THE JOURNAL OF GENE MEDICINE
J Gene Med 2000; **2**: 233–242.

RESEARCH ARTICLE

Improvement of multiple pathophysiological phenotypes of *klotho* (*kl*/*kl*) mice by adenovirus-mediated expression of the *klotho* gene

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Received: 25 November 1999
Revised: 14 March 2000
Accepted: 30 March 2000
Published online: 3 April 2000

Abstract

Background We have established a novel mouse mutant, *klotho* (*kl*), by insertional mutation of a transgene and identified the structural gene. The mouse homozygous for the mutation exhibits multiple pathological conditions resembling age-related disorders in humans and can be regarded as a model of human premature aging syndromes. However, the pathophysiological role of Klotho protein has not been clarified.

Methods A replication-deficient adenoviral vector expressing the membrane form of the mouse *klotho* gene was constructed and we examined Klotho expression *in vitro*. The recombinant adenoviral vector was then administered intravenously into *klotho* mice at 4–5 weeks of age and its therapeutic potential was examined.

Results Expression of Klotho protein was observed in the adenoviral vector-infected CHO cells. The *klotho* mice infused with the recombinant adenovirus showed a significant extension of life span and gain in body weight at 1 week after treatment. Macroscopic and histological analyses demonstrated the improvement of multiple pathological findings such as restoration from atrophy and cell formation and differentiation in the gonadal cells, immune tissues and subcutaneous fat.

Conclusion We showed that local expression of the *klotho* gene retards or partially improves pathological abnormalities in several organs of *klotho* mice after onset of the phenotypes. Therefore, the recombinant adenovirus vector will provide an important tool for investigating the molecular mechanism of the Klotho protein and give clues to understanding the individual disease mechanisms. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords mouse klotho gene; adenoviral vector; *in vivo* gene transfer; aging

Introduction

A novel mouse mutant, *klotho* (*kl*), was recently established by insertional mutation of a transgene. A defect in the expression of the *klotho* gene leads to a short life span, growth retardation and infertility, and manifests phenotypes similar to age-related disorders in humans, such as arteriosclerosis, osteoporosis, pulmonary emphysema and skin atrophy [1]. The *klotho* gene encodes a novel single-pass membrane protein of 1014 amino acids. The extracellular region consists of two internal repeats, mKL1 and mKL2, which shares homology with β-glucosidases of bacteria and plants and with

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mammalian lactase-phlorizin hydrolase [2]. Expression of the *klotho* gene was observed in the kidney and brain by Northern blot analysis and in several organs including the testes, ovaries, and pituitary gland by the reverse transcriptase-polymerase chain reaction (RT-PCR) technique. mRNA which encodes the soluble isoform containing the N-terminus of the first repeat (mKL1) was also identified in mice and humans [3,4]. Systemic circulating factor(s) such as soluble form of Klotho protein seem to be partly responsible for the physiological role of the protein since pathology caused by the absence of *klotho* expression is observed in organs where the gene is naturally not expressed (lung, stomach, skin and bone). Blood plasma exchange between wild-type and heterozygous *klotho* mice (*+/kl*) by parabiosis experiments resulted in restoration of endothelial dysfunction in heterozygous *klotho* mice [5]. This finding also suggests the existence of circulating factor(s) mediated by Klotho protein. However, the pathophysiological role of Klotho protein has not been clarified.

In this study, we examined the therapeutic potential of Klotho protein to *klotho* (*kl/kl*) mice by a recombinant adenoviral transfer and expression of the *klotho* gene. Adenoviruses are one of the most efficient vectors for gene transfer in cultured cells and in whole animals. Although the adenoviral vector induces immune responses in animals and expression of a transgene is transient in many cases, there are some examples of pathophysiological symptoms being transiently rescued by the local introduction of a recombinant adenovirus, such as the introduction of the leptin gene in obese (*ob/ob*) mice [6] and the ornithine transcarbamylase gene in Otc^{spf} mice [7]. We constructed a replication-deficient adenoviral vector expressing the membrane form of the *klotho* gene under the control of the CAG promoter [8] and administered it intravenously into *kl/kl* mice at 4–5 weeks of age. The results showed that local expression of the *klotho* gene by a recombinant adenovirus retards or partially improves several pathological abnormalities in several organs of *klotho* mice after onset of the phenotypes.

