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Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease

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Glycogen storage disease type II (GSDII; Pompe disease), caused by inherited deficiency of acid α-glucosidase, is a lysosomal disorder affecting heart and skeletal muscles. A mouse model of this disease was obtained by targeted disruption of the murine acid α-glucosidase gene (*Gaa*) in embryonic stem cells. Homozygous knockout mice (*Gaa* –/–) lack *Gaa* mRNA and have a virtually complete acid α-glucosidase deficiency. Glycogen-containing lysosomes are detected soon after birth in liver, heart and skeletal muscle cells. By 13 weeks of age, large focal deposits of glycogen have formed. Vacuolar spaces stain positive for acid phosphatase as a sign of lysosomal pathology. Both male and female knockout mice are fertile and can be intercrossed to produce progeny. The first born knockout mice are at present 9 months old. Overt clinical symptoms are still absent, but the heart is typically enlarged and the electrocardiogram is abnormal. The mouse model will help greatly to understand the pathogenic mechanism of GSDII and is a valuable instrument to explore the efficacy of different therapeutic interventions.

INTRODUCTION

Heart disease and skeletal muscle disorders are life threatening and often incurable. They have become the subject of a gamut of therapeutic studies in which animal models play a crucial role, be it for the development of drug, transplantation, enzyme or gene therapy. Here, we report on the generation of a knockout mouse model of glycogen storage disease type II (GSDII; Pompe disease) with obvious involvement of heart and skeletal muscle.

GSDII is a fatal disorder with a characteristic progressive loss of skeletal and/or heart muscle function (1). Early and late onset subtypes are distinguished. The infantile or generalized form of the disease presents in the first few months of life with cardiomyopathy and hypotonia. There is moderate enlargement of the liver, without serious impairment of liver function. Patients die before the age of 2 years by cardiorespiratory failure (2,3). Milder forms of the disease are characterized by skeletal muscle weakness in the absence of cardiac symptoms and are easily misdiagnosed as muscular dystrophy or limb girdle dystrophy (4). Most patients become wheelchair bound and/or dependent on artificial ventilation. There is no effective therapy for any form of this disease.

GSDII is inherited in an autosomal recessive mode. At the very basis of this disease are mutations in the acid α-glucosidase gene (*GAA*) (5,6) that fully or in part prohibit the biosynthesis of acid α-glucosidase and thereby the degradation of lysosomal glycogen. The clinical phenotype is largely determined by the combination of mutant alleles and the resulting level of residual acid α-glucosidase activity (7,8). Infantile patients show virtually no activity, whereas older and more mildly affected patients have enzyme activity levels up to 25% of the control value (8).

The extent of lysosomal glycogen storage is concordantly different. Infantile patients have massive storage in many tissues including heart, skeletal muscle, smooth muscle, liver, kidney and the peripheral and central nervous system (1,3,9–12). In late onset GSDII, there is minimal to no storage of glycogen in tissues other than skeletal muscle (13).

Despite the fact that this disease has been known for many years, questions concerning its pathogenesis have remained. This was one reason for us to invest in the generation of a laboratory mouse model. A second reason was the need to have a versatile animal model for testing the efficacy of innovative therapeutic developments such as enzyme and gene therapy.

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RESULTS

Targeted disruption of the *Gaa* gene and generation of *Gaa* (–/–) mice

The rationale for making a mouse model of GSDII via targeted disruption of the acid α-glucosidase (*Gaa*) gene is the 84% identity of the murine and human acid α-glucosidase amino acid sequences (14) indicating conservation of enzyme function. For this purpose, a 6.8 kb *Asp*718 fragment containing exon 5–14 of the murine *Gaa* gene was isolated from a 129 genomic library. To disrupt the murine *Gaa* gene, a *neo* cassette was inserted in the unique *Eag*I site in exon 13 of the genomic fragment. The *tk* cassette for counter selection was positioned at the 5′ end. Both cassettes are orientated in the same direction as the *Gaa* gene (Fig. 1A). The construct was introduced into the E14 embryonic stem (ES) cell line by electroporation, and 137 clones were picked after positive and negative selection. Twenty-two clones were positive for homologous recombination according to PCR analysis as described in Materials and Methods (targeting frequency of 16%). Seven of these were tested further by Southern blotting, after *Nco*I digestion. The blot was hybridized with the *neo* sequence as probe resulting in a 2.6 kb fragment indicative of homologous recombination (data not shown). The clones were karyotyped. Three clones were euploid and had correct homologous recombination. Two of these (15.1D and 17.5D) were injected into blastocysts. Six chimeric mice were identified by coat colour, five males and one female. The males were mated with C57BL/6 and FVB females. Four males appeared to be germline transmitters. The heterozygous offspring were intercrossed, and their offspring were tested by PCR analysis with primers m8–m9 and nested PCR to discriminate between homozygous knockout (–/–), heterozygous knockout (+/–) and wild-type (+/+) offspring (Fig. 1B). Genotyping of 274 offspring from heterozygous crosses revealed a frequency of 22.6% for homozygous knockout mice compatible with the expected Mendelian frequency (25%). The α-glucosidase knockout mice, both females and males, are fertile and produce normal litter sizes. No differences were observed between mice derived from the two different clones or the different inbred strains.

