Modeling lethal X-linked genetic disorders in pigs with ensured fertility

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Genetically engineered pigs play an indispensable role in the study of rare monogenic diseases. Pigs harboring a gene responsible for a specific disease can be efficiently generated via somatic cell cloning. The generation of somatic cell-cloned pigs from male cells with mutation(s) in an X chromosomal gene is a reliable and straightforward method for reproducing X-linked genetic diseases (XLGDs) in pigs. However, the severe symptoms of XLGDs are often accompanied by impaired growth and reproductive disorders, which hinder the reproduction of these valuable model animals. Here, we generated unique chimeric boars composed of mutant cells harboring a lethal XLGD and normal cells. The chimeric boars exhibited the cured phenotype with fertility while carrying and transmitting the genotype of the XLGD. This unique reproduction system permits routine production of XLGD model pigs through the male-based breeding, thereby opening an avenue for translational research using disease model pigs.

blastocyst complementation | chimera | disease model pig | gene knockout | somatic cell cloning

D isease model animals developed via genetic engineering have played an indispensable role in the study of human monogenic diseases. In addition to various rodent models, genetically modified pigs have recently been generated as models for intractable hereditary diseases and rare genetic disorders (1, 2). Mutations in genes homologous to those responsible for such disorders have been reported to cause similar symptoms in pigs to those in human patients, while genetic mutations do not necessarily produce the same disease phenotype in rodents (1, 3, 4).

Methods involving somatic cell cloning are highly effective for generating pigs with genetic mutations (5), and this technique is commonly used due to the limited availability of porcine embryonic stem (ES)/induced pluripotent stem (iPS) cells with proven totipotency, i.e., chimera formation ability (6, 7). Nuclear transfer using male cells carrying mutation(s) in an X chromosomal gene enables the direct "cell-to-animal" generation of models for X-linked genetic diseases (XLGDs). In fact, feasible production of model pigs reproducing XLGDs such as severe combined immunodeficiency (SCID) (8, 9), Duchenne muscular dystrophy (DMD) (10), and hemophilia (11) has been reported. Among these XLGD model pigs, severe symptoms starting at the neonatal or juvenile stage develop in many cases.

Male individuals congenitally exhibiting severe symptoms of XLGDs are prone to neonatal lethality, impaired growth, and reproductive disability, complicating the propagation of disease model animals (8–10). For instance, the DMD model pigs reported previously showed premature lethality (10). Thus, severe symptoms often hinder the distribution of valuable disease model pigs. However, this problem can be overcome by the cryopreservation of sperm, which enables extensive distribution and long-term storage of these valuable genetic resources. Therefore, in the present study, we developed a method for generating unique chimeric boars that carry the mutation responsible for an XLGD in both somatic and germ cells while maintaining competence for growth and reproduction. We showed that complementation of a male cloned embryo carrying an X-linked mutation ($X^{KO}Y$) with normal female embryonic cells ($X^{WT}X^{WT}$) can give rise to a viable chimeric boar comprising both mutant and normal cells ($X^{KO}Y \leftrightarrow X^{WT}X^{WT}$). Normal $X^{WT}X^{WT}$ cells compensated for the impaired function of $X^{KO}Y$ cells in the tissues/organs of the chimeric pig. We demonstrated that induced chimerism rescued the lethal congenital symptoms of gene-knockout (KO) pigs for three types of XLGDs: ornithine transcarbamylase deficiency [OTCD, Online Mendelian Inheritance in Man (OMIM) no. 311250] (12, 13), SCID (OMIM no. 300400) (9), and DMD (OMIM no. 310200) (10). Chimerism induced in a gene-deficient embryo has been demonstrated to compensate for the traits of genetic disorders in rodents (14–16).

Chimeric pigs that had been directed to grow into fertile boars as a result of intersex chimerism (17, 18) were able to produce next-generation progeny carrying the mutation. Reproduction of the chimeric boars demonstrated that their germ cells were derived from the original cloned embryos harboring the lethal

Significance

The development of therapies for rare and intractable genetic disorders represents a significant unmet medical need. Disease model pigs characterized by physiological, anatomical, and pathogenetic similarities to humans allow translational studies to be performed, yielding valuable data that can be extrapolated to patients. The establishment of an efficient reproduction system is a key element in the practical application of disease model pigs, which often suffer from reproductive inability due to severe symptoms. Here, we showed that the valuable trait of genetically modified disease model pigs can be maximized by generating unique chimeric boars composed of mutant and normal cells.

