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ORIGINAL ARTICLE

Genetic diversity, growth and heart function of Auckland Island pigs, a potential source for organ xenotransplantation

Andreas Lange[1,2] | Ivica Medugorac[3] | Asghar Ali[1,2] | Barbara Kessler[1,2] | Mayuko Kurome[1,2] | Valeri Zakhartchenko[1,2] | Sabine E. Hammer[4] | Andreas Hauser[1] | Joachim Denner[5] | Britta Dobenecker[6] | Gerhard Wess[7] | Paul L. J. Tan[8] | Olga Garkavenko[8] | Bruno Reichart[9] | Eckhard Wolf[1,2,10] | Elisabeth Kemter[1,2]

[1]Chair for Molecular Animal Breeding and Biotechnology, Gene Center and Department of Veterinary Sciences, LMU Munich, Munich, Germany

[2]Center for Innovative Medical Models (CiMM), LMU Munich, Munich, Germany

[3]Population Genomics Group, Department of Veterinary Sciences, LMU Munich, Munich, Germany

[4]Institute of Immunology, Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria

[5]Institute of Virology, Free University of Berlin, Berlin, Germany

[6]Chair for Animal Nutrition, Department of Veterinary Sciences, LMU Munich, Munich, Germany

[7]Clinic of Small Animal Medicine, Center for Clinical Veterinary Medicine, LMU Munich, Munich, Germany

[8]NZeno Limited, Auckland, New Zealand

[9]Walter-Brendel-Center for Experimental Medicine, LMU Munich, Munich, Germany

[10]Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, LMU Munich, Munich, Germany

Correspondence
Eckhard Wolf and Elisabeth Kemter, Chair for Molecular Animal Breeding and Biotechnology, Gene Center and Department of Veterinary Sciences, LMU Munich, Munich, Germany.
Email: ewolf@genzentrum.lmu.de and kemter@genzentrum.lmu.de

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Abstract

One of the prerequisites for successful organ xenotransplantation is a reasonable size match between the porcine organ and the recipient's organ to be replaced. Therefore, the selection of a suitable genetic background of source pigs is important. In this study, we investigated body and organ growth, cardiac function, and genetic diversity of a colony of Auckland Island pigs established at the Center for Innovative Medical Models (CiMM), LMU Munich. Male and female Auckland Island pig kidney cells (selected to be free of porcine endogenous retrovirus C) were imported from New Zealand, and founder animals were established by somatic cell nuclear transfer (SCNT). Morphologically, Auckland Island pigs have smaller body stature compared to many domestic pig breeds, rendering their organ dimensions well-suited for human transplantation. Furthermore, echocardiography assessments of Auckland Island pig hearts indicated normal structure and functioning across various age groups throughout the study. Single nucleotide polymorphism (SNP) analysis revealed higher runs of homozygosity (ROH) in Auckland Island pigs compared to other domestic pig breeds and

Eckhard Wolf and Elisabeth Kemter share equal last author contribution.

demonstrated that the entire locus coding the swine leukocyte antigens (SLAs) was homozygous. Based on these findings, Auckland Island pigs represent a promising genetic background for organ xenotransplantation.

KEYWORDS
Auckland Island pigs, genetics, growth, heart, PERV, xenotransplantation

1 | INTRODUCTION

Cardiac xenotransplantation has made significant progress, demonstrating consistent long-term survival of genetically multi-modified (GM) pig hearts in both heterotopic abdominal[1] and orthotopic transplantations in baboons.[2–4] Recently, compassionate clinical xenotransplantations involving 10× GM pig hearts were performed in two patients with terminal heart diseases. The patients survived for 8 and 6 weeks, respectively.[5–7] In addition to appropriate genetic modifications to overcome xenograft rejection and coagulation dysregulation (reviewed in refs. [8, 9]), other important steps for successful cardiac xenotransplantation involve perfusion preservation of the pig hearts,[10] non-nephrotoxic immunosuppression of the recipients involving T cell co-stimulation blockade ([1]; reviewed in ref. [11]), and controlling the post-transplantation growth of the pig hearts (reviewed in refs. [9, 12]).

The size of the xenotransplant is of importance for its survival and functionality in the recipient.[13] Post-transplantation heart growth can be regulated by early weaning of glucocorticoids, lowering the recipient's blood pressure, and using the rapamycin prodrug temsirolimus. This drug blocks the activation of the mechanistic target of rapamycin (mTOR) and prevents cardiomyocyte hypertrophy.[2] A genetic strategy to reduce the growth of the donor pigs and their hearts is the inactivation of the growth hormone receptor gene (*GHR*-KO).[14,15] This modification, in conjugation with other genetic modifications, significantly prolonged the survival of orthotopic cardiac xenografts in baboons.[4] *GHR*-KO was also one of the modifications in the 10x GM donor pigs used in the already mentioned clinical compassionate use cardiac xenotransplantations.[5,7] However, *GHR*-KO may induce various physiological changes in the donor pigs, such as juvenile hypoglycemia,[16] pronounced obesity,[14] alterations in multiple metabolic pathways of the liver,[17] and structural, proteomic, and functional changes in the anterior pituitary gland.[18] Notably, proteomic analysis of the hearts of *GHR*-KO pigs revealed only marginal changes compared to wild-type littermates.[15] Nevertheless, using a donor pig breed with organ sizes comparable to humans is preferable compared to the larger pig breeds, whose body weights and organ sizes exceed those of humans by several-fold.

GM Yucatan minipigs have been utilized for preclinical organ xenotransplantation trials,[19–21] but this breed is prone to an increased occurrence of ventricular septum defects.[22,23] In addition, Yucatan minipigs are known for high expression and release of PERV.[24] An alternate option is the use of the Auckland Island pig, a breed of feral domestic pigs that originated on the Auckland Islands. In 1807, Captain Abraham Bristow first released these pigs on the Auckland Islands, with at least two subsequent releases until the mid-19th century (reviewed in ref. [25]). In 1999, the Rare Breeds Conservation Society of New Zealand removed 17 feral pigs from the main Auckland Island (Figure 1A). Analysis of mitochondrial D-loop DNA from a subset of these animals suggested their European origin.[26] Wild-type Auckland Island pigs are free of numerous viruses[27] and animals maintained under designated pathogen-free (DPF) conditions already served as donors for clinical encapsulated islet xenotransplantation trials[28,29] with no indication of transmission of infectious agents to the recipients.[30,31] Due to their appropriate size for humans, they are also good candidates as donors for organ xenotransplantation. Here, we analyze the genetic diversity, SLA haplotypes, growth, and heart size and function of a small colony of Auckland Island pigs established at the Center for Innovative Medical Models (CiMM), LMU Munich, Germany.

