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Transgenic mouse expressing human mutant α-galactosidase A in an endogenous enzyme deficient background: a biochemical animal model for studying active-site specific chaperone therapy for Fabry disease

Satoshi Ishii[a,b,1], Hidekatsu Yoshioka[c], Kazuaki Mannen[c], Ashok B. Kulkarni[d], Jian-Qiang Fan[a,*]

[a]*Department of Human Genetics, Mount Sinai School of Medicine, Fifth Avenue at 100th Street, New York, NY 10029, USA*
[b]*Usuki Bio Research Center, Usuki-shi, Oita, Japan*
[c]*Oita Medical School, Oita, Japan*
[d]*National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland, USA*

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Abstract

Fabry disease is an inborn error of glycosphingolipid metabolism caused by the deficiency of lysosomal α-galactosidase A (α-Gal A). We have established transgenic mice that exclusively express human mutant α-Gal A (R301Q) in an α-Gal A knock-out background (TgM/KO mice). This serves as a biochemical model to study and evaluate active-site specific chaperone (ASSC) therapy for Fabry disease, which is specific for those missense mutations that cause misfolding of α-Gal A. The α-Gal A activities in the heart, kidney, spleen, and liver of homozygous TgM/KO mice were 52.6, 9.9, 29.6 and 44.4 unit/mg protein, respectively, corresponding to 16.4-, 0.8-, 0.6- and 1.4-fold of the endogenous enzyme activities in the same tissues of non-transgenic mice with a similar genetic background. Oral administration of 1-deoxygalactonojirimycin (DGJ), a competitive inhibitor of α-Gal A and an effective ASSC for Fabry disease, at 0.05 mM in the drinking water of the mice for 2 weeks resulted in 13.8-, 3.3-, 3.9-, and 2.6-fold increases in enzyme activities in the heart, kidney, spleen and liver, respectively. No accumulation of globotriaosylceramide, a natural substrate of α-Gal A, could be detected in the heart of TgM/KO mice after DGJ treatment, indicating that degradation of the glycolipid in the heart was not inhibited by DGJ at that dosage. The α-Gal A activity in homozygous or heterozygous fibroblasts established from TgM/KO mice (TMK cells) was approximately 39 and 20 unit/mg protein, respectively. These TgM/KO mice and TMK cells are useful tools for studying the mechanism of ASSC therapy, and for screening ASSCs for Fabry disease.

Keywords: Transgenic; α-Galactosidase A; Fabry disease; Active-site specific chaperone; 1-deoxygalactonojirimycin

1. Introduction

Fabry disease is a lysosomal storage disorder resulting from the deficient activity of lysosomal α-galactosidase A (α-Gal A, EC 3.2.1.22), an enzyme responsible for the catabolism of glycosphingolipids, predominantly globotriaosylceramide (Gb3) [1]. In classically affected hemizygotes with Fabry disease, who generally have little or no detectable α-Gal A activity, the accumulation of Gb3 in the lysosomes of endothelial cells leads to clinical manifestations that include hypohidrosis, angiokeratoma, acroparesthesia, corneal opacity, and vascular diseases of the heart, kidneys and central nervous system. Death typically occurs in the fourth or fifth decade of life as a result of renal failure, or cardiac or cerebrovascular diseases [2]. In contrast,

* Corresponding author. Fax: +1 732 745 9769.
E-mail address: jian-qiang.fan@mssm.edu (J.-Q. Fan).
[1] Current address: Department of Agricultural and Life Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080-8555, Japan.

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cardiac Fabry patients who have measurable residual enzyme activity are usually asymptomatic until their late thirties and eventually suffer from heart failure in the fifth or sixth decade of life [2]. Their clinical manifestations are often limited to the heart [3,4], and typically present with left ventricular hypertrophy [5,6]. Recently, enzyme replacement therapy has been developed and approved for the treatment of Fabry disease [7,8]. The treatment is effective in lowering the accumulation of Gb3 in kidneys and reducing pain in classically affected Fabry patients. However, reduction of the glycosphingolipid storage in the heart is rather inefficient and slow [9].

The majority of cardiac Fabry patients has missense mutations in the α-Gal A gene (GLA), although alternative splicing mutations and small deletions have also been observed [10,11]. It is hypothesized that the cardiac missense mutations cause a deficiency in enzyme activity primarily because of misfolding of the mutant enzyme in the early biosynthesis pathway and premature degradation of the protein in the endoplasmic reticulum associated degradation (ERAD) [12,13]. We recently proposed a novel therapeutic strategy for the treatment of Fabry disease using competitive inhibitors of α-Gal A as active-site specific chaperones (ASSCs) to restore the normal folding of mutant enzymes, thus preventing excessive degradation in the ERAD [13,14]. Cultivation of patients' cells with competitive inhibitors of α-Gal A (e.g., 1-deoxygalactonojirimycin, DGJ) at sub-inhibitory concentrations resulted in a substantial increase in both residual enzyme activity and the amount of cellular protein, and also accelerated transport of the mutant enzyme to the lysosomes [13]. This strategy is particularly effective in raising the level of residual enzyme activity in cells established from cardiac Fabry disease patients. In order to further evaluate the mutant enzyme rescue and pharmacodynamics of drug, an animal model expressing human mutant α-Gal A is necessary.