XLGD trait and that the X chromosomes carrying the mutant genes were transmitted to progeny in a Mendelian fashion.

The present study demonstrates that fertile chimeric boars that latently carry an XLGD trait can transmit the disease-causing mutations to progeny and will thereby play a pivotal role in expanding the practical use of novel disease model pigs.

Results

Gene-Knockout Cloned Pigs Exhibiting X-Linked Disorders. In the present study, we used model pigs for three types of XLGDs: OTCD, SCID (9), and DMD (10). *OTC*-KO pigs, a model for a congenital metabolic disorder characterized by hyperammonemia, were newly developed in this study by means of genome editing and somatic cell cloning. Cloned pigs were generated via nuclear transfer of primary culture cells carrying a truncated OTC gene (5-bp deletion in exon 2, c.186_190delTCTGA; Fig. S1). The cloned *OTC*-KO pigs exhibited high perinatal mortality (6/8) and severe neonatal OTCD symptoms (2/2), including hyperammonemia, high urine levels of orotic acid, tremors, and early postpartum mortality (Table 1 and Movie S1).

To produce cloned embryos and animals for the other two models, we used cell lines carrying the mutations *IL2RG* $X^{KO}Y$ (9) and *DMD* $X^{KO}Y$ (10), which were established in our previous studies. *IL2RG*-KO pigs exhibited SCID symptoms such as T and NK cell deficiency (8). These pigs are prone to systemic infection under conventional housing conditions and, therefore, rarely develop into fertile adults.

DMD-KO pigs exhibited progressive dystrophic changes in their skeletal muscles, accompanied by increased serum creatine kinase levels, impaired mobility, muscle weakness and a maximum life span of 3 mo, due to respiratory impairment (10). In addition, characteristic age-related proteome changes were apparent in this model (19).

Complementation of Gene-Knockout Cloned Pigs. Cloned embryos derived from the three types of XLGD model pigs were complemented (18) with normal embryonic cells to produce chimeric boars (Fig. 1A). First, cloned embryos generated from male *OTC*-KO cells were injected at the morula stage with blastomeres from female cloned morulae expressing the humanized Kusabira-Orange gene (*huKO*) to generate chimeric blastocysts (Fig. 1 B and C). A total of four piglets, including two chimeras (*OTC* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$), were obtained after the transfer of 70 blastocysts to a recipient gilt (Table 2 and Table S1). The chimerism of the piglets was confirmed based on coat color (Fig. 1D) and genotyping (Fig. S2). The remaining two animals were nonchimeric males derived from *OTC*-KO embryos (Table 2 and Table S1).

The *OTC* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimera was born healthy, grew up normally with normal blood ammonia level (Fig. S3 A and B and Table S4), and was fertile after maturity. Mating of the chimeric boar with a total of seven WT females yielded 74 F_1 offspring in 7 litters (pregnancy rate: 100%; Table S7). All but one of the female offspring exhibited an *OTC* $X^{KO}X^{WT}$ genotype, and the male siblings were all wild type. The cause of X chromosome deletion ($X^{WT}O$) in one exceptional female was unknown (Table S7). These results indicated that the germ cells of the chimeric boar had arisen from the *OTC* $X^{KO}Y$ cloned embryos, such that the X chromosomes harboring the mutant *OTC* were transmitted to the F_1 progeny in a Mendelian fashion. We previously reported that a chimeric pig embryo composed of male and female embryonic cells developed into a fertile boar, yielding sperm exclusively from the male cells (17, 18).