Analysis of gene products of GSDII knockout mice

No correct *Gaa* mRNA is produced by cultured cells of homozygous knockout mice as illustrated by reverse transcription-PCR analysis using primer set m8–m4 (Fig. 1C). Instead, there is an abnormal messenger in which acid α-glucosidase sequences are linked to sequences of the *neo* cassette (Fig. 1C; primer set n2–m4). RT-PCR with primers RT2 in exon 4 and n1 in the *neo* cassette revealed expression of a correctly spliced recombinant messenger (data not shown). For each RT reaction, the amplification of α-actin mRNA was performed as control. In accordance with the absence of a normal acid α-glucosidase transcript, the enzyme activity is virtually fully deficient in the cultured cells and in the organs of knockout mice, the exception is the intestine due to the sucrase–isomaltase activity in this tissue (Table 1). Cells and tissues of heterozygous mice have intermediate activity levels. The data in Table 1 are activities measured with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside (4MU) at a suboptimal pH of 3.6 (instead of 4.3) to reduce the interference of neutral α-glucosidase isozymes. The deficiency of acid α-glucosidase in organs of homozygous knockout mice was confirmed by Western blotting. Figure 1D shows expression of a 65 kDa acid α-glucosidase (arrow) in all tissues of wild-type mice and an additional 70 kDa form in skeletal muscle, brain and intestine. No trace of acid α-glucosidase is detectable in tissues of homozygous knockout mice, while the method is sensitive enough to detect 3% of wild-type activity. The cultured cells are also devoid of acid α-glucosidase, as demonstrated by immunofluorescence microscopy (Fig. 2A and B).

Table 1. Acid α-glucosidase activity in tissues of wild-type, heterozygous and knockout mice

Tissue	Acid α-glucosidase activity (nmol 4MU/mg/h)		
	Wild-type	Heterozygote	Knockout
Cultured cells	129.0	73.2	2.5 (1.9)
Liver	29.2	13.7	0.6 (2.0)
Spleen	7.4	3.5	0.2 (2.6)
Kidney	31.9	19.5	0.6 (1.9)
Intestine	70.1	53.0	51.9 (74.0)
Thymus	9.8	3.4	0.3 (2.1)
Lung	8.4	3.0	0.3 (3.6)
Heart	5.6	1.8	0.2 (3.8)
Triceps	8.7	4.7	0.2 (1.8)
Femoral muscle	7.8	4.5	0.2 (2.6)
Sural muscle	6.2	4.8	0.2 (2.6)
Tongue	10.3	6.3	0.1 (1.3)
Cerebellum	39.6	17.7	0.1 (0.4)
Cerebrum	47.0	21.0	0.2 (0.5)

Enzyme activity was assayed in cell lysates and tissue homogenates with the artificial 4MU substrate at pH 3.6 and expressed per mg protein. Measurements were performed in duplicate, and figures represent the mean of two independent experiments. The figures in parentheses are the activities in knockout mice as a percentage of wild-type mice.

Phenotype of the GSDII knockout mice

Clinical signs. At birth, the homozygous knockout mice were indistinguishable from their normal littermates in terms of physical appearance. Up until the present time (9 months after birth), this situation has not changed. The mice still lack overt clinical symptoms, but there is evidence of a developing cardiomyopathy. Several knockout mice had an abnormal electrocardiogram at 32 weeks of age with a high voltage QRS complex, as seen in human infantile GSDII (not shown). Radioscopy revealed cardiac enlargement which was confirmed at autopsy (Fig. 3). The heart of a knockout mouse (right) is round, swollen and pale compared with the smaller, oval and darker stained heart of an unaffected littermate (left). A dilatation of both ventricles was observed in cross-section. The pale colour of the heart was described earlier for Corriedale sheep with GSDII (15). The condition is called round heart disease in affected Nicholas turkeys (16).

Tissue pathology. Animals were sacrificed at 8 days, 6 weeks and 13 weeks after birth to search for pathological changes. No macroscopic abnormalities of organs were noted at these time points, but microscopical changes were seen early after birth. At 8 days, the femoral muscle is morphologically unaffected (Fig. 2C; stained with methylene blue), but some muscle fibres contain groups of