Materials and methods

Recombinant adenoviral vector

An E1 and E3 deleted adenoviral vector expressing *klotho* cDNA was constructed by the COS-TPC method [9]. Briefly, the 3.2 kb *Not*I-*Xba*I fragment [the 5′ *Not*I site was derived from the *Eco*RI (*Not*I) Adapters (Gibco-BRL, Rockville, MD, USA) used for cDNA cloning] containing the complete open reading frame of the membrane form of mouse *klotho* cDNA clone was blunt-ended and inserted into the *Swa*I site of the cosmid pAxCAwt [10] to yield cosmid pAxCAmKlo. The *klotho* gene was controlled by the CAG promoter comprising a cytomegalovirus immediate early enhancer, a chicken β-actin/rabbit β-globin hybrid promoter and terminated by the rabbit β-globin poly (A) signal [8]. A recombinant virus was generated by cotransfecting 293 cells with cosmid pAxCAmKlo and *Eco*T22I digested Ad5-dlX [11] DNA-terminal protein complex. The virus designated AxCAmKlo was propagated to high titer in 293 cells and purified by two cesium chloride density gradient centrifugations [12]. The viral preparations were dialyzed against phosphate-buffered saline (PBS) containing 10% glycerol and stored at -80°C until required. Titers of the virus stocks were determined by end-point dilution using 293 cells and expressed as plaque forming units (pfu) per ml. AxCALacZ carrying the *Escherichia coli* β-galactosidase gene [13] was prepared and used as an adenoviral control vector.

Cell cultures

The human 293 cell line derived from embryonic kidney (ATCC CRL-1573; Rockville, MD, USA) [14] was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS). The *dhfr*-deficient Chinese hamster CHO DUKX B11 cell line [15] was grown in α-minimum essential medium (α-MEM; Gibco-BRL) supplemented with 5% heat-inactivated FCS. Media were supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. These cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Immunoprecipitation and western blot analysis

To detect Klotho protein expression by immunoprecipitation and western blot analysis, cells were lysed in a RIPA buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and complete™ protease inhibitors (Boehringer-Mannheim GmbH, Mannheim, Germany). The cell lysate was incubated with the anti-Klotho monoclonal antibody KM2076 for 1 h at room temperature. KM2076 was established by immunizing rats with a partial fragment of human recombinant Klotho protein (amino acids 55–261) [16]. Protein G-Sepharose beads (Amersham Pharmacia Biotech, Tokyo, Japan) were used to collect the antigen–antibody complexes. The immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and proteins were electrophoretically transferred onto polyvinylidine difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Western blot analysis was performed using the same antibody described above and the protein was detected by the enhanced chemiluminescence western blotting system (ECL western blotting analysis system; Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Animals

Mice homozygous for the mutation in the *kl* locus (*kl/kl*) were generated by crossing heterozygous (*+/kl*) parent

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mice, as *kl/kl* mice are infertile. Mice were allowed food *ad libitum* and had free access to water. Animal studies were performed in accordance with recommendations for the proper care and use of laboratory animals.

In vivo administration of adenoviruses

kl/kl mice at 4–5 weeks of age were used for the *in vivo* experiments. Mice were injected in the tail vein with a purified recombinant adenovirus, diluted to 100 μl with PBS using a 29-gauge needle.