Cloned embryos generated from the male *IL2RG* $X^{KO}Y$ cells (light-brown coat color) were complemented with female WT cloned embryos (black coat color). The host *IL2RG* $X^{KO}Y$ embryos at the morula stage were injected with morula blastomeres to produce chimeric blastocysts (Table S2). The transfer of 62 blastocysts to a recipient female gave rise to six male offspring, including two (33.3%) chimeras (*IL2RG* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$), which were confirmed based on coat color (Fig. 1A and Fig. S3C) and genotyping (Fig. S2). The four *IL2RG*-KO cloned males (Table 2) among the siblings exhibited the same SCID symptoms reported previously (9). The chimeric males were not physiologically aberrant (Table S5) and were confirmed via FACS analysis to possess a normal lymphocyte composition (Fig. 2A). Both chimeric males were grown into fertile boars and yielded F_1 offspring after mating with WT females (Table S8). All of the F_1 females exhibited the *IL2RG* $X^{KO}X^{WT}$ genotype, indicating that the germ cells of the chimeric boar originated from the *IL2RG* $X^{KO}Y$ embryos and that the X chromosome carrying the mutant gene was transmitted to the F_1 progeny in a Mendelian fashion, as observed in the case of the *OTC* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimera.

Cloned morulae derived from *DMD* $X^{KO}Y$ cells (white coat color) were complemented with blastomeres from WT (black coat color) cloned morulae to generate chimeric blastocysts. Among five offspring obtained after the transfer of 76 blastocysts to a recipient gilt, three chimeric males were identified (Fig. 1A, Table 2, Fig. S2, and Table S3). The clone siblings included two *DMD*-KO males (Table 2 and Table S3). One of the chimeric males (#61) behaved normally and grew to sexual maturity (Fig. S3 A and D), although it showed mild symptoms of DMD, such as muscle weakness of the legs and shortness of breath after walking for a long time, as the pig grew (Movie S2). Testes were harvested from the chimeric male at 7 mo of age to cryopreserve epididymal sperm. The frozen sperm were capable of efficiently

Table 1. Phenotypic features of *OTC* $X^{KO}Y$ (OTCD) pigs

		Pigs	
Data items		Founder *OTC* $X^{KO}Y$ clones	F_2 *OTC* $X^{KO}Y$	F_2 WT male siblings
No. of animals obtained		8*	18[†]	22[‡]
Average birth weight [range], g		345 ± 91.9[§] [231–463]	574 ± 101.0[§] [405–787]	1,110 ± 343.3[§] [405–1,636]
Blood ammonia [range], μg/dL	Day 0	661.0 [421, 901]	651.5 ± 612.9[§] [160.6–1,690.3]	86.7 ± 39.6[§] [31.5–117.6]
	Day 1	N/A	1922.4 ± 539.3[§] [1,313.9–2,662.8]	66.1 ± 18.9[§] [61–108.4]
Urinary orotic acid [range], μmol/mg Cre.		0.427 [0.116, 0.738] (Day 0)	27.92 ± 17.4[§] [7.612–46.926] (Day 1)	0.317 ± 0.28[§] [0.19–0.731] (Day 1)

Data are presented as the mean ± SD. N/A, not applicable; Cre., creatinine.

*Blood ammonia and urinary orotic acid were measured in two live animals. Statistical comparisons with other groups were not performed due to the small number of animals.

[†]Blood ammonia and urinary orotic acid were measured in five and four live animals, respectively.

[‡]Blood ammonia and urinary orotic acid were measured in 11 and 3 selected animals, respectively.