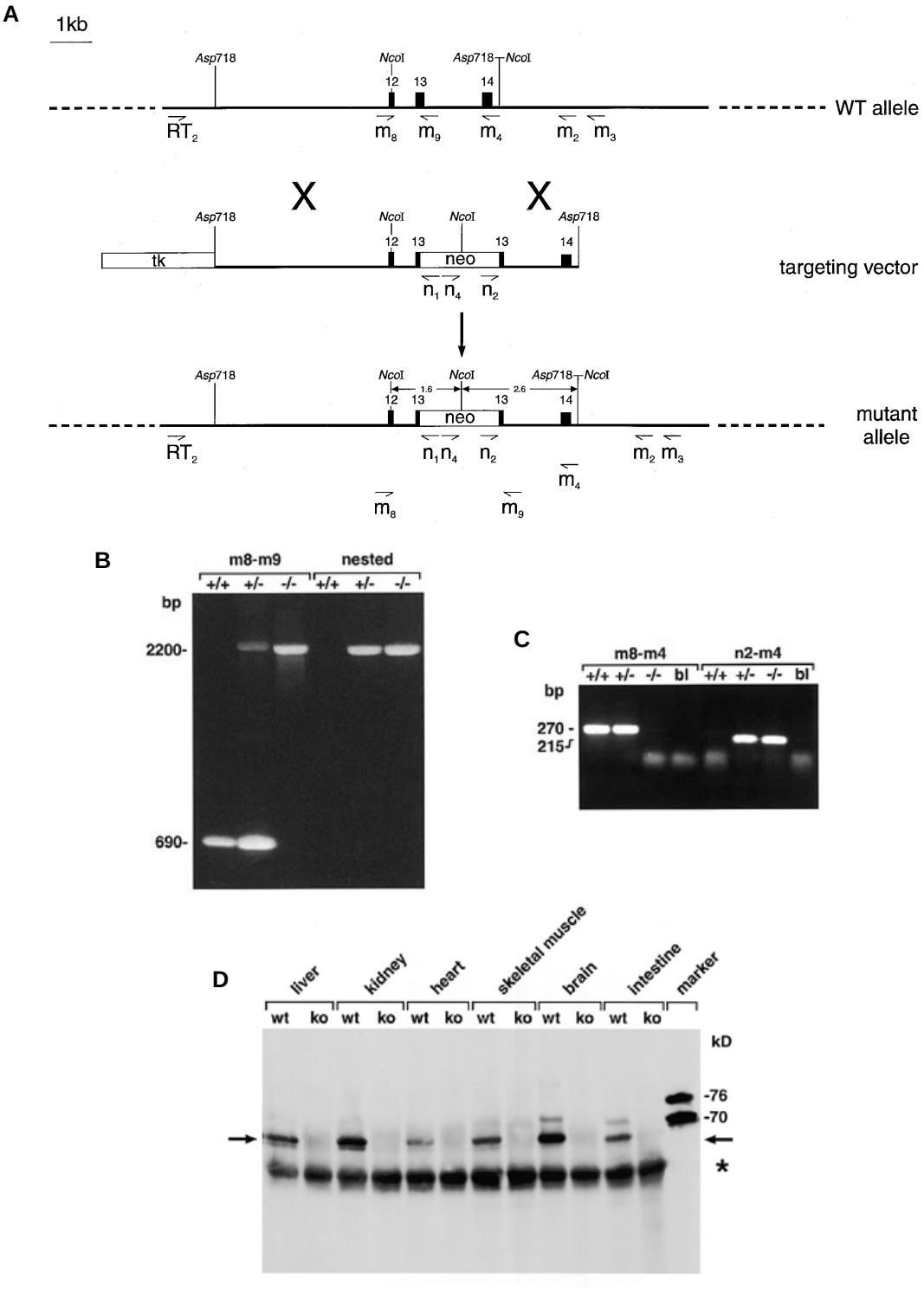
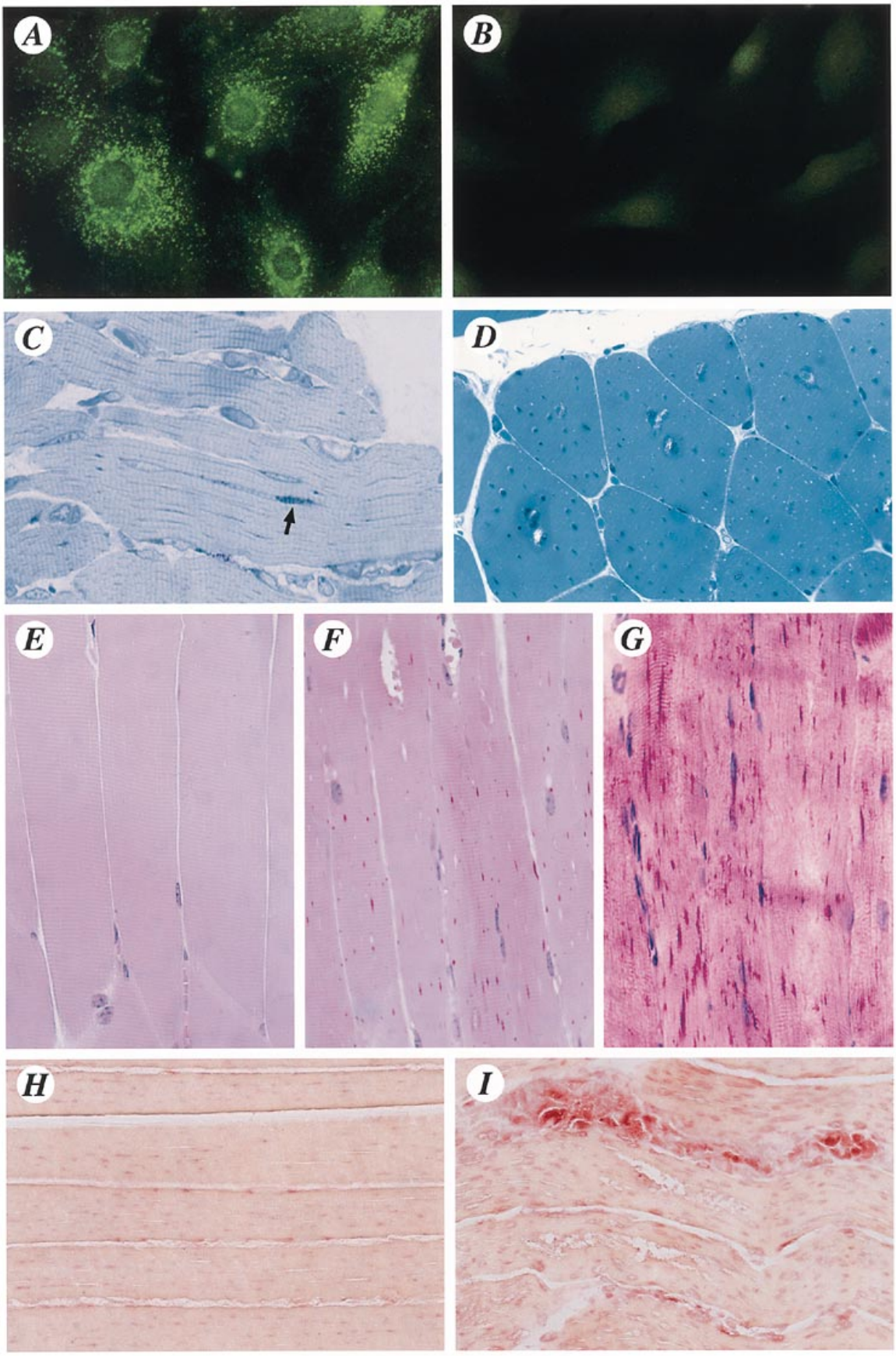