RT-PCR

Poly $(A)^+$ RNA (500 ng) was extracted from various mouse tissues using the Quick Prep mRNA Purification Kit (Amersham Pharmacia Biotech) and reverse transcribed with random 9-mers and oligo dT-adapter primers using AMV reverse transcriptase XL (Takara Shuzo Co., Kyoto, Japan). As much as 5% of reverse-transcribed materials were amplified with LA-Taq DNA polymerase (Takara Shuzo Co.) using a 5′ primer for the exon in the CAG promoter region (5′-CTGTCTCATCATTTTG GCAAAG -3′) and a 3′ primer for mouse *klotho* cDNA (5′-TTAAGGC-GATAGACACCCGG-3′). Conditions for amplification were 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 90 s. Control RT-PCR reactions were carried out using a primers pair for GAPDH (Control Amplimer Set; Clontech, Palo Alto, CA, USA). The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

Histology

Various tissues from wild-type, *kl/kl* mice and *kl/kl* mice injected with a recombinant adenovirus were fixed with 10% formaldehyde, embedded in paraffin, sectioned into 4-μm slices and stained with hematoxylin–eosin staining and/or von Kossa staining.

Results

Expression of the *klotho* gene in an AxCAmKlo-treated CHO cell line

We constructed a recombinant adenovirus (AxCAmKlo) carrying the membrane form of mouse *klotho* cDNA under the control of the CAG promoter (Figure 1). The adenovirus is derived from the human type 5 with the E1A, E1B and E3 regions deleted and is defective in replication. To examine the recombinant adenovirus-mediated gene transfer and expression of the *klotho* gene *in vitro*, CHO cells were infected with AxCAmKlo at a multiplicity of infection (MOI) of 100 or 200. Three days after infection, cells were harvested and the expression of Klotho protein was examined by immunoblot analysis (Figure 2A). The molecular mass of the 130 kDa protein was detected in AxCAmKlo-infected cells using a specific antibody against Klotho protein. The protein of the same mobility was also confirmed in a transfected CHO cell line producing Klotho protein.

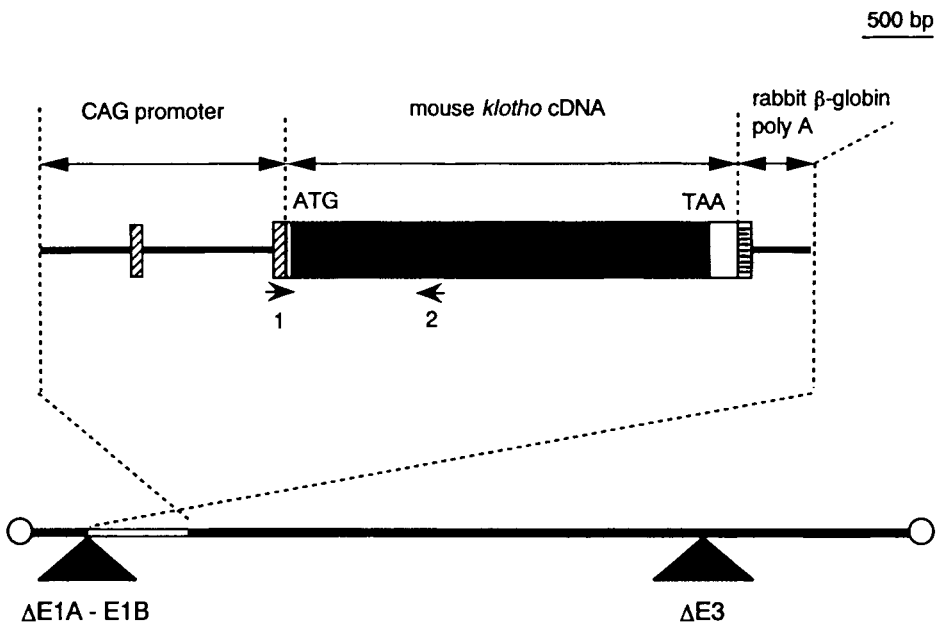

Figure 1. Schematic representation of a recombinant adenovirus expressing the membrane form of mouse *klotho* cDNA. The hatched areas indicate the exons of the CAG promoter, the unshaded and filled areas indicate the untranslated regions and coding sequence of a mouse *klotho* cDNA respectively, and the gray shaded area indicates the 3′ flanking region of rabbit *β*-globin gene including a polyadenylation signal. 1, 2: the sense and antisense primer pair for RT-PCR (arrow). An adenoviral genome is derived from human adenovirus type 5 with deletions of E1A, E1B (1.3–9.3 mu) and E3 (79.6–84.8 mu) regions as reported previously [9]