[§]Values with different superscript within the same line differ significantly ($P < 0.05$).
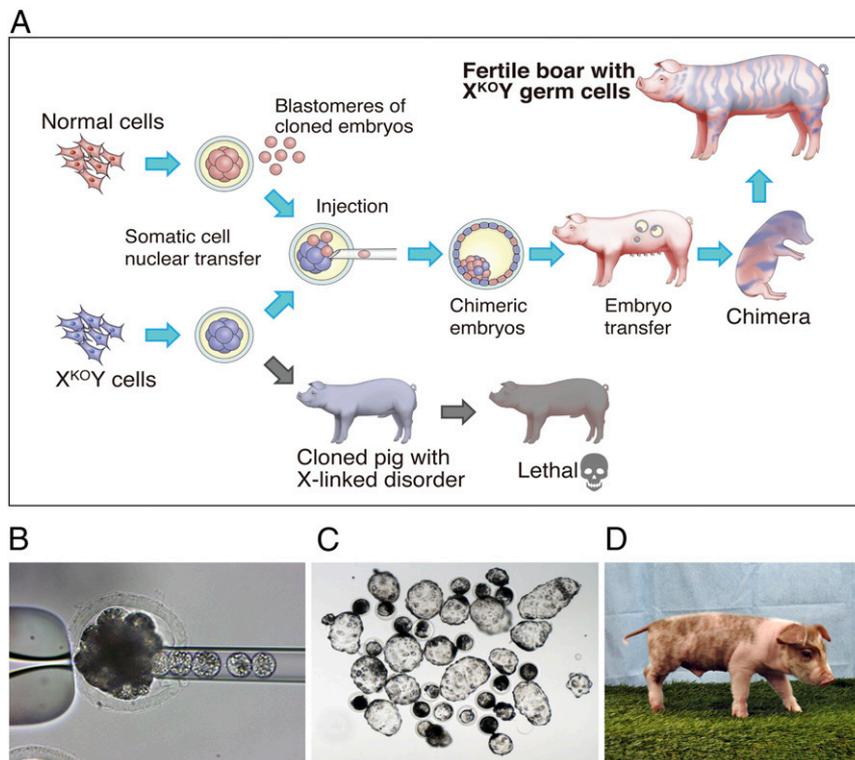
Fig. 1. Rescuing the lethal traits of X-linked genetic disorders via chimerism. (*A*) Cloned embryos generated from $X^{KO}Y$ cells gave rise to male pigs exhibiting lethal X-linked disorders, including OTCD, SCID, and DMD. Injection of the $X^{KO}Y$ cloned embryos with embryonic blastomeres with normal genetic traits yielded chimeric embryos. Fertile chimeric boars producing $X^{KO}Y$ germ cells were obtained after transferring the chimeric embryos to surrogate sows. (*B* and *C*) Microinjection of the cloned embryos with blastomeres (*B*) yielded chimeric blastocysts (*C*). (*D*) A chimeric boar (*OTC* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$) showing normal growth and reproductive ability was obtained.

producing blastocysts through in vitro fertilization. Transfer of the in vitro-fertilized blastocysts yielded 12 piglets, including 7 (58.3%) heterozygous mutant females ($X^{KO}X^{WT}$) and 5 (41.7%) WT males (Table S9). The other chimeric male (#62) exhibiting lower chimerism died at the age of 3 mo after showing impaired motility, accompanied by severe weakness of the leg muscles and constant shortness of breath (Movie S3). The *DMD*-KO cloned males among the siblings died by 5 wk of age, consistent with our previous study describing the phenotypes of the original *DMD*-KO clone pigs (Movie S4) (10).

Phenotypic Features of the Chimeric Pigs Carrying the Mutant Gene. In the *OTC* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimera, blood ammonia levels were maintained within the normal range throughout growth (Table S4), and the pig reached maturity by 7 mo of age. This pig retained normal reproductive ability at the age of 3.5 y.

The *IL2RG* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ boars displayed a normal lymphocyte composition after growing into adulthood (6 mo old, Fig. 2*A*). They appeared physiologically normal and remained healthy until up to 3 y of age under conventional rearing conditions, including vaccinations (Table S5). A surgical intervention for testis collection at 7 mo of age did not cause infection, indicating normal immunological function, even after sexual maturity.

Histological analysis of the tissues of the *DMD* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimera (#62) that died at 3 mo of age revealed that the proportion of WT cells was very limited (Fig. S4). The lower proportion of WT cells in this animal was supported by their coat color chimerism (Movie S3). The high proportion of mutant cells in the skeletal muscles and diaphragm was suspected to be the cause of this individual's impaired motor function and breathing.

In contrast, the chimeric boar (#61) that grew to maturity was characterized by a higher proportion of WT cells in its tissues according to both its coat color chimerism (~40%; Fig. S3*D*) and histological analysis (Fig. 3). Therefore, the milder impairment of motor and aspiratory function was likely due to a lower proportion of DMD-deficient cells in the tissues of this animal. However, blood creatine kinase (CK) levels in the chimeric boar (75,460 U/L) were comparable to those of DMD patients, suggesting progressive muscle degradation in this animal (Table S6).