Mice deficient in α-Gal A (KO mice) were established by disruption of the murine α-Gal A gene [15]. Despite the accumulation of Gb3 in various tissues of the KO mice, including the kidneys, heart and liver, the mice were clinically normal and had a normal life span. These mice have served as an excellent model for the studies of enzyme replacement [16] and gene therapy [17,18], because an increase in α-Gal A activity and a reduction of Gb3 accumulation were the primary end-points of these studies. However, this mouse model is not suitable for the study of ASSC therapy, since expression of human mutant enzyme is required for such a study. Transgenic mice (TgM mice) expressing human mutant α-Gal A (R301Q) were also generated previously [19]. Since endogenous α-Gal A activity can interfere with the transgene product in the ASSC study, they are not the most suitable model either.

We report here the establishment and characterization of homozygous transgenic mice in a murine α-Gal A knock-out background (TgM/KO mice) by crossbreeding TgM mice [19] with KO mice [15]. Although TgM/KO mice do not exhibit a disease phenotype due to a high enzyme activity expression level caused by a strong β-actin promoter, these mice exclusively express human mutant α-Gal A, and serve as an excellent biochemical model for ASSC study. In addition, fibroblast lines that stably express human mutant enzyme at an elevated level were established from TgM/KO mice. These cells are useful for the study of the biological mechanism of ASSC therapy, and for the further screening of ASSCs for the treatment of Fabry disease.

2. Materials and methods

2.1. Establishment of TgM/KO mice

Fabry KO mice were generated by disruption of exon 3 of the mouse GLA at the X chromosome [15], and maintained in a C57BL/6 background. Transgenic mice expressing human R301Q α-Gal A (TgM mice) were generated by injecting DNA fragments, consisting of chicken β-actin promoter and human mutant α-Gal A (R301Q) cDNA sequences, into the pronuclei of fertilized eggs taken from superovulated C57BL/6 female mice [19]. To generate an animal model exclusively expressing human mutant α-Gal A in an endogenous enzyme knock-out background (TgM/KO mice), homozygous male TgM mice were bred with homozygous female KO mice to eliminate the endogenous GLA gene, since the mouse GLA is located on the X-chromosome.

2.2. Establishment of fibroblast lines stably expressing human R301Q α-Gal A

To obtain primary embryonic fibroblasts, day 16.5 embryos were isolated from heterozygous TgM/KO female mice mated with heterozygous TgM/KO male mice. After removal of the head and organs, carcasses were minced and dispersed into single cell suspensions in 0.05% trypsin at 37 °C for 30 min. Cells were resuspended in Dulbecco's modified Eagle's medium (DMEM, Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Mediatech) and 5 mg/ml penicillin/streptomycin (Invitrogen Corp., Carlsbad, CA), and were maintained at 37 °C, 5% CO_2. Single fibroblast colonies were selected, and these were designated as TMK cells.

2.3. Genotyping of TgM/KO mice and fibroblasts

Genotypes were determined by multiplex PCR analyses. DNA samples were prepared from ear punch samples digested with protease K. To detect the mouse GLA allele, PCR was performed using a primer set of primer 1 (sense, 5′-ATTTAGGTCCACAGCAAAGG-3′) and primer 2 (antisense, 5′-ACATACCATTCTCCAAGGAT-3′) to amplify a 178-bp fragment. To detect the knock-out construct, primer

1 and primer 3 (antisense, 5′-TCCATCTGCACGAGACTAGT-3′) were used to amplify a 400-bp fragment from the GLA KO allele [15]. PCR analysis of the mouse GLA and KO gene consisted of 30 reaction cycles of 30-s denaturation at 94 °C, 30-s annealing at 59 °C, followed by 30-s elongation at 72 °C.

To detect the transgene (human mutant α-Gal A sequence), a primer set of primer 4 (sense, 5′-GTCCTTGGCCCTGAATAG-3′) and primer 5 (antisense, 5′-TCATTCCAACCCCCTGGT-3′) was used to amplify a 231-bp fragment specific to the transgene. In order to determine heterozygositiy of the transgene in the TgM/KO mice, primers 4 and 5, which are specific to the transgene, and a primer set of primer 6 (sense, 5′-GTCCTTGGCCCTGAATAG-3′) and primer 7 (antisense, 5′-TCATTCCAACCCCCTGGT-3′) specific to an endogenous mouse Galβ1,3GalNAc-specific GalNAc α2,6-sialyltransferase (ST6GalNAc II) gene [20] were included in the same reaction to give a 231-bp band (derived from the transgene) and a 377-bp band (derived from the ST6GalNAc II gene). The PCR consisted of 28 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The amount of the transgene was estimated by comparison of its band density with that of the reference gene (ST6GalNac II).

2.4. Determination of human α-Gal A mRNA expression in TMK fibroblasts

The mRNA expression of the transgene (human α-Gal A) was determined by a competitive RT-PCR method as described previously [11]. The concentrations of total RNA samples prepared from TMK fibroblasts and human skin fibroblasts were determined by absorbance at 260 nm. DNA competitor was prepared by PCR amplification of a λ-DNA provided by the Competitive DNA Construction Kit (Takara Bio Inc., Otsu, Shiga, Japan) using sense and antisense primers of 5′-GGTTGGAATGACCCAGATAGTACGGTCATCATCTGACAC-3′ and 5′-TGCGATGGTATAAGAGCGCAGTTAATCGAACAAGAC-3′. Reverse transcription (RT) was performed with 0.3 μg of total RNA in 30 μl of the reaction mixture, using the RNA PCR Kit (Takara Bio). Aliquots (5 μl) of the RT reaction were used for PCR amplification. Target (324 bp) and competitor (275 bp) DNA fragments were amplified with primers 5′-GGTTGGAATGACCCAGATA-3′ and 5′-TGCGATGGTATAAGAGCG-3′, along with the DNA competitor at the indicated concentrations. The PCR amplification consisted of 30 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s.