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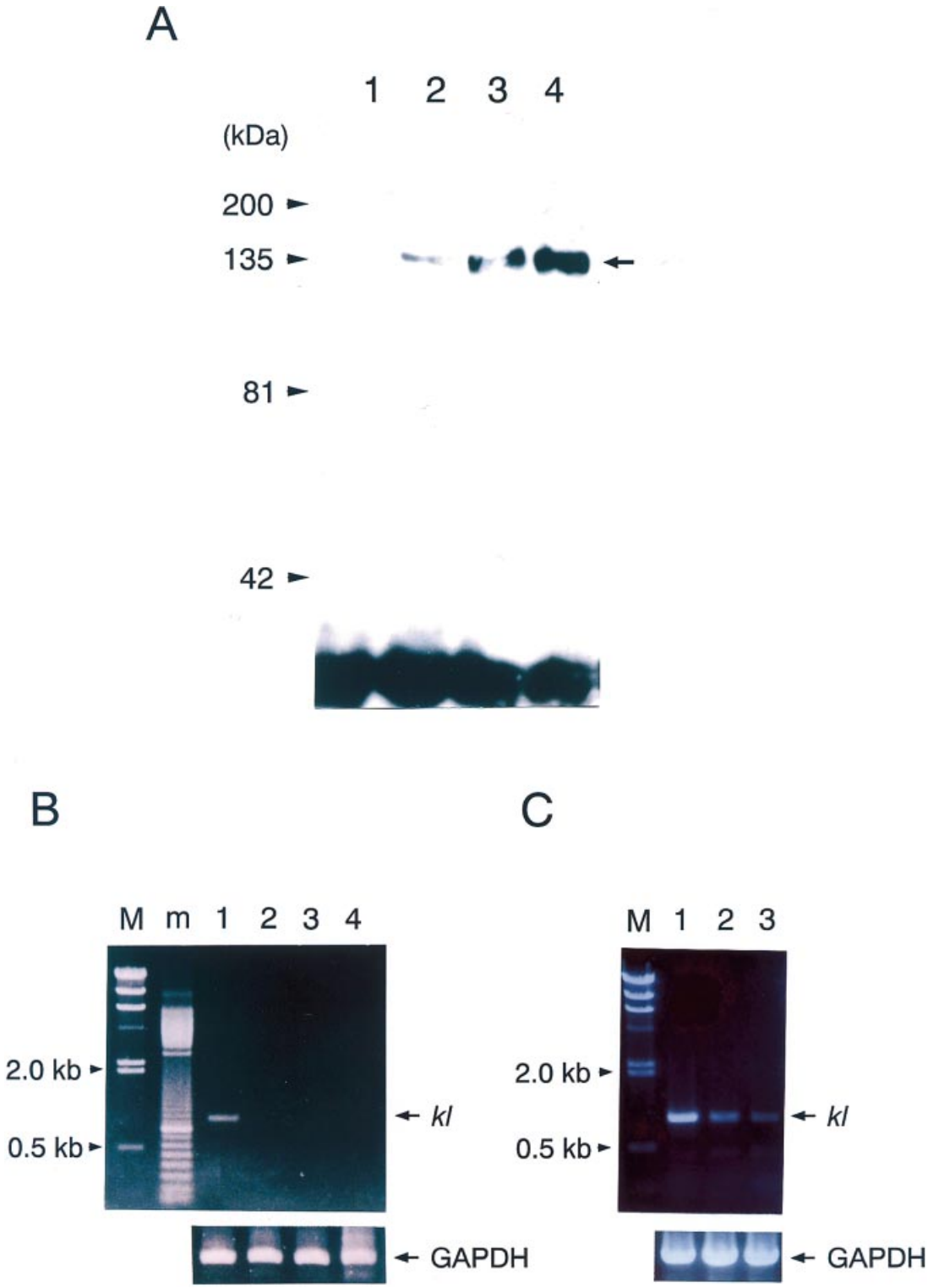