Figure 1. Generation of α-glucosidase-deficient mice by homologous recombination. (**A**) Partial structure of the murine *Gaa* gene (top), the targeting vector (middle) and the predicted structure of the homologous recombined locus (bottom). *Asp*718 and *Nco*I are the relevant restriction sites. The primer positions are indicated. (**B**) Analysis of genomic DNA from tail. The left panel with the primer combination m8–m9 discriminates between wild-type (+/+), heterozygous (+/–) and homozygous knockout (–/–) mice, containing respectively only the wild-type (690 bp), the wild-type and targeted (2200 bp) and only the targeted allele. The nested PCR (right panel) with n4–m3 as the first and n2–m2 as the second primer set identifies the targeted allele (2200 bp) in the heterozygous (+/–) and homozygous (–/–) knockout mice. (**C**) RT-PCR analysis of mRNA extracted from cultured cells of wild-type (+/+), heterozygous (+/–) and homozygous knockout (–/–) mice using primer sets m8–m4 and n2–m4. The first primer set shows the absence of normal mRNA in homozygous knockout mice (–/–), the second the presence of a fusion product of *neo* and acid α-glucosidase sequences. (**D**) Western blot analysis of *Gaa* expression. Acid α-glucosidase was immunoprecipitated from tissue homogenates with rabbit anti-human acid α-glucosidase antibodies and applied to SDS–PAGE. After subsequent blotting, acid α-glucosidase was visualized with the same antibodies and chemiluminescence staining. Human placental acid α-glucosidase was applied as molecular marker. Arrows indicate the position of (cross-reacting) murine acid α-glucosidase in wild-type (wt) tissues. Tissues of knockout mice (ko) are deficient. The immunoglobulin used for immunoprecipitations stains in all lanes (*).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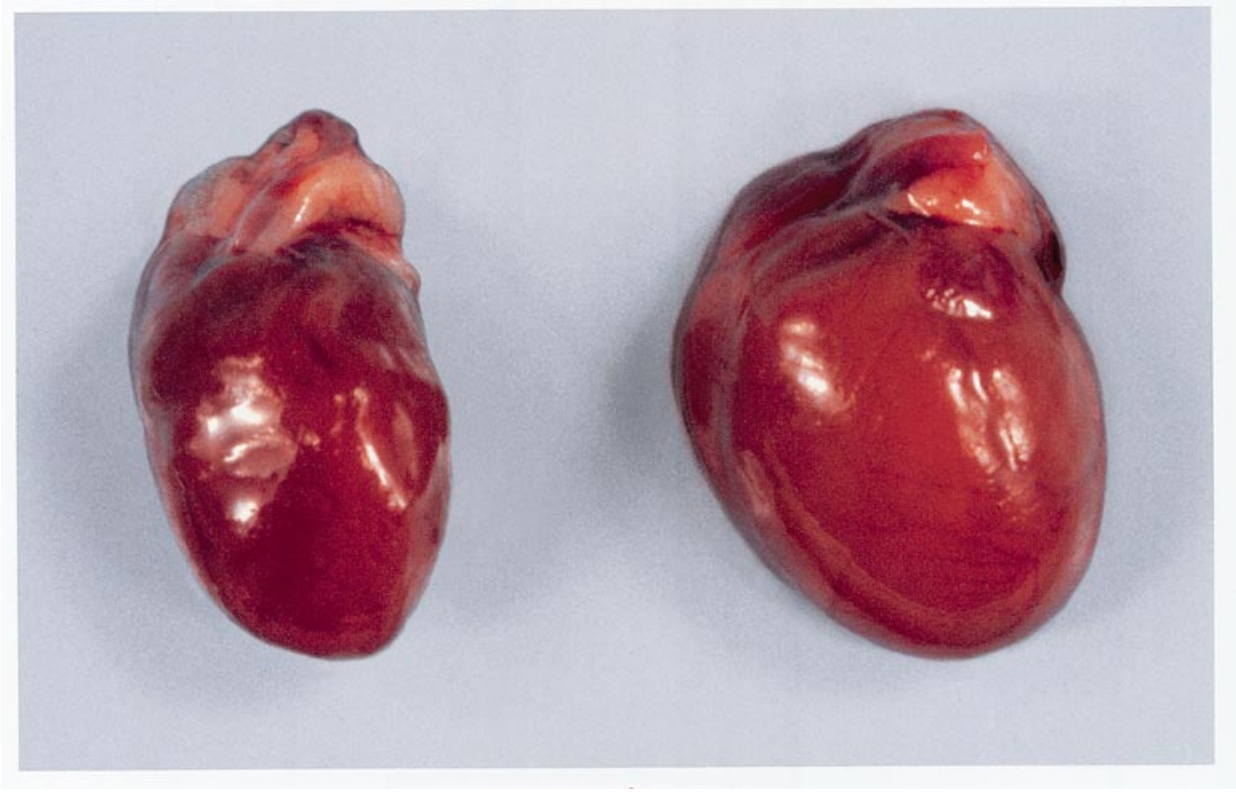

Figure 3. Comparative morphology of the hearts of wild-type (left) and knockout (right) littermates at 32 weeks of age. Note the ventricular hypertrophy and the pale colour of the heart of the afflicted animal.

dark granules (arrow) not present in muscle fibres of wild-type mice of the same age. Similar granules were observed by Cardiff *et al*. (9) in muscle of a patient with GSDII. Conclusive information on the lysosomal accumulation of glycogen was obtained by electron microscopy. Figure 4A shows glycogen deposits surrounded by a membrane in femoral muscle of 8-day-old mice. At 6 weeks after birth, the majority of fibres contains numerous glycogen deposits, as illustrated in cross-section (Fig. 2D). Large vacuoles are seen in the centre of some fibres. Electron microscopy performed at 6 weeks confirms this picture (not shown). The progressive nature of glycogen storage is also illustrated in Figure 2F and G showing femoral muscle stained with periodic acid–Schiff (PAS) reagent. Arrays of PAS-positive material are observed 6 weeks after birth, in between and parallel to the fibrils (Fig. 2F). At 13 weeks of age, the PAS-positive arrays are more numerous and start to form a continuity along the fibrils. Muscle fibres from wild-type animals do not stain with PAS (Fig. 2E).