2 | MATERIALS AND METHODS

2.1 | Import of Auckland Island pigs and establishment of a breeding herd

Primary kidney cell lines were established at NZeno Limited from a male (M1) and a female (W1) piglet previously selected to be free of porcine endogenous retrovirus C (PERV-C) using standard procedures.[32] Cryopreserved cells were shipped to LMU Munich, where they were thawed and used for somatic cell nuclear transfer (SCNT).[33] Cloned embryos were laparoscopically transferred to estrous synchronized recipient gilts. Pregnancy diagnosis was done by ultrasound 3 weeks after embryo transfer and subsequently at regular intervals. Animals were maintained under specific-pathogen-free (SPF) conditions in the CiMM (www.lmu.de/cimm).[34] They were housed in groups, separated by sex, and kept under a 12-h light/dark cycle. They were fed a custom-made diet tailored to their needs (Table S1), designed by the Chair of Animal Nutrition and Dietetics, LMU Munich. All experiments were performed after the permission of the Government of Upper Bavaria under file name ROB-55.2-2532.Vet_02-19-195.

2.2 | AO blood group and SLA genotyping

Determination of AO blood group was performed by multiplex polymerase chain reaction (PCR). Alleles of SLA class I and II genes were determined by Sanger sequencing of PCR products using cDNA of

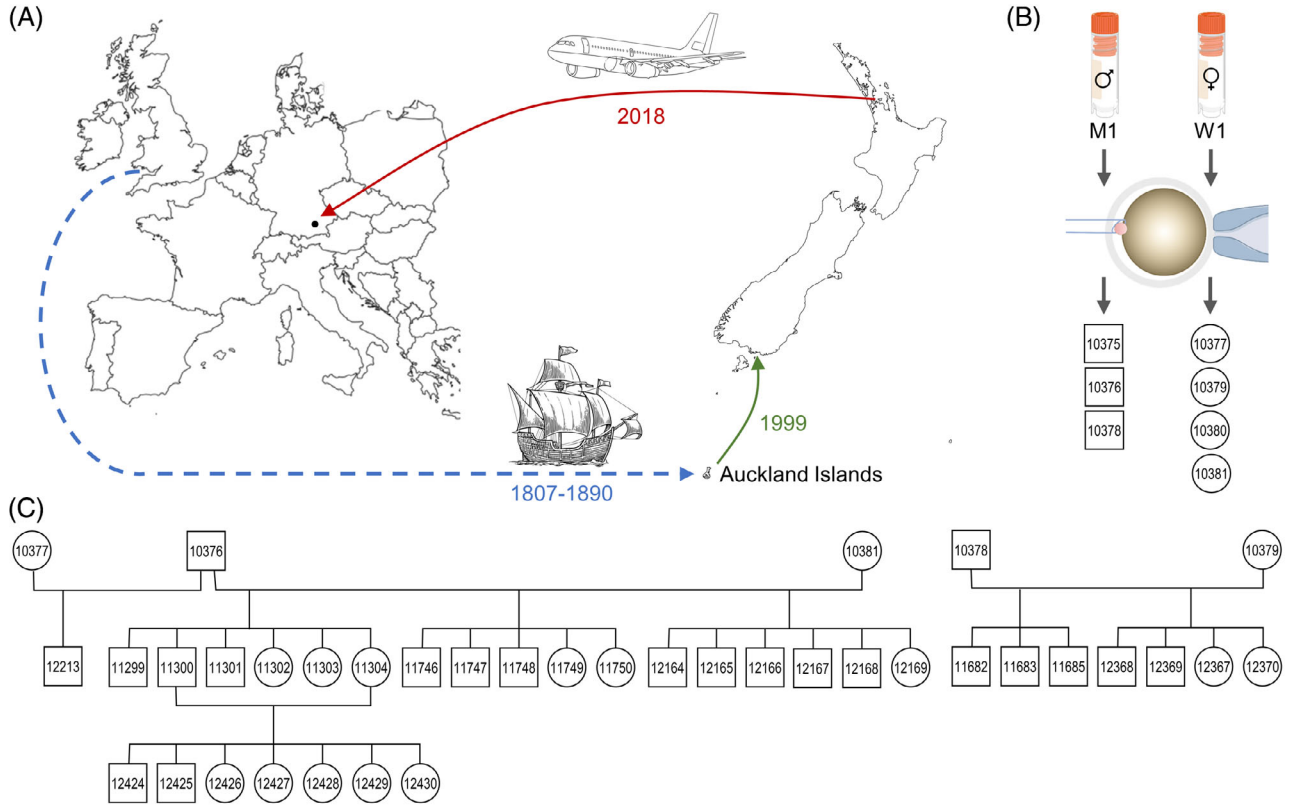

FIGURE 1 Establishment of an Auckland Island pig breeding colony in Munich, Germany. **(A)** History of the transfer of Auckland Island pigs to the Auckland Islands, from there to Invercargill, and of cells to Munich. **(B)** Establishment of male and female founder pigs from a male and a female PERV-C free Auckland Island pig cell line by somatic cell nuclear transfer (SCNT). **(C)** Pedigree of the animals used for phenotypic characterization.

PBMCs and/or spleen and heart tissue as templates. Primers were designed first in conserved regions based on alignments of all known alleles in the Immuno-Polymorphism Database (IPD)-MHC database (https://www.ebi.ac.uk/ipd/mhc/group/SLA/), and if indicated in allele-specific regions (primers listened in Table S2).