Figure 2. Expression of the *klotho* gene in CHO cells and *kl/kl* mice after adenovirus-mediated gene transfer. A: western blot analysis of Klotho protein in CHO cells. CHO cells infected with AxCAmKlo were lysed, immunoprecipitated and analyzed by immunoblot analysis using a monoclonal antibody specific for Klotho protein (KM2076). Lane 1: uninfected, lane 2: a transfected CHO cell line producing Klotho protein (CHO^{kl}), lane 3: AxCAmKlo (MOI 100), lane 4: AxCAmKlo (MOI 200). The solid arrow shows the position of recombinant Klotho protein. Molecular weight markers are shown on the left. B: RT-PCR analysis of *klotho* RNA from different tissues of adenovirus-treated *kl/kl* mice. Tissue samples were collected 7 days after viral infusion. The expression of the *klotho* transgene (*kl*, 982 bp) was detected in the liver. For positive controls, mouse GAPDH cDNA was amplified from the same RNA preparations. Lane 1: liver; lane 2: kidney; lane 3: brain; lane 4: spleen; M: *λ/Hin*dIII molecular size marker, m: 100 bp ladder marker. C: RT-PCR analysis of *klotho* mRNA in the liver. Tissue samples were collected 1, 2 or 3 weeks after viral infusion. Lane 1: 1 week; lane 2: 2 weeks; lane 3: 3 weeks after viral infusion; M: *λ/Hin*dIII molecular size marker

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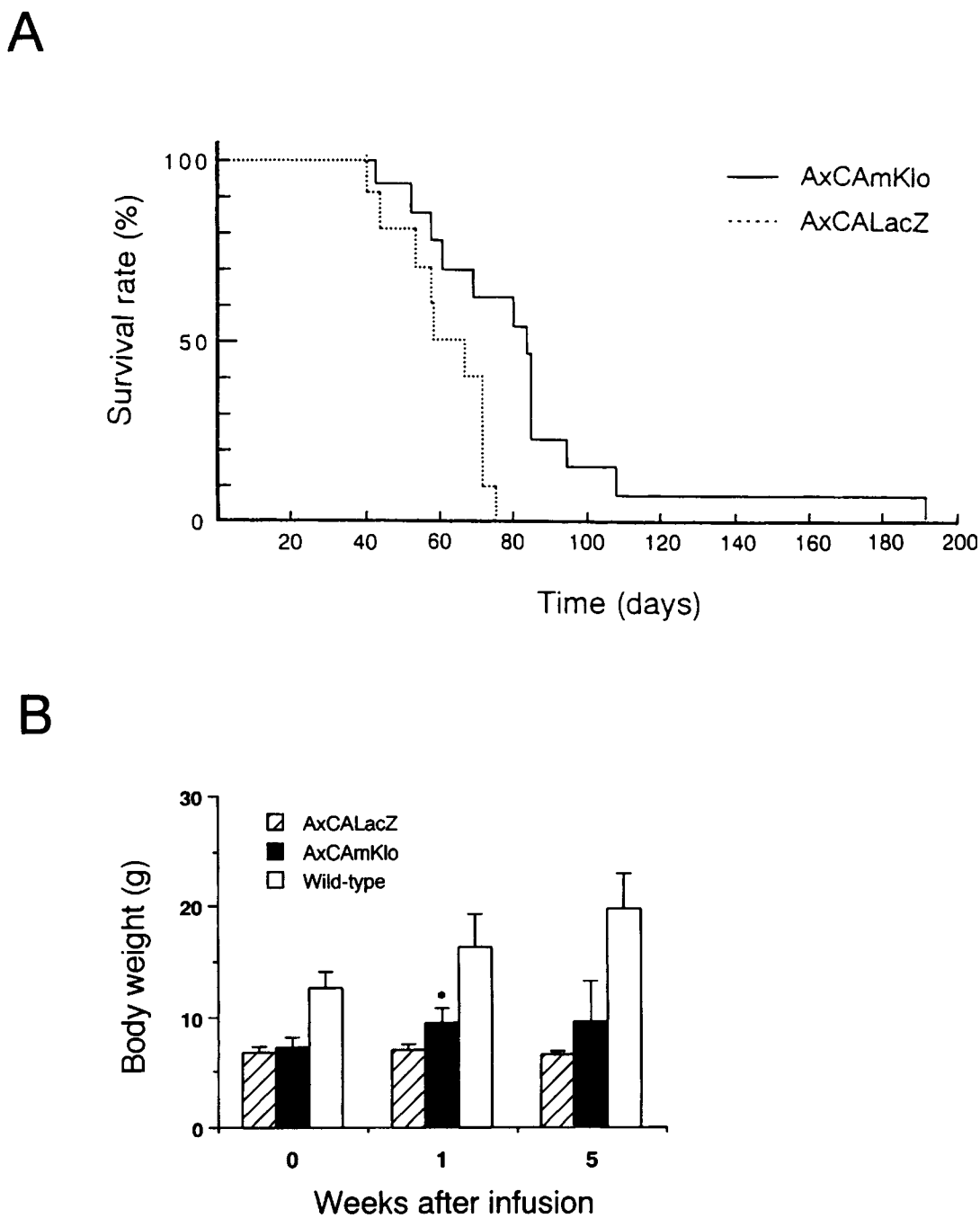