2.5. Administration of DGJ to TgM/KO mice

TgM/KO mice were supplied with fresh tap water ad libitum and rodent pellets. DGJ (Toronto Research Chemicals, Toronto, Canada) was administrated to one group of male TgM/KO mice (n=3) in tap water as a 0.05 mM solution, without any other substances. The consumption of drinking water was approximately 5 ml/day/mouse, and the DGJ dosage was estimated to be approximately 3 mg/kg/day. After 2 weeks, the animals were sacrificed, and organs were quickly removed and rinsed with phosphate-buffered saline (PBS). Tissue homogenates were subjected to enzyme assay and lipid extraction.

2.6. Enzyme assay and protein determination

All samples were kept on ice and processed as rapidly as possible. Approximately 10% (w/v) tissue homogenates were prepared in water using a micro-homogenizer (Physcotron, Niti-on, Inc., Chiba, Japan). Cell pellets from culture fibroblasts (6-cm culture dish) were homogenized in water (200 μl) using the Physcotron. The supernatant obtained from the homogenate after centrifugation at 10,000×g for 5 min was used in enzyme assays.

Typical α-Gal A activity was assayed with a mixture (50 μl) of 4-methylumbelliferyl α-D-galactoside (5 mM) as substrate and *N*-acetyl-D-galactosamine (75 mM) as inhibitor for α-*N*-acetylgalactosaminidase (α-Gal B) in 0.1 M sodium citrate buffer (pH 4.6) at 37 °C for 30 min. The reaction was stopped by addition of 1.2 ml of 0.1 M glycine-NaOH buffer (pH 10.7). The released 4-methylumbelliferone was determined by fluorescence measurement at 360 and 450 nm as excitation and emission wavelengths, respectively. One unit of enzyme activity was defined as the amount of enzyme that releases 1 nmol of 4-methylumbelliferone per hour. The protein concentration was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as a standard.

2.7. Western blot analysis

Western blot analysis was performed with anti-α-Gal A antibody produced in rabbits [21] and HRP-conjugated anti-rabbit IgG antibody produced in goat (Pierce Biothechnology, Rockford, IL). Cell homogenate containing approximately 30 μg of protein was applied to a 10% polyacrylamide gel. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred electorophoretically to a PVDF (Immobilon P) membrane (Millipore, Billerica, MA) and visualized with SuperSignal® Chemiluminescent Substrate (Pierce).

2.8. Detection of neutral glycosphingolipids by TLC analysis

Neutral glycosphingolipids were extracted from mice tissue and analyzed as follows. Crude lipids were extracted from the heart (0.15 g) of a TgM/KO or KO mouse, treated with or without DGJ in drinking water (0.05 mM) for 2 weeks, using a mixture of chloroform–methanol (2:1, v/v). Glycosphingolipids were dried under a stream of nitrogen

and then subjected to mild alkaline treatment with 1 ml of 0.1 N KOH in methanol at 40 °C for 2 h. After neutralizing the solution with glacial acetic acid, glycosphingolipids were further subjected to the Folch's partition (chloroform–methanol–H_2O, 8:4:3 in v/v/v) and recovered in the lower phase, and then applied to TLC plates. TLC analyses were performed with high-performance thin-layer chromatography (HPTLC)-Silica gel 60 plates (Merck & Co., Inc., Whitehouse Station, NJ) using a solvent system of chloroform–methanol–water (60:35:8, v/v/v). Glycosphingolipids were detected by spraying orcinol–sulfuric acid reagent, followed by heating of the plate at 110 °C. The Gb3 standard purified from human erythrocytes [22] was a gift from Dr. Y. Kushi (Obihiro University Agriculture and Veterinary Medicine, Japan).

3. Results and discussion

3.1. Establishment of TgM/KO mice

The breeding strategy for the generation of homozygous TgM/KO mice is described as the follows, and their genotype was confirmed by PCR analysis. Since GLA is located on the X chromosome, male TgM mice were mated with female GLA KO mice to yield male offspring in a GLA knock-out background. The male offspring were further bred with female GLA KO mice to generate breeding pairs of heterozygous TgM/KO mice, and interbreeding of these heterozygotes provided the homozygous TgM/KO mice.

The mouse GLA gene and KO construct were detected by PCR amplification using a mixture of three primers: primer 1, a sense primer for both the mouse GLA and KO gene; primer 2, an antisense primer specific for mouse GLA; and primer 3, an antisense primer specific for the KO construct. Only a 400-bp fragment derived from the KO allele was observed in lanes 1, 2, and 3, indicating that these mice are KO mice (Fig. 1A). In contrast, only a 178-bp fragment (derived from the mouse GLA) was seen in lane 4, indicating that this mouse retains the endogenous GLA. In order to determine homozygosity of the TgM/KO mice, further PCR analyses were performed with one reaction containing primers 4 and 5 that amplify a 231-bp band from the transgene, and primers 6 and 7 that amplify a 377-bp band from a reference gene (a mouse house keeping gene, ST6GalNAc II gene) [20]. A 231-bp band in lanes 2 and 3 (Fig. 1B) indicates that these mice are transgenic. The ratio of the 231-bp band to 377-bp band is higher in lane 3, indicating that the mouse could be a homozygote. The lower ratio in lane 2 suggests that the mouse is probably a heterozygote. Homozygosity of the transgene was further confirmed by outcrossing the transgenic mice (e.g., no. 3 mouse) to non-transgenic (Non-Tg) mice, i.e., 100% transmission of the transgene to the offspring was ensured.