2.3 | Genome-wide homozygosity analysis

To investigate the length and distribution of runs of homozygosity (ROH) in Auckland Island pigs, we performed SNP genotyping using the PorcineSNP60 v2 BeadChip, which contains 64 232 SNPs covering the pig genome almost uniformly. A total of 93 Auckland Island pigs, including two founder animals (M1 and W1 in Figure 1B) and their offspring (Figure 1C), were genotyped. In addition to these Auckland Island pigs, we downloaded from Yang et al.[35] the SNP genotypes of four of the world's most popular commercial breeds: Duroc, Landrace, Large-White and Pietrain. This study analyzed four Duroc, seven Landrace, two Large-White, and three Pietrain subpopulations. We pooled these and excluded animals that showed high unbiased additive genetic relatedness.[36] After iteratively excluding highly related animals (one per each highly related pair), we were left with 40 Duroc, 40 Landrace, 36 Large-White, and 25 unrelated Pietrain pigs. After the creation of "ped" and "fam" input files, ROH analyses were performed with PLINK using 47 234 SNPs informative in a global set of pig breeds. Identical parameter settings were used for Auckland Island pigs and four cosmopolitan breeds: "–homozyg-window-snp 100 –homozyg-window-het 1". To visualize the distribution of ROHs, the PLINK output file was converted into a diagram similar to a Manhattan plot, where the homozygous segments (ROH) are given the value 1 and the non-homozygous ones are given the value 0.

2.4 | Growth measurements and echocardiography

Echocardiography was performed at 8, 12, 16, 22, 30, 40, and 155 weeks of age, and the animals were weighed each time before the examination. For echocardiography, animals were anesthetized using ketamine (20 mg/kg; Ursotamin, 100 mg/mL, Serumwerke Bernburg) and azaperone (2 mg/kg; Azaporc, 40 mg/mL, Serumwerke Bernburg) and maintained using propofol (4 mg/kg/h; Propofol 2%, 20 mg/mL, MCT Fresenius). To eliminate any discomfort, meloxicam (0.4 mg/kg; Metacam, 20 mg/mL, Boehringer Ingelheim) was applied. Front and rear leg height, crown-rump length, head length, thoracic circumference, and abdominal circumference were measured and pictures were taken in right lateral recumbency on top of a reference board.

Anesthetized pigs were placed on an echocardiography examination table with a hole (Eickemeyer, Germany) in right lateral recumbency. First, a 6-channel electrocardiogram (Eickemeyer Veterinär PC-EKG,

Eickemeyer) was generated over 5 min and saved electronically. Then, transthoracic echocardiography was performed as described previously[37,38] using an Esaote MyLab X8 Vet ultrasound machine with a P 2–9 phased array probe for animals up to 15 kg and a P 1–4 phased array probe for larger animals. The probe was placed underneath the pig in the right 3rd to 5th intercostal space behind the front leg. Echocardiography was performed using the standard planes defined for dogs and cats,[39] slightly modified for use in pigs. Functional analyses were performed with the color flow (CF), pulse wave (PW), and continuous wave (CW) in tissue Doppler imaging. In addition, apical views were generated with the probe placed caudally in the 4th to 6th intercostal space.

Transesophageal echocardiography was performed with an ST2612 Phased Array TEE-Probe from Esaote in animals over 25 kg. In that case, animals were kept in the right lateral recumbent position. The aortic outflow tract and the mitral valve including the left atrium were shown and Doppler measurements of both valves were taken.

Altogether three cardiac cycles were analyzed using the internal software of the Esaote MyLab X8 Vet. Left ventricular (LV) function was obtained in B-mode, and long axis (LAX) LV dimensions were obtained in M-mode. LV ejection fraction (EF) and fractional shortening (FS) were calculated according to the Teichholz method.[40] Aortic valve and the left atrial measurements were done, and aortic valve leaflets were watched in B-mode. The dimensions of the pulmonary valve were measured in B-mode, and the flow through it was shown using pulse wave Doppler; mitral flow and aortic flows were measured in the same way.

2.5 | Blood sampling and clinical chemistry

Blood samples were collected from the jugular vein at 12, 22, 40, and 155 weeks of age. One milliliter blood was sampled in an EDTA vial (Sarstedt) and 5 mL was sampled in a serum vial (Sarstedt). EDTA-treated blood was stored at 4°C immediately after sampling for hematology analysis. Hematological parameters were measured using an Abaxis VetScan HM5. A manual differentiation of leukocytes in blood smears was performed by an experienced lab technician. For serum preparation, the blood was stored at room temperature for 20 min, and centrifuged at 1500× *g* and 4°C for 20 min. The serum was then transferred to collection tubes and stored at 4°C before analysis. The samples were analyzed for standard clinical chemical parameters with a Cobas 311 Analyser System (Roche Diagnostics International AG) and adapted test kits.

2.6 | Necropsy

Necropsies were performed immediately after the last examination at 22, 40, or 155 weeks of age. Animals still under anesthesia were injected with fentanyl (7 µg/kg; Fentadon, 50 µg/mL, Dechra), ketamine (Ursotamin, 100 mg/mL, Serumwerke Bernburg), and xylazine (Xylazine, 20 mg/mL, Serumwerke Bernburg). After 5 min, they were exsanguinated by cutting their carotid artery. Necropsy was followed by a standardized sampling protocol.[41] Thoracic and abdominal organs were weighed to the nearest gram. The heart size was measured at the left coronary from the base to the apex. The heart width was also measured at the left coronary artery from side to side. The heart circumference was measured at the base of the heart. Both ventricles were opened and the rest of the blood was removed to get an accurate weight. Tissue samples for histology were taken 2 cm above the apex and stored in 4% formaldehyde, 4% paraformaldehyde, or methacarn. The size and weight of the kidneys were measured. The kidneys were cut longitudinally and vertically to look for pathologies. Samples for histologic examination were taken 1 cm next to the hilus from the cortex and medulla.

2.7 | Data analysis

Body weight and size data, heart rate as well as echocardiographic data were analyzed using PROC MIXED (SAS 8.2; SAS Institute Inc.), taking the fixed effects of age, sex, and the interaction age*sex as well as the random effect of the individual animal into account. Heart weight and size data were analyzed using PROC GLM (SAS 8.2), taking the fixed effects of age, sex, and the interaction age*sex into account. Linear regressions and correlations between heart and body weights of male and female Auckland Island pigs were calculated using GraphPad Prism.