Figure 3. Survival and growth of AxCAmKlo-treated *kl/kl* mice. A: survival curve of *kl/kl* mice treated with AxCAmKlo and AxCALacZ. Kaplan–Meier curves were established for each group and survival was compared with the log-rank test. Survival of AxCAmKlo-treated *kl/kl* mice ($n=13$) is significantly different ($p<0.05$) from AxCALacZ-treated *kl/kl* mice ($n=10$). B: comparison of body weight of wild-type, AxCALacZ-treated and AxCAmKlo-treated *kl/kl* mice. Data of AxCAmKlo-treated ($n=13$) and AxCALacZ-treated ($n=10$) *kl/kl* mice at 1 week were significantly different ($^*p<0.005$) by the unpaired Student's *t*-test. The results show mean ± S.E.M. (bars)

Adenovirus-mediated expression of *klotho* in the *kl/kl* mouse liver

Administration of recombinant adenovirus via the tail vein leads to the expression of a transgene mainly in the liver [17,18]. We therefore examined *klotho* gene expression in *kl/kl* mice infected with AxCAmKlo in several tissues including the liver. Seven days after infusion of 5×10^8 pfu of the virus into *kl/kl* mice, poly $(A)^+$ mRNAs were isolated from several tissues and expression of *klotho* mRNA was examined by RT-PCR analysis using primers that amplified mRNA of the *klotho* transgene and did not amplify that of the endogenous *klotho* gene. The presence of *klotho* mRNA was detected at least 3 weeks in the liver, but not in the kidney, brain or spleen (Figure 2B, C). Thus the *klotho* transgene was efficiently expressed in the liver upon AxCAmKlo infection.