As another sign of progressive cellular pathology, the muscle fibres of the homozygous knockout mice become positive for acid phosphatase, a lysosomal marker with increased activity in various lysosomal storage disorders and commonly used as diagnostic marker for GSDII (13). Figure 2I illustrates this phenomenon at 13 weeks of age, but it is already notable at 6 weeks. Similar observations were made in sural muscle, triceps and skeletal muscle of the tongue.

Pathological signs are also manifest in heart muscle of knockout mice. At 8 days after birth, small fields with glycogen-containing lysosomes are observed by electron microscopy (Fig. 4B and C). Loss of organized structure of heart muscle cells is seen at 13 weeks of age in knockout mice (Fig. 5B) compared with wild-type (Fig. 5A). Large vacuoles have formed inside the cells. The vacuoles are PAS positive, indicating that they are filled with glycogen (Fig. 5C), and acid phosphatase-positive (red colour), indicating that they are actually lysosomes (Fig. 5D).

Other tissues and cells also show lysosomal glycogen storage. This was observed in liver of 8-day-old mice using transmission electron microscopy (not shown). PAS-positive granules were seen in smooth muscle cells of blood vessels (Fig. 5E), in Schwann cells (Fig. 5F) and in anterior horn cells (Fig. 5G).

DISCUSSION

Animal models of human diseases have proven their value for obtaining insight into pathogenic mechanisms and for testing therapeutic drugs and innovative treatment protocols. It was for use in these two applications that we set out to generate a mouse model of GSDII by targeted disruption of the acid α-glucosidase

Figure 2. Cultured cells from the tail of wild-type (**A**) and knockout mice (**B**) stained for the presence of acid α-glucosidase using rabbit anti-human acid α-glucosidase antibodies. (**C**) Methylene blue staining of a semi-thin (1 μm) longitudinal section of femoral muscle of an 8-day-old knockout mouse. (**D**) Same staining of a semi-thin cross-section of the same type of muscle of a knockout mouse at 6 weeks of age. (**E** and **F**) PAS staining of longitudinal section of GMA-embedded femoral muscle of a wild-type (E) and knockout mouse (F) at 6 weeks of age. (**G**) PAS staining of a longitudinal cryo-section of femoral muscle of a 13-week-old knockout mouse. (H and I) Acid phosphatase staining of the same muscle of wild-type (**H**) and knockout (**I**) mice at 13 weeks of age.

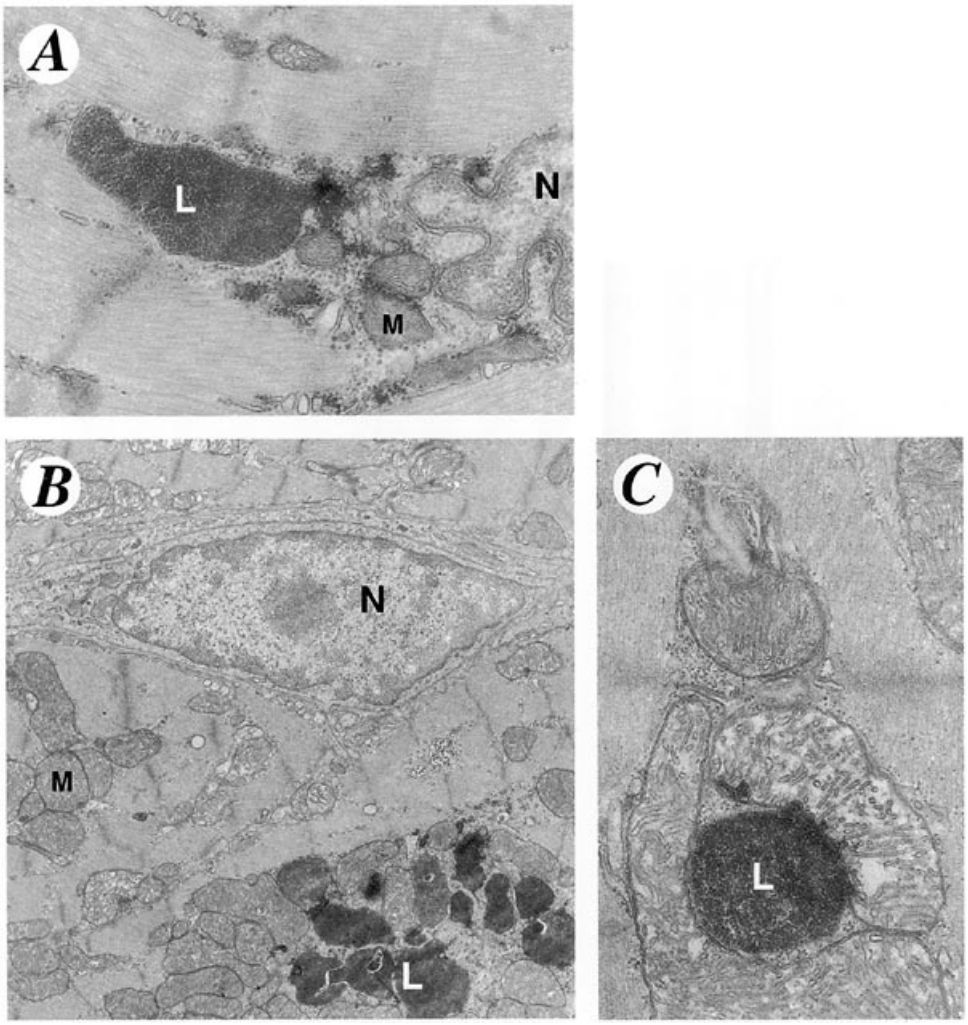

Figure 4. Electron micrographs showing lysosomal glycogen storage in an 8-day-old knockout mouse. (**A**) Femoral muscle fibre. (**B** and **C**) Heart muscle fibre. Glycogen particles are dark and densely packed in lysosomes, and freely dispersed in cytoplasm. (L) lysosomes, (M) mitochondria, (N) nucleus.

gene. The naturally occurring animal models of GSDII are less suitable for this purpose because of the physical dimension of the animal, the long generation time and the small littersize (cattle) (17–19) or the evolutionary distance from humans (quail) (20). Strains of sheep (15), dogs (21,22), cats (23) and turkeys (16) with the disease have to our knowledge not been established.