3 | RESULTS

3.1 | Establishment of an Auckland Island pig colony at CiMM, LMU Munich

Male and female primary kidney cell lines (M1 and W1), previously selected to be PERV-C-free, were obtained from NZeno Limited (Figure 1A). A total of 373 male and female embryos, generated by SCNT, were transferred to three recipients. Two of the recipients became pregnant and gave birth to a total of three male and four female piglets (Figure 1B). All of the seven liveborn piglets could be raised to adulthood. Two boars (#10376, #10378) and three sows (#10377, #10379, #10381) served as founders of the breeding colony at CiMM, LMU Munich (Figure 1C). These founder pigs were naturally mated to produce F1 offspring, and later an F2 generation was also produced. Two founder pigs, 25 F1 offspring (17 males, eight females), and seven F2 offspring (two males, five females) were used for phenotypic analysis (Figure 1C), and a higher number of animals was used for SNP analysis.

3.2 | Growth characteristics of Auckland Island pigs in Munich

The body dimensions of Auckland Island pigs were regularly measured as shown in Figure 2(A). The height at withers increased from approximately 30 cm at 8 weeks to approximately 70 cm at 155 weeks of

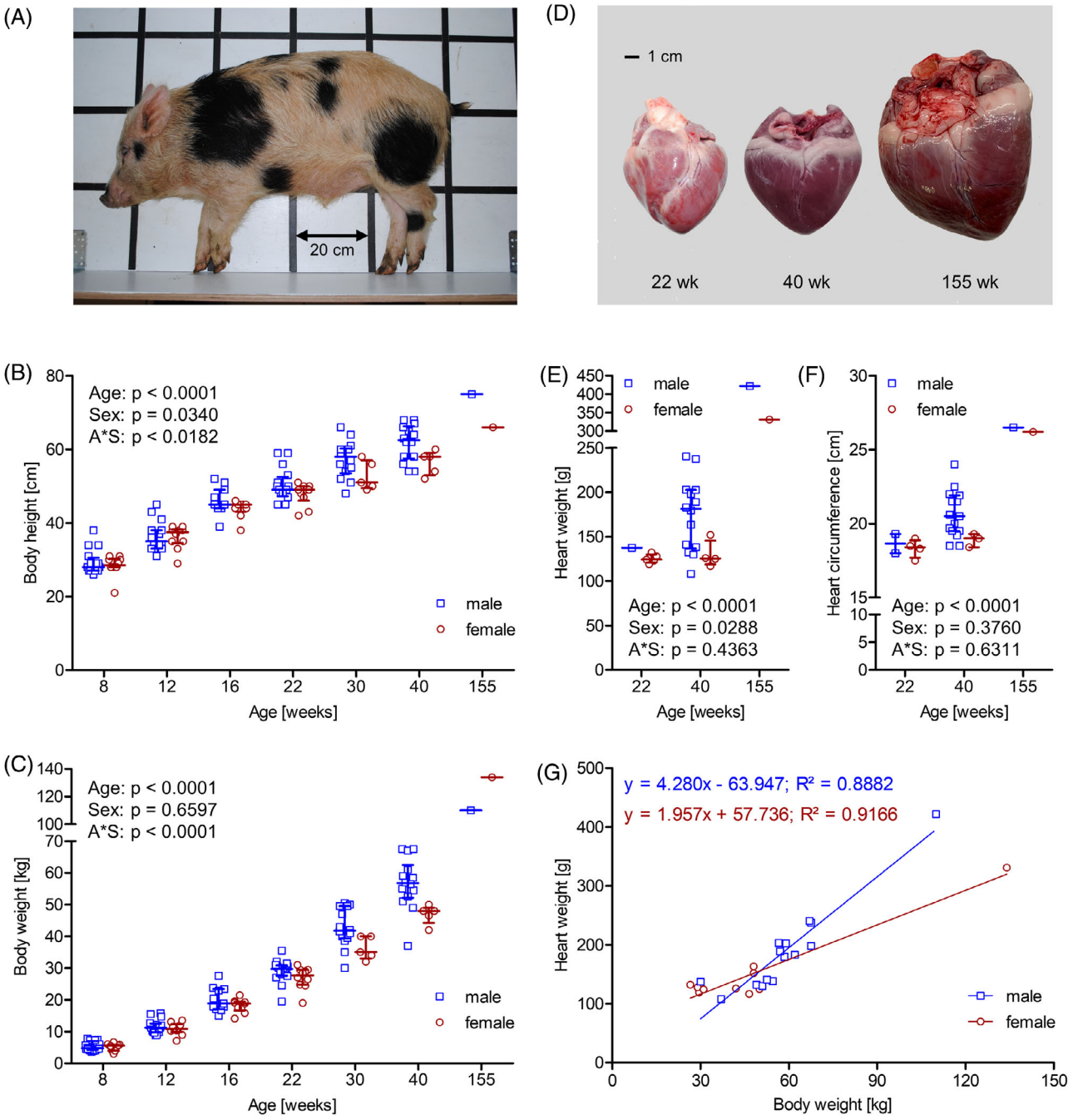

FIGURE 2 Body and cardiac growth of Auckland Island pigs in Munich. **(A)** Grid for the measurement of body dimensions. **(B)** Body height. **(C)** Body weight. **(D)** Representative hearts at different ages. **(E)** Heart weight. **(F)** Heart circumference. **(G)** Regression between body weight and heart weight. Significant effects of Age, Sex and the interaction Age*Sex (A*S) are indicated.

age. From the age of 40 weeks, females were significantly smaller than males (Figure 2B). The body weight of both male and female Auckland Island pigs increased from 3 kg to >100 kg within the same period, with males having greater body weight than females (Figure 2C). Additional parameters of body growth are presented in Table S3.

Representative hearts at 20, 40, and 155 weeks of age are shown in Figure 2(D). The heart weights ranged between 119 to 137 g in the 20-week group and between 108 to 240 g in the 40-week group. The hearts of the two 155-week-old animals weighed 331 and 422 g. The corresponding data for heart circumference are shown in Figure 2(E). The age-related increase in heart weight was correlated with body weight, with a trend of higher relative heart weights in males than in females (Figure 2F). Histologically, the hearts were free of any pathologies.