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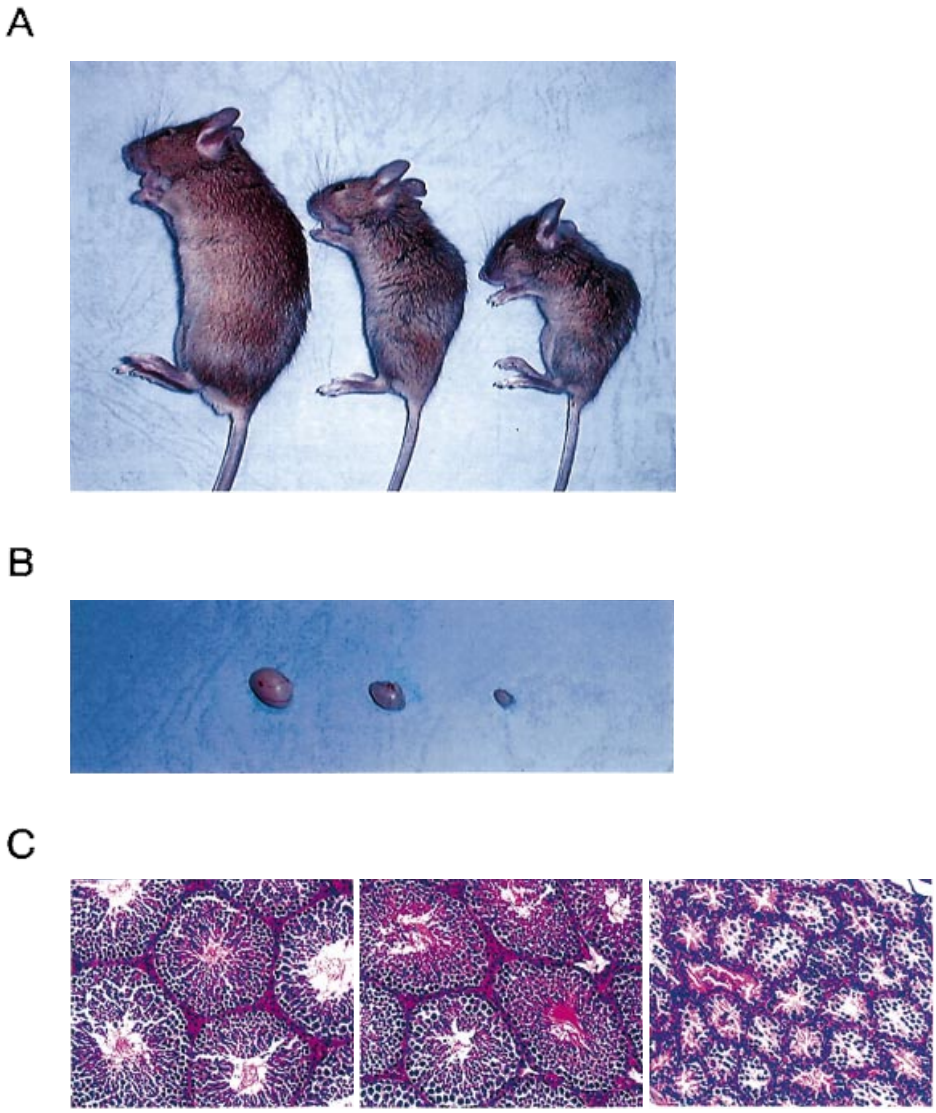

Figure 4. Appearance and histological analysis of AxCAmKlo-treated *kl/kl* mice. A: morphological analysis of a wild-type (left), an AxCAmKlo-treated *kl/kl* mouse (middle) and a *kl/kl* mouse (right) at 10 weeks of age. B: separated testes from a wild-type (left), an AxCAmKlo-treated *kl/kl* mouse (middle) and a *kl/kl* mouse (right) at 10 weeks of age. C: histological analysis of sectioned testes from a wild-type (left), an AxCAmKlo-treated *kl/kl* mouse (middle) and a *kl/kl* mouse (right) at 10 weeks of age. Sperm can be observed in an AxCAmKlo-treated *kl/kl* mouse (middle)