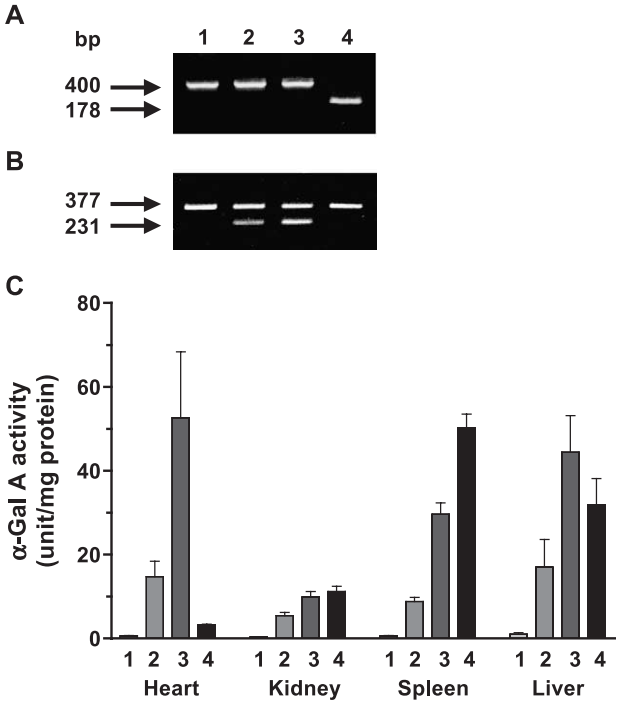

Fig. 1. Characterization of TgM/KO mice. The genotypes of mice were determined by multiplex PCR analyses for the KO construct (A) and the transgene (B). (A) Primers 1, 2 and 3 were used altogether for the PCR reaction. The 400- and 178-bp bands were derived from the KO construct and the endogenous GLA allele, respectively. (B) Primers 4, 5, 6, and 7 were used in the PCR reaction. The 377-bp band was amplified from the ST6GalNAc II gene [20] as a reference. The 231-bp band is derived from the transgene. (C) α-Gal A activity in tissues of various mice. The values are an average of three mice for each group and the standard deviations were shown as error bars. Column 1, KO mice; 2, heterozygous TgM/KO mice; 3, homozygous TgM/KO mice; and 4, Non-Tg mice.

TgM/KO mice appeared to be healthy, and had a life span similar to that of KO mice, typically 1.5 to 2 years. No abnormality could be found in their tissues, and behavior was comparable to that of Non-Tg mice. The α-Gal A activities in the major tissues of homozygous TgM/KO mice were higher than those of heterozygotes (Fig. 1C). The enzyme activities in homozygous and heterozygous TgM/KO mice were, respectively, 52.6 and 14.7 U/mg protein in the heart, 9.9 and 5.4 U/mg protein in the kidney, 29.6 and 8.8 U/mg protein in the spleen, and 44.4 and 17.0 U/mg protein in the liver. Because enzyme activities in the tissues of KO mice were minimal, the enzyme activities in TgM/KO mice were derived from the transgene. In comparison to endogenous α-Gal A activity, the enzyme activities in the tissues of homozygous TgM/KO mice were 16.4-, 0.8-, 0.6-, and 1.4-fold of those in the heart of Non-Tg mice with a similar background (3.2 U/mg protein), kidney (11.2 U/mg), spleen (50.2 U/mg), and liver (31.8 U/mg), respectively. Since TgM mice contain a single copy of the transgene [19], the relatively high level of mutant enzyme activity in TgM/KO mice is presumably caused by the strong chicken β-actin promoter, which compensates for the lower mutant enzyme activity. Enzyme activity was particularly higher in

the heart and muscles of TgM/KO mice (data not shown). Since high protein expression was also observed in the heart of transgenic mice using the β-actin promoter [23,24], this result could be attributed to the properties of the promoter, the expression of which may be favored in the heart.

3.2. Establishment and characterization of TgM/KO fibroblasts

TgM/KO fibroblasts (TMK cells) were established from the embryos of heterozygous female TgM/KO mice mated with male heterozygous TgM/KO mice. Their genotypes and α-Gal A activity of the TMK cells were determined. All cells were confirmed to be murine α-Gal A-deficient by PCR analysis using primers 1, 2 and 3 (data not shown). As shown in Fig. 2A, the amplification of a 231-bp band with DNA extracted from TMK1, TMK2, TMK4 and TMK5 cells indicates that they contain the transgene. TMK3 fibroblasts gave no 231-bp fragment, and their α-Gal A activity was minimal (Fig. 2B), indicating that these cells are derived from KO embryos. In comparison to the reference band (377 bp), the ratio of the 231-bp to 377-bp band was higher in TMK1 cells, and lower in TMK2, TMK4 and TMK5 cells (Fig. 2A). Because nonquantitative PCR analysis may cause misleading in the determination of homozygosity, the enzyme activities in these cell lines were further assayed. TMK1 cells had the highest level of α-Gal A activity (39 U/mg protein), and TMK2, TMK4 and TMK5 cells had approximate half this amount of enzyme activity (16–25 U/mg protein, Fig. 2B). These results indicate that TMK1 is a homozygous TgM/KO fibroblast line, whereas TMK2, TMK4 and TMK5 cells are heterozygous with respect to the human transgene in a knock-out background.