The weights of hearts, kidneys and other organs are presented in Table S4. Macroscopically, the kidneys displayed a good ratio of cortex to medulla and no cysts or signs of dilation. No histological changes were observed. Of all the animals investigated, there was one case

of pericarditis of unknown origin. Another animal had liver cirrhosis, whereas its littermates, living under the same housing conditions, showed no alterations in the liver.

3.3 | Cardiac function of Auckland Island pigs in Munich

Cardiac dimensions and functional parameters were determined by echocardiography (Figure 3A). Cardiac parameters of different age groups are summarized in Table S5. Reference values were determined by using the 95% confidence interval (CI).[42] Size-dependent parameters, such as left ventricular inner diameter in diastole (Figure 3B) and aortic diameter (Figure 3C) increased with age, with a trend of a smaller aortic diameter in females than in males. The mean left ventricular ejection fraction (LVEF) increased from 58% at 8 weeks of age to 70% in the 40-week group, with a trend of a smaller LVEF in females than males of the younger age group (Figure 3D). Other functional parameters, such as flow parameters of all valves, E and A wave, isovolumic contraction time (IVCT) and isovolumic relaxation time (IVRT) remained constant among the different age groups (Table S5). In some animals, trivial, hemodynamically insignificant tricuspid regurgitations were seen. However, no visible morphologic alterations were observed, especially no incompetencies.

3.4 | Homozygosity analysis of the Auckland Island pig colony at CiMM

A total of 93 Auckland Island pig DNA samples, including the cell lines M1 and W1, two founder animals (#10375 und #10379) and their offspring, were SNP genotyped. The SNP genotyping data from Auckland Island pigs was also compared to the above-described 47 234 SNPs identified in the global dataset from four pig breeds to estimate the expected heterozygosity and runs of homozygosity across five breeds. The total length of ROH (Mb) across the autosomal genome and their genome proportion was estimated for each animal. ROH analysis for the M1 and W1 cell lines and some chosen offspring including the ones with the lowest and highest proportion of genome in ROHs are visualized in Figure 4(A). The two relatively long chromosomes 6 (170.8 Mb) and 13 (208.1 Mb) showed only relatively short ROHs in the female (W1) and no ROHs in the male (M1) cell line. Consequently, chromosomes 6 and 13 in our Auckland Island pig colony have a very low proportion of ROHs, 4.4 %, and 6.2 %, respectively. On the other hand, the two relatively short chromosomes 17 (63.5 Mb) and 18 (55.9 Mb) have very high proportions of ROHs (94.0 % and 64.5 %, respectively) in our Auckland Island pig colony in Munich.

The simple statistics of ROHs across the breeds are shown in Table 1. Auckland Island pigs clearly showed the lowest heterozygosity (0.123), followed by Duroc (0.281), Pietrain (0.320), Large-White (0.331), and Landrace (0.336). In the 93 Auckland Island pigs analyzed here, identical haplotypes were inherited from both parents and thus form long tracts of homozygous genotypes, which on average add up to 626.2 Mb (27.6 %) over the entire genome (Table 1). This was 2.8-4.4 times higher than for the four intensively selected and economically most important pig breeds. Other ROH summary statistics such as the number of ROHs per animal and the number of SNP per ROH (Table 1) also confirm that our Auckland Island pig colony is many times more homogeneous than long-term and intensively selected commercial breeds.

3.5 | Uniformity of AO blood group and SLA genotype of Munich Auckland Island pigs

The AO blood group of Auckland Island pigs is uniformly O within the breeding herd. The SNP analysis revealed that all Munich Auckland Island pigs were also uniform in their SLAs. Of note, ROHs of SNPs were present between SSC7 19.5-30.8 Mb encompassing the 2.6 Mb SLA gene clusters region. The constellation of SLA class I and SLA class II genes is shown in Figure 4(B).

3.6 | Clinical-chemical reference values of Auckland Island pigs in Munich

The clinical-chemical reference values of all groups are presented in Table S6. Compared to reference values of German Landrace pigs,[43] Auckland Island pigs had higher creatinine kinase, hemoglobin, and hematocrit values, but lower leukocyte counts with up to 54% lymphocytes.

4 | DISCUSSION

Xenotransplantation of solid organs from pigs to humans is being explored as an alternative to human organ allotransplantation, aiming to alleviate the critical issue of transplantable human organ shortages. Substantial research has been done to overcome key challenges in organ xenotransplantation, including immune rejection due to molecular incompatibilities across species, variations in organ sizes between pigs and humans, and the potential transmission of pathogens. A widely adopted approach to tackle these challenges is the creation and utilization of genetically modified (GM) pigs for xenotransplantation. Identifying an optimal donor pig breed for organ xenotransplantation has proven difficult. In this current study, we propose that Auckland Island pigs may be a superior option for pig-to-human organ xenotransplantation.

Utilizing male and female Auckland Island pig primary kidney cell lines, we developed a breeding colony of Auckland Island pigs at LMU Munich through SCNT. All founder animals resulting from the initial SCNT (comprising three males and four females) exhibited good health and displayed no cloning artifacts, affirming the cloning efficiency of this highly inbred pig breed. By subsequent breeding of the founder animals, we successfully established F1 and F2 progenies and raised them to adulthood without encountering any significant health issues. We assessed the viability of these Auckland Island pigs as potential