Evidence that the knockout mouse described in this study stands as a model for human GSDII is based on the following findings. Analysis of genomic DNA demonstrates the presence of the disrupted *Gaa* gene and the absence of the normal gene. The normal messenger is not present either. Instead, there is a messenger in which *neo* sequences are linked to acid α-glucosidase sequences. Furthermore, tissues and cultured cells of homozygous knockout mice are fully deficient in acid α-glucosidase as judged by immunoblotting or immunocytochemistry. Acid α-glucosidase activity is profoundly deficient in all organs except intestine. The residual activity in the latter organ is explained by the presence of the intestinal enzymes sucrase and isomaltase, which share structural homology with acid α-glucosidase. They act on the same artificial substrate with maximal activity at neutral pH, but exhibit residual activity at pH 3.6 (24). Heterozygous knockout mice have 50% of the normal acid α-glucosidase activity, as expected for an autosomal recessive trait. The best evidence that this knockout mouse represents a model of human GSDII is the progressive lysosomal glycogen storage and the ensuing cellular pathology.

There are several arguments to classify this mouse model as representative for the early-onset infantile form of GSDII. mRNA synthesis is not detectable, the acid α-glucosidase deficiency is virtually complete and the heart muscle is evidently involved. The tissue pathology of the mice also points to infantile GSDII. Knockout mice have, from birth onwards, a generalized and progressive lysosomal storage of glycogen. At 13 weeks of age, many muscle fibres show structural abnormalities. Large PAS-positive vacuoles have formed between the contractile elements. These vacuoles are in essence expanded lysosomes as evidenced by the fact that they are acid phosphatase positive. Moreover, there is accumulation of glycogen in motor neurons and Schwann cells, as only observed in tissue specimens of patients with infantile GSDII (11–13). However, in contrast to most babies born with infantile GSDII, the knockout mice do not develop overt clinical symptoms before adulthood.