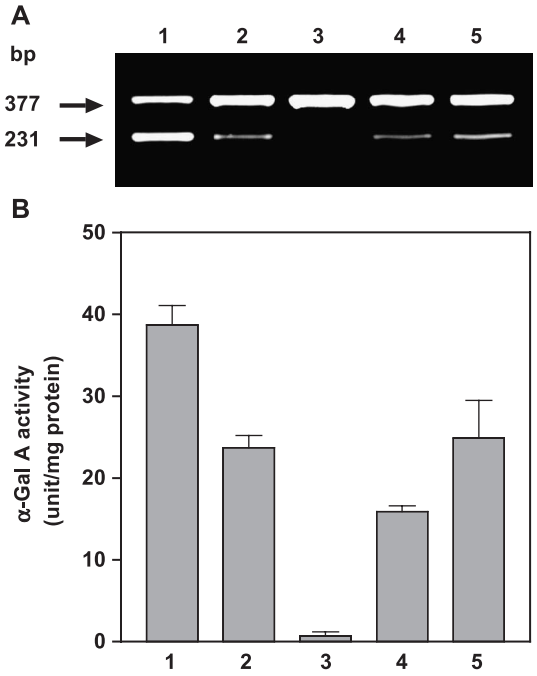

Fig. 2. Characterization of TMK fibroblasts. TMK cell lines were prepared from day 16.5 embryos interbred between heterozygous TgM/KO mice. (A) Genotyping of the transgene. Primers 4, 5, 6, and 7 were used for the PCR reaction in the same tube. The 377- and 231-bp bands were amplified from the ST6GalNAc II gene and the transgene, respectively. (B) Intracellular α-Gal A activity in the TMK cell lines. The values are an average of three samples and the standard deviations were shown as error bars. Lane and column 1, TMK1; 2, TMK2; 3, TMK3; 4, TMK4; and 5, TMK5.

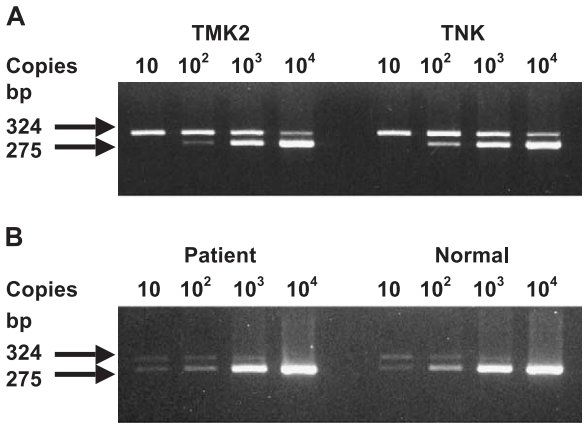

Fig. 3. α-Gal A mRNA expression in transgenic mouse and human fibroblasts. The mRNA expression levels in transgenic (A) and human skin fibroblasts (B) were determined by a competitive RT-PCR method. After reverse transcription, the target (324 bp) and competitor (275 bp) fragments were amplified in the presence of a DNA competitor at indicated concentrations. The mRNA expression level of α-Gal A was estimated from the relative intensities of target and competitor bands. (A) Transgenic mice fibroblasts heterogeneously containing the human mutant and wild-type α-Gal A gene (TMK2 cells and TNK cells, respectively). (B) Fibroblasts established from a Fabry patient with R301Q mutation and a normal subject.

Mouse fibroblasts expressing human wild-type α-Gal A in an endogenous knock-out background (TNK cells) were also established from embryos obtained by crossbreeding male homozygous human wild-type α-Gal A transgenic mice [23] with GLA KO female mice. The enzyme activity in heterozygous TNK cells was determined to be 590.3±26.0 U/mg protein. mRNA levels of human α-Gal A in TMK2 and TNK cells were determined by competitive RT-PCR, and results indicated that expressions in these two cell lines were similar (Fig. 3A), suggesting that the lower enzyme activity in TMK2 cells (approximately 5% of TNK cells) is caused by the gene mutation.

The α-Gal A activities of human skin fibroblasts established from a Fabry patient with the R301Q mutation and from a normal control subject were determined to be 2.0 and 47.6 U/mg protein, respectively. When compared to the band intensity of the DNA competitor, the human α-Gal A mRNA level in TMK2 fibroblasts and human skin fibroblasts were estimated to be approximately 500 and 50 copies, respectively (Fig. 3B). This is in agreement with intracellular enzyme activity levels, which are approximately 10-fold higher activity in TMK2 cells than in human fibroblasts.