Transmission of the Mutant Trait to Offspring. The $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric boars transmitted the causal mutant gene of their respective XLGDs to all of the F_1 female progeny (Tables S7–S9). In general, the phenotypes of the female carriers rarely showed expression of the X-linked mutation. The F_1 females produced in this study mostly expressed no signs of the X-linked conditions. However, a very small proportion (2/33) of the $OTC^{+/-}$ females sporadically expressed hyperammonemia, and some of these animals fell into comas and were killed for analysis. The onset dynamics of the $OTC^{+/-}$ female pigs resembled those of female carriers of the mutant OTC gene in humans (20, 21).

Table 2. Production efficiency of live $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric pigs via blastocyst complementation

Model types	$X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric male	$X^{KO}Y$ clone	$X^{WT}X^{WT}$ clone
OTCD*	1 (1)	2	0
SCID*	2	4	0
DMD*	2 (1)	2	0

Numbers in parentheses indicate additional stillborn chimeras.
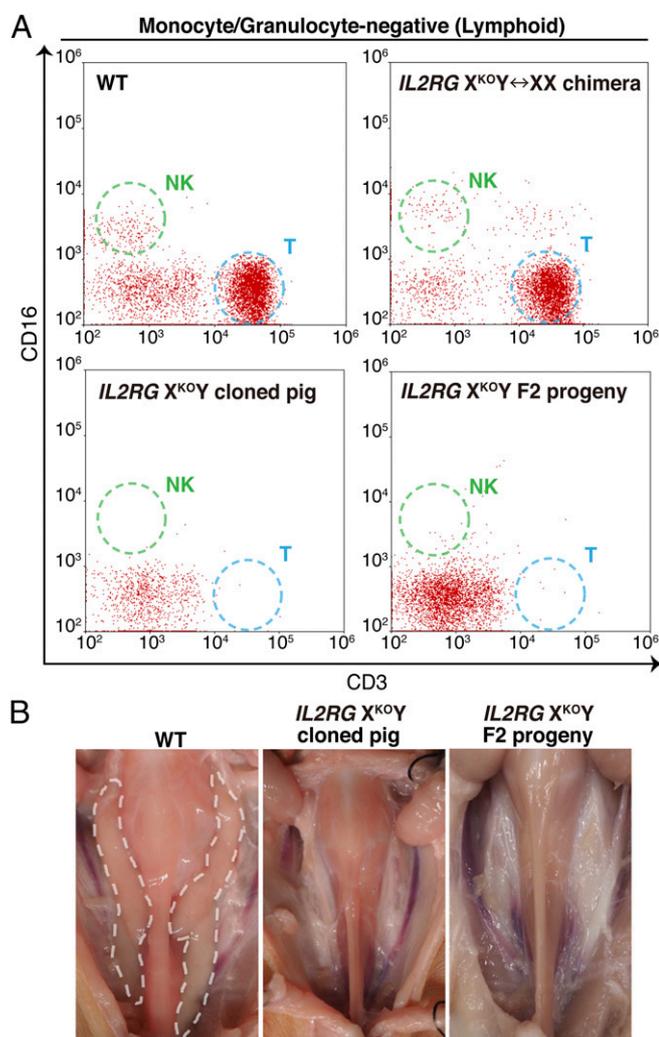*The offspring in one litter are indicated for each model.

Fig. 2. Phenotype of the *IL2RG* -$X^{KO}Y$ and *IL2RG* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric pig. (*A*) Flow cytometric analysis of the *IL2RG* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric pig. Flow cytometric analysis of the peripheral blood of a WT and *IL2RG* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric boar demonstrated restoration of the T and NK cell populations, which were absent in the cloned *IL2RG* $X^{KO}Y$ pigs. The F_2 *IL2RG* $X^{KO}Y$ progeny of the *IL2RG* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric boar exhibited the same phenotype as the founder *IL2RG* $X^{KO}Y$ cloned pigs. The dot plots show CD3 and CD16 [in the nonmyeloid fraction, i.e., monocyte/granulocyte (M/G)-negative] cells, indicating the differentiation of the T cell and NK cell subpopulations, respectively. (*B*) Macroscopic observation of thymi in the *IL2RG* -$X^{KO}Y$ pigs. Athymic phenotype of the *IL2RG* -$X^{KO}Y$ cloned pig was inherited by the F_2 progeny obtained through sexual reproduction of the *IL2RG* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric boar.