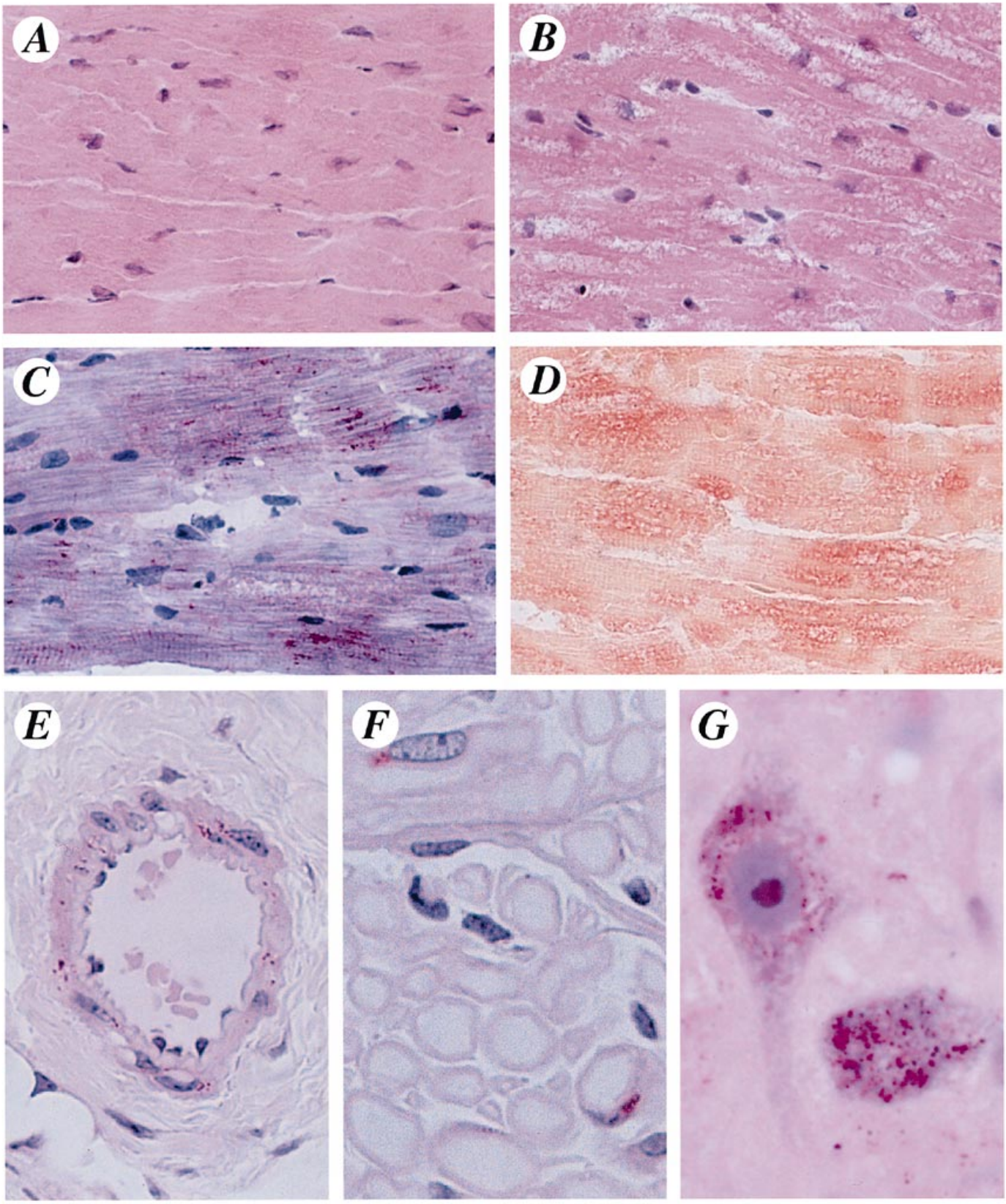

Figure 5. Tissue pathology and glycogen storage in knockout mice. (**A–D**) Heart muscle of 13-week-old mice. Haematoxylin–eosin staining shows fibre damage in tissue of the knockout (B), but not the wild-type (A) mouse. Vacuoles in between the muscle fibres of the knockout mouse are PAS- (C) and acid phosphatase- (D) positive. (**E–G**) Smooth muscle cells of blood vessels at 6 weeks of age (E), Schwann cells of peripheral nerves at 6 weeks of age (F) and anterior horn cells at 3 weeks of age (G) also contain PAS-positive granules.

The knockout mouse model resembles in biochemical, pathological and clinical aspects the naturally occurring animal models of GSDII. Afflicted Brahman and Shorthorn cattle (17–19), Corriedale sheep (15), Lapland dog (21,22,25), Japanese quail (20,26,27) and Nicholas turkey (16) have both skeletal muscle and heart muscle involvement, like the knockout mice. Also, lysosomal glycogen storage in neurons and Schwann cells was described for all species, except turkey (28). Biochemical data are only available for cattle, dog and quail. Afflicted cattle and dogs have a complete acid α-glucosidase deficiency like the knockout mouse model (29,30). For quail, it is unclear to what extent lysosomal acid α-glucosidase activity contributes to the total measured α-glucosidase activity (31–33).

Clinical parameters are more difficult to compare due to the great diversity of the species, but it is interesting to compare the clinical course of the disease in relation to the duration of the gestation period, the time to maturity and the normal life span of the species (Table 2). There is evidently no correlation between the length of the clinical course and the time to maturity or the life span. However, there is a striking similarity between all species including humans, with regard to the length of the pathologic process, which in Table 2 is defined as the arithmetic sum of the duration of the gestation period and the life expectancy of the affected individual. Perhaps this is not completely unexpected if one considers that the pathological process starts for inherited lysosomal storage disease in principle at the (one cell) embryonic stage. If the absolute rather than the relative age of the species is counted when comparing clinical phenotypes, it can be concluded that all animal models resemble the human infantile form of GSDII. This principle may hold in a similar way for animal models of other lysosomal storage diseases, with a comparatively late onset of symptoms (34–36). For our *Gaa* knockout mouse model, it implies that clinical symptoms are likely to emerge at around 1 year of age.

Meanwhile, the mouse model can be used for the development of therapeutic regimens. Approaches to consider are diet, drugs, transplantation, enzyme and gene therapy. The combination of easy to measure glycogen concentration and acid α-glucosidase activity gives the GSDII mouse model a wider applicability. It can serve to test the effectiveness of various gene targeting vectors for diseases of heart and skeletal muscle in general.

MATERIALS AND METHODS

Construction of targeting vector

The mouse *Gaa* cDNA (GenBank accession No. U449351) was isolated from a mouse liver cDNA library using human acid α-glucosidase cDNA as probe. With the murine cDNA as probe, 11 kb of the mouse *Gaa* gene consisting of exons 3–16 was isolated subsequently from a mouse 129 genomic library. A 6.8 kb *Asp*718 genomic fragment, including exons 5–14, was then cloned into the *Kpn*I site of pBluescript with an adapted polylinker, containing *Cla*I, *Sal*I, *Kpn*I, *Cla*I, *Sac*I restriction sites. A *neo* expression cassette with a length of 1.6 kb was isolated from pPGKneopA (37) by digestion with *Eco*RI and *Xho*I, blunt ends were generated and the fragment was inserted in the blunted unique *Eag*I site in exon 13 of the *Gaa* gene. The *HSV-tk* expression cassette, isolated from pHA140 (38) by digestion with *Sal*I, was introduced in the *Sal*I site 5′ of the construct. Both cassettes were inserted in the sense orientation with respect to the transcriptional orientation of the *Gaa* gene. The structure of the targeting vector is shown in Figure 1A.

Gene targeting in embryonic stem cells

After *Cla*I digestion, the targeting construct (15–20 μg) was separated from the vector by agarose gel electrophoresis and isolated via electroelution. It was introduced into E14 ES cells by electroporation, essentially as described by Bakker *et al*. (39). Briefly, transfected cells were cultured in conditioned medium, and double selection with G418 (200 μg/ml) and FIAU (2 μM) was started 24 h after electroporation. Clones were picked 10–14 days after electroporation and screened for homologous recombination by nested PCR amplification. Two primer sets were used to amplify a 1.6 kb fragment that includes the 3′ recombination junction. The first PCR (35 cycles, each consisting of 45 s at 94°C, 1 min at 59°C and 2.5 min at 68°C) was performed with primers n4 (sense, GACGAGTTCTTCTGAGGGGATCA), complementary to a sequence in the *neo* cassette, and m3 (antisense, GCCTTCCTCATGGCCTGCTG), complementary to a sequence in exon 15 immediately downstream of the *Asp*718 restriction site. In the nested PCR (25 cycles, with the same temperature program) primers n2 (sense, GTGGGCTCTATGGCTTCTGAGG) and m2 (antisense, GCTGAACCTGTACGGCTCCTG) were used (Fig. 1A). Correctly recombined clones were karyotyped according to standard procedures and tested by Southern blotting. Genomic DNA was digested with *Nco*I and a *neo* fragment was used as probe.

