemistry analyzer (FUJI DRI-CHEM 7000, FUJIFILM, Tokyo, Japan).

Histological analysis of skeletal muscle

After DYS-HAC-cloned pigs were euthanized under general anesthesia, the skeletal muscles (biceps femoris) were dissected, mounted on cork bases using tragacanth gum (Fujifilm Wako Pure Chemical, Osaka, Japan), and frozen by isopentane cooled in liquid nitrogen. Histological analyses were performed on 8-μm-thick frozen sections. After the observation of EGFP expression in the frozen section under fluorescence microscopy (BZ-X710, Keyence, Osaka, Japan), immunofluorescence staining for dystrophin was performed using the same frozen section. The histological serial sections were stained with H&E using a standard technique. For immunofluorescence, the sections were incubated with Protein Block (X0909, Dako, Glostrup, Denmark) for 30 min at 25°C and then treated with Mouse Monoclonal Antibody Dystrophin (NCL-DYS2, 1:50 dilution, Leica Biosystems, Wetzlar, Germany), which reacts with both human and pig dystrophin, for 1 h at room temperature. After the removal of excess antibody, the sections were incubated with Alexa Fluor 594 (ab150112,1:450 dilution, Abcam, Cambridge, UK) for 1 h at room temperature. The slides were visualized under fluorescence microscopy (BZ-X710, Keyence).

Western blot analysis

After DYS-HAC-cloned pigs were euthanized under general anesthesia, the skeletal muscle samples were collected and frozen in liquid nitrogen. Total protein was obtained from the snap-frozen tissues using modified RIPA lysis buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 9% SDS) including protease inhibitors (Nacalai Tesque, Kyoto, Japan). Tissue samples were homogenized and placed at 25°C for 15 min. The protein lysates mixed with the equal volume of 2× Sample Buffer Solution with 2-mercaptoethanol (Nacalai Tesque) were boiled at 95°C for 5 min. After being cooled down, the samples were centrifuged at 12,000 × *g* for 15 min at 25°C, and the supernatants were recovered. The protein samples were resolved on NuPAGE 3%–8% Tris-acetate gel with Tris-acetate SDS running buffer (Thermo Fisher Scientific) at 100 V for 2 h in an ice-cold bath. Proteins were transferred to a PVDF membrane (Cytiva, Marlborough, MA, USA) using Mini Trans-Blot Electric Transfer Cell (Bio-Rad) at 30 V for 1 h and subsequently at 100 V for 1 h in an ice-cold bath. The membrane was washed with Tris-buffered saline supplemented with 0.1% Tween 20 (TBS-T), then incubated with blocking buffer (Blocking One, Nacalai Tesque) at 25°C for 30 min and then with primary antibodies for dystrophin (Abcam, ab15277, 1:2,000) at 4°C overnight. Membranes were washed with TBS-T and incubated with secondary antibodies for goat anti-rabbit IgG-HRP (Santa Cruz, sc-2004, 1:5,000) at 25°C for 1.5 h. After washing three times with TBS-T, signals were detected using an ECL Prime Western Blotting Detection Reagent (Cytiva) in the iBright FL1500 Imaging System (Thermo Fisher Scientific). For the detection of β-tubulin as loading controls, the membrane was incubated in stripping buffer (Thermo Fisher Scientific) at 37°C for 10 min, blocked, and reprobed with primary antibodies for β-tubulin (Proteintech, Rosemont, IL, USA, 66240-1-Ig, 1:20,000) at 25°C for 1 h and with secondary antibodies for goat anti-mouse IgG-HRP (Santa Cruz, sc-2005, 1:5,000) at 25°C for 1 h. The intensity of the protein band was analyzed using the ImageJ software (https://imagej.nih.gov/ij/index.html), and dystrophin expression was quantified by normalization to β-tubulin. HiMark

Pre-stained Protein Standard (Thermo Fisher Scientific) was used as a protein ladder.

Control materials

Control data on the immunohistochemistry were obtained using materials collected in our previous study.[28] Control data on body weight, survival rates, and blood components described in the present manuscript were obtained in our previous study.[28] These data are unpublished. For RT-PCR analysis and fluorescence microscopy of organs, the control materials were produced by natural mating of WT boars and *DMD*-heterozygous KO female pigs.[27,28]

Statistical analysis

The Student's t test was used for statistical analysis of blood parameters. A p value <0.05 was considered to indicate statistically significant results.

DATA AND CODE AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2023.07.021.

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AUTHOR CONTRIBUTIONS

H.N. and Y.K. conceived and designed this study. M.W., Y.K., and H.N. wrote the manuscript. M.W., H. Miyamoto, K.O., K.N., H. Matsunari, K.K., K.H., A.U., S.T., K.U., Y.H., E.K., N.K., M.K., B.K., E.W., Y.K., and H.N. performed the experiments. M.W., Y.K., K.O., and H.N. performed data analysis. All authors reviewed and approved the final manuscript.

DECLARATION OF INTERESTS

H.N. is a founder and shareholder of PorMedTec Co., Ltd. These associations do not alter the authors' adherence to the journal's policies on sharing data and materials.

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