3.3. Enhancement of α-Gal A activity in TMK cells and TgM/KO mice by ASSC treatment

ASSC therapy is designed specifically for the treatment of mutations that cause a misfolding conformation of the mutant protein during the early biosynthetic pathway [13]. In order to examine whether human R301Q enzyme activity can be increased in TMK cells by an ASSC, TMK2 cells were cultivated with DGJ at various concentrations for 4 days. The intracellular α-Gal A activity increased 16.3-, 22.4- and 22.8-fold in cells treated with DGJ at 5, 20 and 100 μM, respectively (Fig. 4A). Mutant enzyme protein was hardly detectable by Western blot analysis in TMK2 cells without DGJ treatment (Fig. 4B). Upon treatment with DGJ, however, the level of mutant α-Gal A protein was increased substantially. Although the increase in enzyme activity reached a plateau when cells were treated with DGJ at a concentration higher than 20 μM, the protein increased in a DGJ-dependent manner up to 100 μM, suggesting that enzyme activity may be inhibited by DGJ at higher concentrations.

Human fibroblasts from Fabry patients have been used in ASSC study [13, 25], but the signal to noise ratio of α-Gal A activity is rather small. Furthermore, the α-Gal A protein was hardly detectable by Western blotting, even in normal human fibroblasts. TMK fibroblasts have a high level of enzyme activity, a high amount of the mutant protein, and respond well to DGJ treatment. This makes them a suitable cell line to study the mechanism of ASSC therapy, and can be a good model for screening ASSCs for Fabry disease.

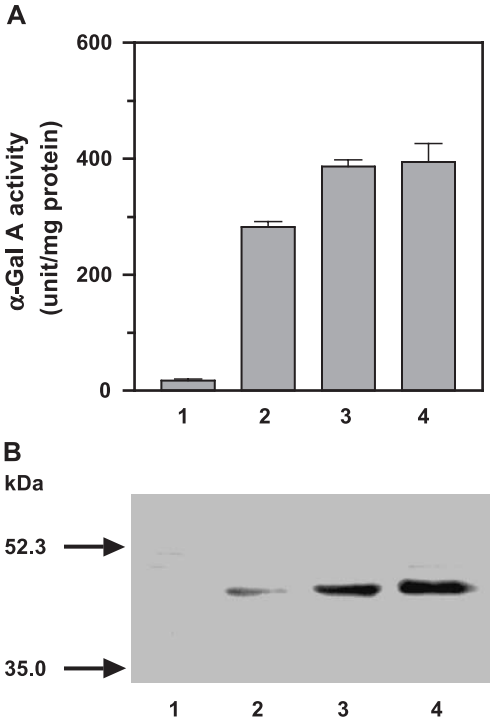

Fig. 4. Enhancement of α-Gal A activity in TMK2 fibroblasts by DGJ. TMK2 fibroblasts were cultured in the presence of DGJ at the indicated concentration for 4 days. After washing the cells twice with PBS, cells were homogenized in water. (A) Enzyme activity in cell homogenate. The values are an average of three samples and the standard deviations were shown as error bars. (B) Western blot of 30 μg of protein in the cell homogenate. DGJ concentrations are in column and lane 1, no addition; 2, 5 μM; 3, 20 μM; and 4, 100 μM.

Table 1
Effect of DGJ administration on α-Gal A activity in mice tissues

	α-Gal A activity (U/mg protein)			
	Heart	Kidney	Spleen	Liver
Control	53.6±12.1	14.7±7.8	26.0±5.6	42.9±7.7
DGJ-treated	740.4±74.2	49.0±5.0	102.4±12.7	110.8±8.5

DGJ was administered (at 0.05 mM) in the drinking water of homozygous TgM/KO mice for 2 weeks. The α-Gal A activities in tissue homogenates were determined as described in Materials and methods. The control group represents TgM/KO mice given tap water without DGJ. Data are the means±S.D. of three mice.

The effect of oral administration of DGJ in TgM/KO mice was also determined (Table 1). After oral administration of DGJ in drinking water (0.05 mM) for 2 weeks, α-Gal A activity increased 13.8-, 3.3-, 3.9- and 2.6-fold in the heart, kidney, spleen and liver, respectively. The R301Q mutation was found from late-onset Fabry patients who typically have a milder form of the disease [21]. A multifold increase in the tissue α-Gal A activity is expected to have a significant impact in the clinical development in these patients. We have used TgM mice to determine the effect of DGJ in our initial study [13]. Because endogenous enzyme activity in TgM mice is also increased slightly by DGJ treatment (data not shown), calculating the net increase in enzyme activity attributed to the transgene requires a subtraction of endogenous enzyme activity determined from

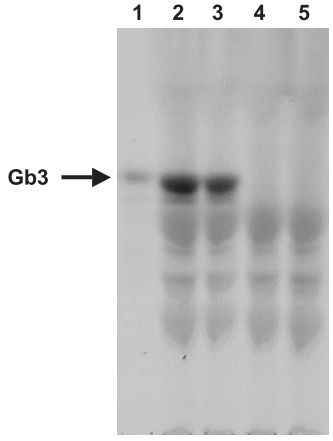

Fig. 5. Thin layer chromatogram of neutral glycosphingolipid fractions from the hearts of KO and TgM/KO mice. DGJ was administrated to KO and TgM/KO mice in drinking water (at 0.05 mM) for 2 weeks. The dosage of DGJ was estimated to be 3 mg/kg/day. Neutral glycosphingolipids were extracted from 0.15 g of heart tissues of KO or TgM/KO mice by Folch's partition. One fifth of the extraction was applied on an HPTLC plate, and developed with chloroform–methanol–water (60:35:8, v/v/v). Lane 1, Gb3 standard (8 μg); lanes 2 and 3, KO mice without or with DGJ treatment; lanes 4 and 5, TgM/KO mice without or with DGJ treatment.

a parallel study with Non-Tg mice. Since TgM/KO mice exclusively express the human mutant enzyme, they are more useful in the determining changes in mutant α-Gal A activity in the tissues of mice after drug administration.