All but one (6/7, 85.7%) of the $IL2RG^{+/-}$ F_1 females grew into healthy, fertile adults (Fig. S5 *A* and *B*). The cause of the sudden death of one animal at 2 mo of age was unknown.

Repetition of Phenotypic Symptoms in F_2 Offspring. The $OTC^{+/-}$ and $IL2RG^{+/-}$ F_1 females were mated with WT boars to produce F_2 progeny (Table 3 and Tables S10–S13). Half of the male and female F_2 progeny exhibited the $X^{KO}Y$ and $X^{KO}X^{WT}$ traits, respectively, indicating that the X chromosome with the KO locus was faithfully inherited by the F_2 progeny from the carrier female in a Mendelian fashion (Tables S10 and S12). Backcrossing of $X^{KO}X^{WT}$ females with the chimeric boar should theoretically yield $X^{KO}X^{KO}$ animals. In fact, *IL2RG* $X^{KO}X^{WT}$ females backcrossed with the *IL2RG* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric boar gave birth to $X^{KO}X^{KO}$, $X^{KO}X$, $X^{KO}Y$, and XY siblings in a single litter

(Table S13). However, *OTC* $X^{KO}X^{KO}$ animals were not obtained, indicating prenatal lethality of the homozygous KO trait (Table S11).

The *OTC* $X^{KO}Y$ and *IL2RG* $X^{KO}Y$ males in the F_2 progeny exhibited the same symptoms as the founder $X^{KO}Y$ cloned pigs. All of the *OTC*-KO F_2 males showed typical OTCD symptoms, including hyperammonemia, high urine levels of orotic acid, tremor, and neonatal death (Table 1 and Movie S1).

Flow cytometry analysis of blood samples from *IL2RG*-KO F_2 males revealed an absence of T and NK cells, as observed in the founder *IL2RG*-KO cloned pigs (Fig. 2*A*). Deficiency of the thymus was also confirmed through autopsy of full-term fetuses (Fig. 2*B*). The *IL2RG*-KO F_2 males were prone to infection and suffered from severe complex infections by 3 wk after birth (Fig. S5 *C–E*).

These data clearly demonstrated that $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric boars can serve as stud males to transmit X-linked mutant genes to progeny while also providing valuable mutation-bearing sperm for cryopreservation, thereby playing a pivotal role in the reliable propagation of XLGD models.

Discussion

Diverse types of disease model pigs have been generated in recent years following the development of genome-editing technologies (22–24). The direct injection of genome-editing molecules into zygotes (25–27) is a feasible strategy for generating genetically engineered pigs. This method cannot, however, exclude the birth of offspring with unfavorable mutations (25, 28, 29). This implies the production of unnecessary animals for research (Fig. S6), which should be avoided from an animal welfare point of view.

Alternatively, the production of gene KO pigs via nuclear transfer of somatic cells carrying a mutation in a specific gene is a reliable and efficient strategy (5, 29, 30). Using this method, animals with the most appropriate mutation type can be exclusively generated by analyzing nuclear donor cells before producing cloned offspring. The production of cloned pigs provides a proof of concept for the outcome of a targeted gene mutation in a straightforward fashion. However, the symptoms that appear in cloned gene KO pigs can be influenced by epigenetic dysregulation (31), which is prone to arise in cloned animals. In this regard, a cloned pig with a mutant disease-causing gene is often flawed as a disease model.

Thus, the generation of disease model pigs through somatic cell cloning (i.e., via asexual reproduction) implies one obvious drawback. In addition, disease model pigs should preferably be reproduced via practical and feasible procedures, such as natural mating, in vitro fertilization, and artificial insemination. Such sexual reproduction systems using a stud male or his sperm have proven highly efficient for proliferating valuable animals throughout the history of animal husbandry.

In the development of an XLGD model, however, it is difficult to generate a healthy, fertile boar (9, 10). Therefore, the generation of a fertile chimeric boar that produces germ cells carrying the mutation(s) responsible for an XLGD has significant implications for the production and promotion of disease model pigs. Cryopreserved sperm carrying the mutant gene enable on-demand reproduction of the models (Fig. S6), thereby easing the maintenance of valuable lines, compared with the cost of maintaining females with a heterozygous mutation ($X^{KO}X^{WT}$). Furthermore, female germ cells (oocytes) of disease model pigs with juvenile/ premature lethality may be produced by blastocyst complementation using $NANOS3^{-/-}$ host embryos, which are characterized by depletion of germ cells in the ovary (32).