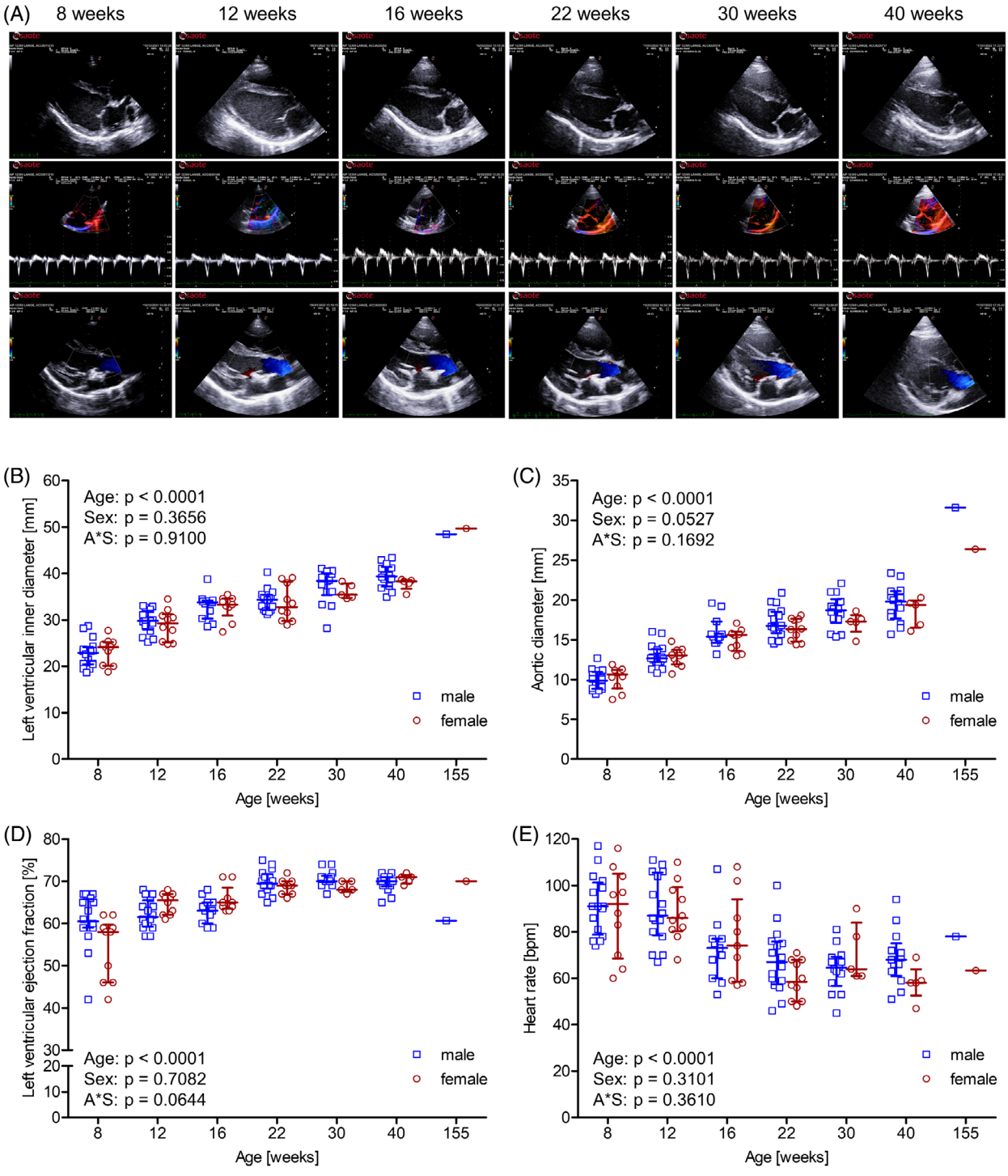

FIGURE 3 Echocardiography of Auckland Island pigs in Munich. **(A)** Representative longitudinal echocardiography images of animal #12369 at the right parasternal four chamber long axis view (upper panel), tissue Doppler images of lateral mitral valve at apical view (middle panel), and color flow Doppler images of the aortic valve at long axis view (lower panel) at different ages as indicated. **(B)** Left ventricular inner diameter. **(C)** Aortic diameter. **(D)** Left ventricular ejection fraction. **(E)** Heart rate. Significant effects of Age, Sex and the interaction Age*Sex (A*S) are indicated.

organ donors for xenotransplantation. This involved a comprehensive analysis of their body and organ sizes, cardiac function, genetic homozygosity, pathogen level, and the presence of SLA haplotypes.

The primary objective of this current study was to delineate the growth and cardiac function of Auckland Island pigs in Munich and to assess their suitability for human transplantations. In adult humans, a normal heart weighs from 156 to 422 g in women and from 188 to 575 g in men.[44] Notably, larger domestic pig breeds, with adult weights reaching up to 350 kg, have hearts that are too big for human transplantation. To ensure the appropriateness of size, Längin et al. utilized the