3.4. Degradation of Gb3 was not inhibited by DGJ treatment in TgM/KO mice at the effective concentration

DGJ is an effective ASSC for the correction of α-Gal A activity in Fabry fibroblasts and lymphoblasts, and TgM/KO mice, and paradoxically, DGJ is also a potent inhibitor of the enzyme [14]. To rule out the possibility of any adverse biochemical effect caused by DGJ, we examined the amount of Gb3 (a natural substrate of α-Gal A) in TgM/KO mice treated with or without DGJ.

The accumulation of Gb3 in tissues is one of the characteristic features of Fabry disease [2]. Gb3 accumulation was observed in the heart of KO mice, however, no accumulation could be detected in the heart tissue of heterozygous TgM/KO mice (Fig. 5, lane 4), indicating that mutant α-Gal A activity (15 unit/mg protein) in the heart of TgM/KO mice is sufficient to degrade Gb3 normally. To determine whether DGJ administration could disturb the Gb3 degradation, TgM/KO mice were treated with 0.05 mM DGJ in their drinking water (corresponding to a dose of 3 mg/kg/day, the proposed efficacy dose in humans) for 2 weeks. Although the α-Gal A activity in heart homogenates of TgM/KO mice increased from 15 to 221 unit/mg protein at the end of the experiment, clearly indicating the effectiveness of the treatment, no accumulation of Gb3 could be seen in the heart of TgM/KO mice treated with DGJ (Fig. 5, lane 5). These data indicate that administration of DGJ at the proposed dosage does not cause any inhibition of Gb3 degradation, suggesting that DGJ works as an ASSC, not as an inhibitor of the enzyme in mice.

In summary, transgenic mice and cell lines that exclusively express human mutant enzyme in a murine endogenous α-Gal A deficient background were established. These mice and cells responded well to DGJ treatment, and are useful tools for the study of ASSC therapy for Fabry disease. Although the TgM/KO mice lack the disease phenotype, they are an excellent biochemical model for developing therapies for the treatment of lysosomal storage diseases. We have used TgM/KO mice to study the biochemical efficacy of DGJ for the treatment of Fabry disease (results will be reported elsewhere). The TMK cells can be used to screen ASSCs for Fabry disease, and to study the mechanism of ASSC effect on the mutant protein in mammalian cells.

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References

[1] O.R. Brady, A.E. Gal, R.M. Bradley, E. Martensson, A.L. Warshaw, L. Laster, Enzymatic defect in Fabry's disease: ceramidetrihexosidase deficiency, N. Engl. J. Med. 276 (1967) 1163–1167.
[2] R.J. Desnick, Y.A. Ioannou, C.M. Eng, α-Galactosidase A deficiency: Fabry disease, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 3733–3774.
[3] K. Ogawa, K. Sugamata, N. Funamoto, T. Abe, T. Sato, K. Nagashima, S. Ohkawa, Restricted accumulation of globotriaocylceramide in the hearts of atypical cases of Fabry's disease, Human Pathol. 21 (1990) 1067–1073.
[4] W. von Scheidt, C.M. Eng, T.F. Fitzmaurice, E. Erdmann, G. Hubner, E.G. Olsen, H. Christomanou, R. Kandolf, D.F. Bishop, R.J. Desnick, An atypical variant of Fabry's disease with manifestations confined to the myocardium, N. Engl. J. Med. 324 (1991) 395–399.
[5] S. Nakao, T. Takenaka, M. Maeda, C. Kodama, A. Tanaka, M. Tahara, A. Yoshida, M. Kuriyama, H. Hayashibe, H. Sakuraba, H. Tanaka, An atypical variant of Fabry's disease in men with left ventricular hypertrophy, N. Engl. J. Med. 333 (1995) 288–293.
[6] B. Sachdev, T. Takenaka, H. Teraguchi, C. Tei, P. Lee, W.J. McKenna, P.M. Elliott, Prevalence of Anderson-Fabry disease in male patients with late onset hypertrophic cardiomyopathy, Circulation 105 (2002) 1407–1411.
[7] R.S. Schiffmann, J.B. Kopp, H.A. Austin, S. Sabnis, D.F. Moore, T. Wiebel, J.E. Balow, R.O. Brady, Enzyme replacement therapy in Fabry disease, JAMA 285 (2001) 2743–2749.
[8] C.M. Eng, N. Guffon, W.R. Wilcox, D.P. Germain, P. Lee, S. Waldek, L. Caplan, G.E. Linthorst, R.J. Desnick, International collaborative Fabry disease study group, safety and efficacy of recombinant human α-Galactosidase A replacement therapy in Fabry's disease, N. Engl. J. Med. 345 (2001) 9–16.
[9] C.M. Eng, M. Banikazemi, R.E. Gordon, M. Goldman, R. Phelps, L. Kim, A. Gass, J. Winston, S. Dikman, J.T. Fallon, S. Brodie, C.B. Stacy, D. Mehta, R. Parsons, K. Norton, M. O'Callaghan, R.J. Desnick, A phase 1/2 clinical trial of enzyme replacement in Fabry disease: pharmacokinetic, substrate clearance and safety studies, Am. J. Hum. Genet. 68 (2001) 711–722.
[10] C.M. Eng, L.A. Resnick-Silverman, D.J. Niehaus, K.H. Astrin, R.J. Desnick, Nature and frequency of mutations in the α-Galactosidase A gene that cause Fabry disease, Am. J. Hum. Genet. 53 (1993) 1186–1197.
[11] S. Ishii, S. Nakao, R. Minamikawa-Tachino, R.J. Desnick, J.-Q. Fan, Alternative splicing in the α-Galactosidase A gene: increased exon inclusion results in the Fabry cardiac phenotype, Am. J. Hum. Genet. 70 (2002) 994–1002.
[12] S. Ishii, R. Kase, T. Okumiya, H. Sakuraba, Y. Suzuki, Aggregation of the inactive form of human α-Galactosidase in the endoplasmic reticulum, Biochem. Biophys. Res. Commun. 220 (1996) 812–815.
[13] J.-Q. Fan, S. Ishii, N. Asano, Y. Suzuki, Accelerated transport and maturation of lysosomal α-Galactosidase A in Fabry lymphoblasts by an enzyme inhibitor, Nat. Med. 5 (1999) 112–115.
[14] J.-Q. Fan, A contradictory treatment for lysosomal storage disorders: inhibitors enhance mutant enzyme activity, Trends Pharmacol. Sci. 24 (2003) 355–360.