In model pigs for XLGDs, somatic cell-cloned females with $X^{KO}X^{WT}$ mutations often exhibit lethal phenotypes similar to those of $X^{KO}Y$ males (8). This effect is ascribed to skewed X inactivation (33) in the cloned animals and, therefore, complicates the maintenance of cloned $X^{KO}X^{WT}$ females as founders of an XLGD model (8). In any case, maintaining a line of large
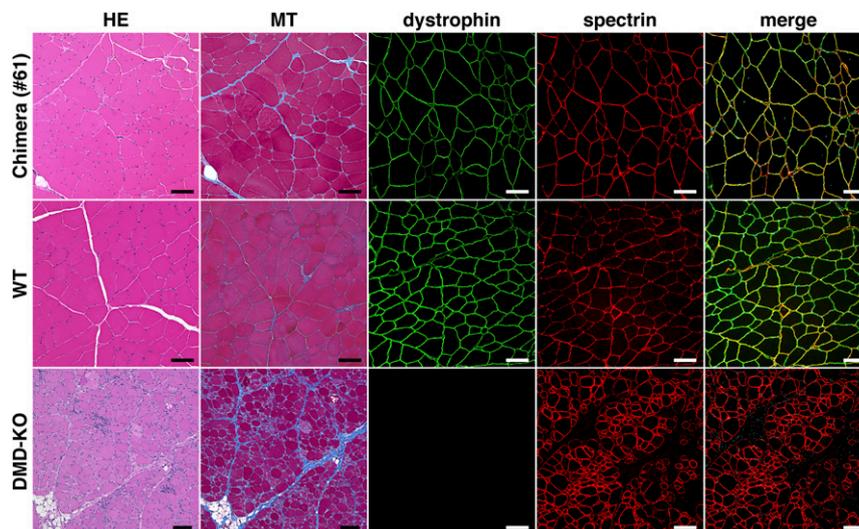
Fig. 3. Histological features of the muscle tissue of the *DMD* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimeric pig. Histological and immunofluorescence analysis of the biceps femoris muscle tissue of the chimeric, WT, and *DMD*-KO pigs. HE, hematoxylin and eosin staining; MT, Masson's trichrome staining. HE and MT staining indicated that a large portion of the muscle tissue of the *DMD* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimera was composed of relatively uniform muscle fibers compared with the tissue of an age-matched WT pig. Histological analysis of the *DMD*-KO clones revealed regressive changes in the muscle tissue, including excessive variation of fiber diameter and regeneration/necrosis of fibers, as shown in our previous study (10). Immunofluorescence analysis demonstrated an extensive distribution of dystrophin-positive fibers in the muscle tissue of the *DMD* $X^{KO}Y \leftrightarrow X^{WT}X^{WT}$ chimera, although the fibers were accompanied by slight variations in size. (Scale bars: 100 μm.)

animals such as pigs using females as breeding stock is too costly, regardless of the survivability of the $X^{KO}X^{WT}$ females (Fig. S6).

OTCD is the most common congenital urea cycle disorder in humans (12). Hyperammonemia in infants causes ataxia, lethargy, and death in severe cases. Furthermore, persistent hyperammonemia in a patient gives rise to intellectual impairment (12). Liver transplantation is the only available curative treatment for this disease. Hence, OTCD model pigs would be a useful research tool not only for developing treatments before liver transplantation but also for investigating novel medical technologies, such as regenerative therapies, to replace liver transplantation. Pathogenesis in women carrying a heterozygous *OTC* mutation is also a clinically serious issue (12). Chimeric boars harboring *OTC* $X^{KO}Y$ germ cells allow the large-scale production of $X^{KO}X^{WT}$ progeny and will therefore have a significant impact on research on the etiology of mutation-carrying females.