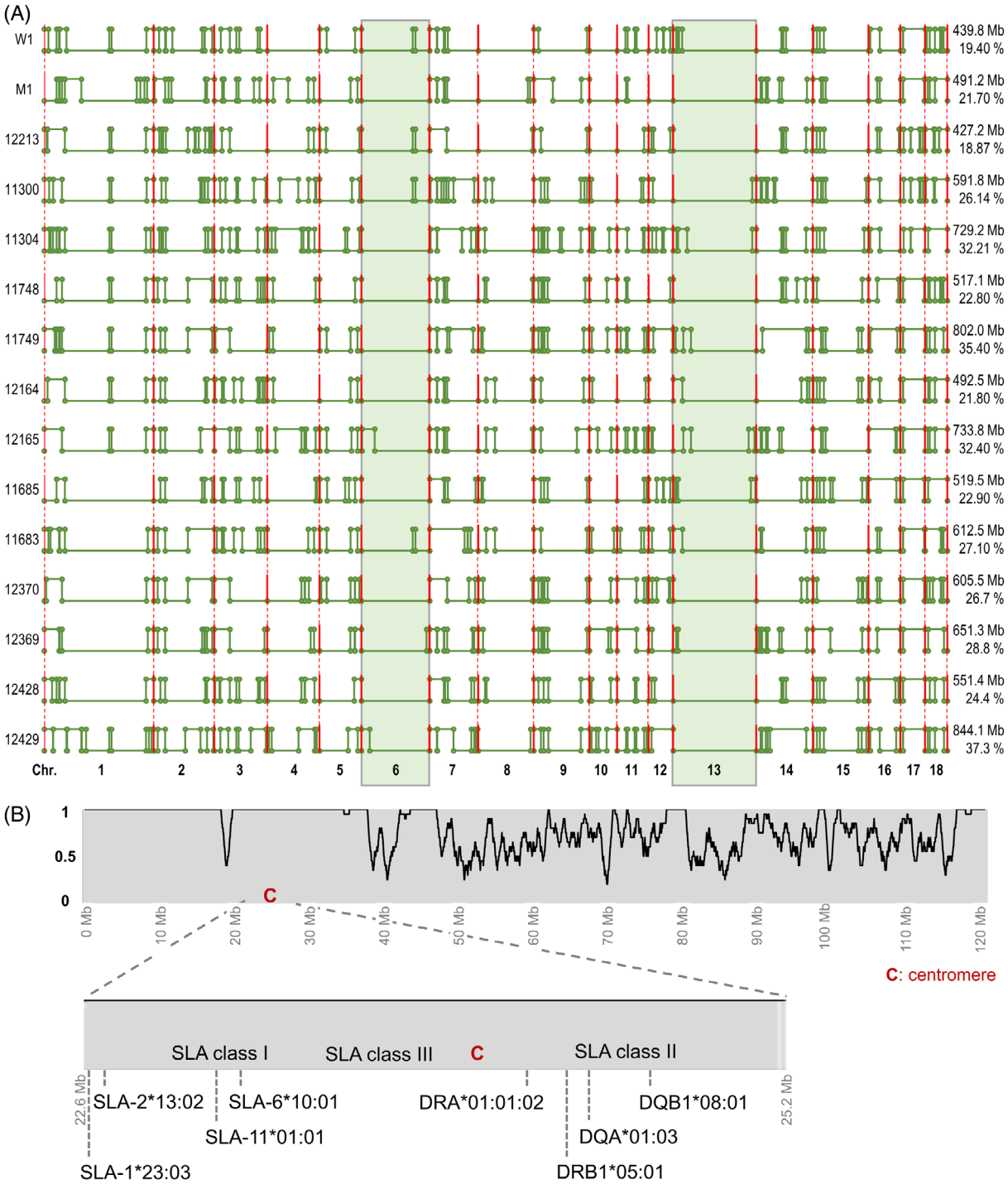

FIGURE 4 **(A)** Runs of homozygosity (ROH) analysis of the initial cell lines, the founder animals, and their offspring. A value of 0 indicates heterozygous genome segments and a value of 1 indicates homozygous segments. The chromosomes are separated by red lines, the chromosome numbers are indicated below the graph. Left site upper number: sum of all ROHs per animal (in Mb); lower number: proportion of genome in ROHs per animal. **(B)** Region of homozygosity of SNP haplotype of male and female Auckland Island pigs on SSC7 harboring the MHC/SLA locus. Haplotype identification for SLA class I alleles was done from cDNA of PBMC, for SLA class II from cDNA of PBMCs and tissue.

hearts of juvenile cross-bred pigs aged 6-12 weeks (a cross between German Landrace and Large White) for transplantation in non-human primates.[45] However, these hearts exhibited post-transplantation growth in preclinical trials, leading to graft failure within 28 days.[45] This data suggests that, even when isolated at an early age, porcine organs retain the capacity to grow in accordance with the specific pig breed. An alternative strategy to address organ size discrepancies involved knocking out the growth hormone receptor (*GHR*) gene in domestic pig breeds, resulting in smaller bodies and organ sizes suitable for human transplantation.[14,15] Nevertheless, *GHR*-KO can

TABLE 1 The simple statistics of runs of homozygous (ROH) across Auckland Island pigs and the four most popular commercial breeds worldwide.

	Auckland Island	Duroc	Landrace	Large-White	Pietrain
Number of animals	93	40	40	36	25
Average number of ROHs per animal (±SD)	**38.3 (±4.2)**	25.8 (±6.1)	16.0 (±6.6)	16.9 (±5.5)	18.2 (±5.5)
Range (Minimum-Maximum)	***30–48***	*16–46*	*1–32*	*7–32*	*10–28*
Average sum of all ROHs per animal (in Mb) (±SD)	**626.2 (±92.6)**	226.4 (±62.5)	144.0 (±76.6)	150.2 (±72.5)	165.7 (±42.9)
Range (Minimum-Maximum)	***427.9–844.1***	*107.0–384.7*	*13.5–346.7*	*44.8–384.5*	*80.7–248.0*
Number of SNP in ROHs per animal (±SD)	**15 299 (±2171)**	5485 (±1496)	3506 (±1839)	3665 (±1729)	4013 (±1017)
Range (Minimum-Maximum)	***10 331 –20 784***	*2727 – 9503*	*325 – 8190*	*1055 - 9007*	*1908 – 5829*
Proportion of genome in ROHs per animal (±SD)	**27.6 (±4.1)**	10.0 (±2.8)	6.4 (±3.4)	6.6 (±3.2)	7.3 (±2.3)
Range (Minimum-Maximum)	***18.9 –37.3***	*4.7 –17.0*	*0.6 –15.3*	*2.0 –17.0*	*3.6-11.0*

Note: Maximal values are in bold.

lead to various pathophysiological complications in donor pigs,[14,16–18] restricting the long-term viability of this approach. In another approach to address the size mismatch issues, GM Yucatan minipigs were used in preclinical organ xenotransplantation trials.[19–21] However, Yucatan minipigs were reported to exhibit a high occurrence of ventricular septum defects.[22,23] Therefore, finding a donor pig breed with organ sizes comparable to those of humans and devoid of functional defects in the organs remains a crucial objective. In the wild, adult Auckland Island boars were reported to weigh an average of 41.7 kg while adult sows weigh about 37.3 kg.[25] On the other hand, provided with a regular diet, adult Auckland Island pigs bred in Munich are similar to those bred by NZeno Limited in New Zealand where they weigh 100–140 kg for adult males and 65–104 kg for adult sows (unpublished communication). Based on our findings, the hearts of adult Auckland Island pigs aged 9–12 months demonstrate an optimal weight and size for potential transplantation into adult humans (Figure 2E, F).

Notably, no additional genetic modifications or pharmacological interventions are deemed necessary to regulate heart growth post-transplantation in humans. Furthermore, throughout the study, Auckland Island pig hearts displayed normal structure and functionality across various age groups, with no structural or functional anomalies observed. Additionally, we can efficiently predict the heart weight using 2D transthoracic echocardiography. Based on these findings, we propose that an Auckland Island pig heart is a superior option for xenotransplantation into humans, and a size-matched heart obtained from an adult Auckland Island pig is anticipated to maintain its size post-transplantation. Additionally, we have presented reference data on the functional parameters of Auckland Island pig hearts. This information will be invaluable in screening potential Auckland Island donor pigs, excluding cardiac abnormalities, and facilitating the selection of source pigs for pre-clinical or clinical trials.