[15] T. Ohshima, G.J. Murray, W.D. Swaim, G. Longenecker, J.M. Quirk, C.O. Cardarelli, Y. Sugimoto, I. Pastan, M.M. Gottesman, R.O. Brady, A.B. Kulkarni, α-Galactosidase A deficient mice: a model of Fabry disease, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 2540–2544.
[16] Y.A. Ioannou, K.M. Zeidner, R.E. Gordon, R.J. Desnick, Fabry disease: preclinical studies demonstrate the effectiveness of α-Galactosidase A replacement in enzyme-deficient mice, Am. J. Hum. Genet. 68 (2001) 14–25.
[17] R.J. Ziegler, N.S. Yew, C. Li, M. Cherry, P. Berthlette, H. Romanczuk, Y.A. Ioannou, K.M. Zeidner, R.J. Desnick, S.H. Cheng, Correction of enzymatic and lysosomal storage defects in Fabry mice by adenovirus-mediated gene transfer, Hum. Gene Ther. 10 (1999) 1667–1682.
[18] T. Takenaka, G.J. Murray, G. Qin, J.M. Quirk, T. Ohshima, P. Qasba, K. Clark, A.B. Kurkarni, R.O. Brady, J.A. Medin, Long-term enzyme correction and lipid reduction in multiple organs of primary and secondary transplanted Fabry mice receiving transduced bone marrow cells, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 7515–7520.
[19] M. Shimmoto, R. Kase, K. Itoh, K. Utsumi, S. Ishii, C. Taya, H. Yonekawa, H. Sakuraba, Generation and characterization of transgenic mice expressing a human mutant α-Galactosidase with an R301Q substitution causing a variant form of Fabry disease, FEBS Lett. 417 (1997) 89–91.
[20] N. Kurosawa, M. Inoue, Y. Yoshida, S. Tsuji, Molecular cloning and genomic analysis of mouse Galb1,3GalNAc-specific GalNAc a2,6-sialyltransferase, J. Biol. Chem. 271 (1996) 15109–15116.
[21] S. Ishii, R. Kase, H. Sakuraba, S. Fujita, M. Sugimoto, K. Tomita, T. Semba, Y. Suzuki, Human α-Galactosidase gene expression: significance of two peptide regions encoded by exons 1–2 and 6, Biochim. Biophys. Acta 1204 (1994) 265–270.
[22] Y. Kushi, M. Shimizu, K. Watanabe, T. Kasama, S. Watarai, T. Ariga, S. Handa, Characterization of blood group ABO(H)-active gangliosides in type AB erythrocytes and structural analysis of type A-active ganglioside variants in type A human erythrocytes, Biochim. Biophys. Acta 1525 (2001) 58–69.
[23] S. Ishii, R. Kase, H. Sakuraba, C. Taya, H. Yonekawa, T. Okumiya, Y. Matsuda, K. Mannen, M. Takeshita, Y. Suzuki, α-Galactosidase transgenic mouse: heterogeneous gene expression and posttranslational glycosylation in tissues, Glycoconj. J. 15 (1998) 591–594.
[24] H. Honda, K. Harada, I. Komuro, F. Terasaki, H. Ueno, Y. Tanaka, K. Kawamura, Y. Yazaki, H. Hirai, Heart-specific activation of LTK results in cardiac hypertrophy cardiomyocyte degeneration and gene reprogramming in transgenic mice, Oncogene 18 (1999) 3821–3830.
[25] N. Asano, S. Ishii, H. Kizu, K. Ikeda, K. Yasuda, A. Kato, O.R. Martin, J.-Q. Fan, In vitro inhibition and intracellular enhancement of lysosomal α-Galactosidase A activity in Fabry lymphoblasts by 1-deoxygalactonojirimycin and its derivatives, Eur. J. Biochem. 267 (2000) 4179–4186.