Generation of chimeric and acid α-glucosidase (–/–) mice (GSDII knockout mice)

Two positive clones were injected into C57BL/6 blastocysts. Chimeric mice were bred to C57BL/6 and FVB females, and the offspring with agouti and grey coats were tested for the transmission of the disrupted allele by the PCR described above. Homozygous mice (–/–) were obtained by interbreeding the heterozygotes (+/–). DNA from the tail was analysed using a PCR (35 cycles, following the same temperature program as described above) with primer m8 (sense, CCTCACTGAAGCTATCGCCTCC) in exon 12 and m9 (antisense, GCAAGATGCTCCCAAGAGCTCC) in exon 13 in combination with the nested PCR described above.

Cell culture from murine tail

Tails were cut into small pieces, and the tissue was dissociated for 15 min at 37°C in 10 mM sodium phosphate, 150 mM NaCl (pH 7.1), containing 0.05% trypsin and 0.02% EDTA. After low speed centrifugation, a fresh solution of trypsin/EDTA was added for another 15 min. Cells and softened tissue were then resuspended in Dulbecco's modified Eagle's medium (DMEM) with 15% fetal calf serum (FCS) and antibiotics and seeded in 25 cm^2 tissue culture flask, pre-coated with FCS and 1 ml of medium. Clonal outgrowth of cells was observed 3 weeks later, and stable cell lines were obtained after 2 months of growth.

Table 2. Normal development and clinical course of GSDII in humans and in animal models

Species	Normal development			Clinical course of GSDII		
	Gestation period	Time to maturity	Average life span	Onset of clinical symptoms	Death	Duration of the pathologic process[a]
Human[b]	9 m	12–15 y	75 y	3 m	6–18 m	15–27 m
Brahman	9 m	2 y	15–20 y	3 m	8–9 m	17–18 m
Shorthorn	9 m	2 y	15–20 y	3 m	7–16 m	16–25 m
Sheep	5 m	8 m	15–20 y	<6 m	6–12 m	11–17 m
Dog	2 m	12 m	15–20 y	4 m	18 m	20 m
Mouse	0.7 m	2 m	2–3 y	>9 m	>9 m	>10 m
Quail	0.5 m	2 m	7–8 y	3–8 m	18 m	19 m

[a]Time span from the one-cell embryonic stage to death.
[b]The infantile form.
m stands for months, y for years.

Analysis of RNA

RNA was isolated from cultured cells with RNAzol· according to the manufacturer's directions (Tel-Test). cDNA synthesis was carried out using the Superscript™ pre-amplification system and random hexamer-primers (Gibco BRL). The cDNA was PCR-amplified with several primer combinations: m8 with m4 (antisense, TGGGTCCAGCGCACACACAGCT in exon 14) amplifying a fragment including the *neo* cassette in the knockout allele, n2 in the *neo* cassette in combination with m4 amplifying the 3′ junction of the *neo* cassette and the *Gaa* gene, RT2 (GAACCAGTTCCTTCAGCTGT sense in exon 4) in combination with n1 (GTGTAGCGCCAAGTGCCAGCG antisense, in the *neo* cassette). Primers AGGTCATCACCATCGGCAATG (sense) and CGTCGTACTCCTGCTTGGTG (antisense) were used for amplifying α-actin, a non-relevant control gene.

Acid α-glucosidase activity and protein assays

Mouse tissues were homogenized in phosphate-buffered saline using an ultra turrax (TP 18–10, 20 000 Upm; 170 W, Janke & Kunkel KG). After removal of the large debris at 10 000 *g* twice for 15 min, the supernatant was stored at –20°C. The supernatants were assayed for α-glucosidase activity with 4MU as substrate (40). The protein content of the supernatant was determined using the bicinchoninic acid (BCA) protein assay (Pierce).

Western blotting

Acid α-glucosidase was immunoprecipitated from the homogenates with rabbit antibodies raised against human placental acid α-glucosidase complexed to protein A–Sepharose 4B as described (41). The Sepharose beads with the enzyme–immunoglobulin complex bound to them were boiled in sample buffer and applied to 10% SDS–PAGE. The proteins subsequently were blotted onto nitrocellulose filters and visualized on these filters with rabbit anti-human placental acid α-glucosidase antibodies using the ECL detection kit (Amersham).

Immunocytochemistry

Acid α-glucosidase in cultured cells was visualized with rabbit polyclonal antiserum raised against human placental acid α-glucosidase in combination with goat anti rabbit IgG conjugated to fluorescein isothiocyanate (FITC).

Histology

For routine histological examination, tissues were either frozen in 2-methylbutane chilled by liquid nitrogen or fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer and embedded in glycolmethacrylate (GMA). Cryostat sections (6 µm) were stained with haematoxylin–eosin (HE), PAS and acid phosphatase. Alternatively, sections (2 µm) from GMA-embedded tissues were PAS stained. For electron microscopy, glutaraldehyde-fixed tissues specimens were post-fixed in 1% OsO_4 with $K_3Fe(CN)_6$ in 0.1 M cacodylate buffer according to De Bruijn (42). Standard procedures were used for epon embedding. Semi-thin (1 µm) and ultrathin sections were cut on an LKB Nova ultratome and stained with methylene blue (semi-thin) and uranylactate in combination with Sato's lead citrate (43) (ultrathin). Ultrathin sections were examined in a Philips CM100 electron microscopy.

Cardiology

Mice were anaesthetized with ether and pulsed radioscopies were made (25/s) with a Siemens radiography apparatus. A surface electrocardiogram (standard Einthoven) was obtained by subcutaneous placement of 27-gauge needles in two front and one hind limb. A 300 Hz filter was used. Data were recorded, digitized on-line using a data acquisition program (WinDaq/ex; Dataq Instrument, Inc., USA) and stored for post-acquisition off-line analysis. The mice were killed with an overdose of hypnodil (Janssen Pharmaceutica B.V.) for *ex vivo* comparison of gross cardiac morphology.

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