Cross-species transmission of potentially zoonotic viruses during pig-to-human xenotransplantation poses a significant concern. In the first pig-to-human heart transplantation, porcine cytomegalovirus/porcine roseolovirus (PCMV/PRV) was detected in the recipient, possibly contributing to the patient's pathological condition.[5] Preclinical trials have indicated that PCMV/PRV can be transmitted to the host, leading to a reduction in xenograft survival time.[46,47] However, early weaning has been shown to eliminate PCMV/PRV.[34] In contrast, porcine endogenous retroviruses (PERVs) are integrated into the pig genome and cannot be eliminated by conventional methods.[48,49] While PERVs can infect human cells in vitro,[50] there have been no reported in vivo infections of human cells by PERVs in xenotransplantation clinical trials.[47] PERV-A and -B can infect both human and pig cells, while PERV-C infects only pig cells.[51,52] Recombination between PERV-A and PERV-C can generate PERV-AC which is 500-fold more infective than the parental PERV-A.[53] Therefore, using PERV-C-free animals is crucial to reduce the risk of PERV infection,[47] as mandated by the Food and Drug Administration (FDA) for xenotransplantation. Various strategies have been implemented to minimize the risk of PERV transmission, including using pig breeds with low or no PERV expression, employing antiretroviral drugs to prevent PERV infection, and utilizing donor pigs with PERV gene knockouts.[47] Notably, Auckland Island pigs are free from a wide range of porcine pathogens, including PERV-C.[47] In clinical trials of Auckland Island pig islet xenotransplantation, no PERVs were transmitted to the human recipients,[30,31,47] making them preferable tissue and organ donors for xenotransplantation.

The founder animals and their offspring exhibited high ROH levels, indicating a high degree of inbreeding. Surprisingly, we did not find any signs of inbreeding depression. This resilience might be attributed to a gradual rise in genomic homozygosity and the elimination of deleterious alleles through natural selection.[54] Notable exceptions were chromosomes 6 and 13, which displayed considerably lower ROH levels compared to other chromosomes. Interestingly, these chromosomes harbor genes associated with the regulation of meat quality traits[55] and characteristics such as litter size and uterine horn length,[56] respectively. A pivotal genomic region is found on chromosome 7,

harboring the major histocompatibility complex (MHC) or swine leukocyte antigen (SLA) complex.[57] SLAs are analogous to human leukocyte antigens (HLAs) and play a crucial role in the immune system. Pregnant women, individuals who have undergone blood transfusions, and transplant recipients develop antibodies against HLAs.[58] In such HLA-sensitized human recipients, anti-HLA antibodies may cross-react with SLAs on the xenograft, resulting in graft failure.[59] This underscores the significance of SLAs in the context of xenotransplantation.

Removal of SLAs from donor pigs has been considered to avoid immune rejection of the xenograft.[60,61] However, SLA deletion can render the pig at risk for infectious complications.[62] Alternatively, site-specific SLA mutations are recommended to avoid the reactivity with anti-HLA antibodies.[63] Moreover, the careful detection of anti-SLA antibodies in the xenograft recipients can assist in reducing cross-reactivity issues. Implementing these strategies requires a precise classification of SLAs in the donor pig breed. SLAs are hyperpolymorphic genes, with over 150 loci encoded in the SLA region at chromosome 7, at least 120 of which are believed to be functional.[63] SLAs are categorized into SLA class I (SLA-1, SLA-2, SLA-3), class II (DRB1, DQB1, DQA), and class III molecules. To date, at least 50 class I and 37 class II SLA haplotypes have been identified,[64] and the database continues to expand. Intriguingly, in our Auckland Island pig colony in Munich, the entire SLA region on chromosome 7 was found to be homozygous in all animals. Although MHC haplotypes could be determined for numerous SLA genes, it failed for some, such as SLA-3. This limitation might be attributed to preliminary pig genome annotations that are currently under revision for the chromosomal SLA gene complex (Sabine E. Hammer, personal communication 2024). Nevertheless, the homozygosity of SLAs in our Auckland Island pigs simplifies potential strategies to overcome SLA-mediated xenograft rejection in preclinical and clinical trials. The blood group of the donor pig is also an important consideration in xenotransplantation trials. Pigs with blood type O are preferred, as they are less likely to elicit a strong immune response in humans, particularly if the recipient's blood type is not O.[65–67] In addition to homozygous SLAs, all Auckland Island pigs in Munich possess the blood group O, reinforcing the notion that these pigs may be a superior organ source for xenotransplantation.

5 | CONCLUSION

Auckland Island pigs exhibit excellent cardiac function, and their heart size closely resembles that of humans. No pathologic alterations were observed in echocardiography, necropsy, or histologic assessments. Since Auckland Island pigs in Munich are free of PERV-C and are kept under designated pathogen-free (DPF) conditions, the risk of infection through xenografts is minimized. Moreover, Auckland Island pigs are homozygous for SLAs which simplifies further strategies to overcome rejection. They posses blood group O, naturally addressing a crucial barrier in xenotransplantation. With minimal genetic modifications, such as the elimination of carbohydrate xeno-antigens, and the expression of human complement pathway regulatory proteins and human thrombomodulin, Auckland Island pigs emerge as a promising organ source for xenotransplantation.

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CONFLICT OF INTEREST STATEMENT

Paul L. J. Tan and Olga Garkavenko are founders of NZeno Limited, Auckland, New Zealand; Bruno Reichart, Eckhard Wolf, and Elisabeth Kemter are founders of XTransplant GmbH, Starnberg, Germany.

ORCID

Sabine E. Hammer https://orcid.org/0000-0003-1475-3938
Andreas Hauser https://orcid.org/0000-0002-3997-7252
Joachim Denner https://orcid.org/0000-0003-3244-6085
Bruno Reichart https://orcid.org/0000-0003-2859-3664
Eckhard Wolf https://orcid.org/0000-0002-0430-9510
Elisabeth Kemter https://orcid.org/0000-0001-7785-7502

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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