X-linked SCID is the most common form of primary immunodeficiency, occurring in one of 50,000–100,000 human births (34–36). This disease is lethal without appropriate treatment, such as hematopoietic stem cell transplantation, due to severe infections, including opportunistic infections. Without appropriate treatment, affected individuals typically die in the first year of life (34, 37). SCID model pigs would be useful for developing therapies for this life-threatening disease. SCID pigs would also be valuable as a large animal model for transplantation studies involving human pluripotent stem cells (PSCs) and/or PSC-derived organoids. In the present study, we generated $X^{KO}X^{KO}$ progeny by mating a chimeric boar carrying IL2RG $X^{KO}Y$ germ cells with IL2RG $X^{KO}X^{WT}$ carrier females. The $X^{KO}X^{KO}$ females may serve as a SCID model.

DMD is the most common form of progressive muscular dystrophy, affecting ~1 in 3,000 boys (38). Patients exhibit progressive proximal muscle weakness of the legs and pelvis in infancy and degeneration of heart and respiratory muscles at a later age, which can cause death (39). Next-generation therapies, including gene editing, exon-skipping drugs, gene therapy, and cell transplantation therapy, are considered promising remedies for this disease (40–42). In this context, the establishment of an effective system for producing DMD model pigs using chimeric boars containing *DMD* $X^{KO}Y$ germ cells will have a significant impact.

In a previous study by our group (10), cloned *DMD*-KO pigs died by 3 mo of age. In the present study, the normal growth of the chimeric boar to adulthood proved that the chimerism induced by exogenous normal cells cured the muscle dysfunction of the DMD pig, at least to some extent. However, the insufficient recovery of muscle function in pigs with lower chimerism may suggest limitations of cell transplantation therapy for DMD.

In contrast, *mdx* mice exhibiting milder DMD symptoms have been rescued by systemic chimerism (15). The symptoms of the *mdx* mice were also improved via i.p. transplantation of encapsulated Sertoli cells (43). Pig models with more severe DMD symptoms are tremendously useful for developing feasible therapies.

Rescuing embryos carrying lethal genetic mutations through the induction of chimerism using normal cells has been reported to give rise to individuals with normalized traits (14, 18, 44). For instance, injecting Rag-2–deficient mouse blastocysts with normal ES cells led to the generation of somatic chimeras with normal B and T lymphocytes (14). The apancreatic phenotype of *Pdx1*-KO mice has also been rescued via chimerism using blastocysts injected with ES/iPS cells (44), and the lethal apancreatic trait of *Pdx1-Hes1* transgenic pigs was shown to be complemented by chimerism with WT cells (18). In the present study, we demonstrated a state-of-the-art embryo technology that rescues the lethal traits of XLGD model pigs and enables the transmission of the mutant gene to subsequent progeny. This chimeric rescue technology, which enables sexual reproduction of mutant cloned pigs produced via asexual somatic cell nuclear transfer (SCNT), will play a pivotal role in expanding the practical applications of disease model pigs.

Table 3. Generation of F$_2$ progeny from *OTC*-$X^{KO}X^{WT}$ and *IL2RG*-$X^{KO}X^{WT}$ F1 females

F$_1$ females*	$X^{KO}Y$	XY	$X^{KO}X$	XX
OTC-$X^{KO}X^{WT}$	18 [22.5%]	22 [27.5%]	20 [25.0%]	20 [25.0%]
IL2RG-$X^{KO}X^{WT}$	5 [16.7%]	9 [30.0%]	9 [30.0%]	7 [23.3%]

*Eight (*OTC*-$X^{KO}X^{WT}$) and three (*IL2RG*-$X^{KO}X^{WT}$) females were mated with WT boars.

Materials and Methods

Detailed description of TALEN design/preparation, OTC-KO cell preparation, mutation analysis, production of cloned embryos/pigs, chimera pig production by blastocyst complementation, and analysis of chimera/cloned pigs are provided in *SI Materials and Methods*.

Animal Care. All of the animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC11-0016, 12-0008, 14-0010, 15-0002, 15-0003).

Statistical Analysis. Statistical analyses were performed with SPSS software (version 23; IBM). Differences in proportional data between the two groups were analyzed with the chi-square test. Numeric data, including body weight

and biochemical parameters, were analyzed with Student's t test or Dunnett's T3 test. The level of significance was set at $P < 0.05$.

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