Effect on life span and body weight of *kl/kl* mice following AxCAmKlo treatment

Differences in the appearance of *kl/kl* mice were not apparent at 3 weeks of age when compared to wild-type mice, after which they began to show growth retardation, gradually became inactive and died prematurely at 8–9 weeks of age. The average life span was 60 days and no *kl/kl* mouse survived for longer than 100 days. *kl/kl* mice which were administered the control vector AxCALacZ carrying the reporter β-galactosidase gene at 4–5 weeks of age at 5×10^8 pfu died between days 40 and 74 and the life span was not found to have been extended (60.4 days, $n = 10$). In contrast, *kl/kl* mice treated with 5×10^8 pfu of AxCAmKlo showed significantly prolonged survival (> 83.3 days, $n = 13$) compared with the AxCALacZ-treated *kl/kl* mice (Figure 3A). Four mice which survived fomore than 13 weeks were killed for histological analysis.

kl/kl mice were found to gain body weight more slowly than wild-type mice about 2 weeks after birth [1]. At the time of viral injection, *kl/kl* mice weighed 48% less than age-matched wild-type mice (*kl/kl* mice, 7.0 ± 0.9, $n = 23$; wild-type, 13.4 ± 1.6 g, $n = 12$). In AxCALacZ-treated *kl/kl* mice, no difference of body weight was observed as with untreated *kl/kl* mice. However, *kl/kl* mice treated with AxCAmKlo revealed significantly greater weight gain in comparison with the control vector, AxCALacZ at 1 week after treatment (Figure 3B). Mean body weight of AxCAmKlo-treated mice was not decreased at 5 weeks after treatment.

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Macroscopic and histological analyses of tissues from *kl/kl* mice injected with AxCAmKlo

We compared a wild-type, an AxCAmKlo-treated and an untreated *kl/kl* mouse at 10 weeks of age. In morphological analysis, appearance and the size of testis of an AxCAmKlo-treated *kl/kl* mouse showed a drastic increase compared with those in a *kl/kl* mouse, but smaller than that of a wild-type littermate (Figure 4A, B). Histological analysis revealed that sperm maturation, which was not observed in a *kl/kl* mouse, was recognized in an AxCAmKlo-treated *kl/kl* mouse (Figure 4C). Subsequently, four *kl/kl* mice surviving to 13, 15 and 27 weeks of age after AxCAmKlo infusion were designated as numbers 13-1 (male), 13-2 (female), 15 (female) and 27 (male), respectively, and killed for the examination of pathological phenotypes. Age-matched wild-type mice and *kl/kl* mice at 4–10 weeks of age were used as references, as these mice had survived up to or over the longest life span of *kl/kl* mice. In macroscopic analysis, the size of genital organs in numbers 13-1 and 27 were indistinguishable from wild-type littermates. The white adipose tissue, which is not detected in *kl/kl* mice, was recognized in all four mice. Spleen and thymus, which are shrunken with the presence of the thymus being barely detectable in *kl/kl* mice at 6–9 weeks old, were recovered (data not shown). Histological analyses revealed that multiple pathological findings observed in *kl/kl* mice were ameliorated. In the genital organs of *kl/kl* mice, no sperm maturation or secondary follicles were observed. However, a full complement of spermatogenic cells were found to exist in the testes of numbers 13-1 and 27 (Figure 5A), and secondary follicles and Graafian follicles were observed in the ovaries of numbers 13-2 and 15 (Figure 5B). Subcutaneous fat was barely detectable in *kl/kl* mice, but fat cells and the development of the subcutaneous fat layer were observed in the killed mice (Figure 5C). In the aorta, medial calcification was observed, but was less advanced than that in *kl/kl* mice (Figure 5D). In the trabecular bone, although the density was higher in the epiphyseal part of the petrous bone in *kl/kl* mice, no significant differences between number 27 and wild-type mice were observed in sections of the distal ends of the femora stained with hematoxylin–eosin (Figure 5E).

Discussion

In the present study, we construct a recombinant adenovirus expressing the membrane form of the mouse *klotho* gene and examined its role for the improvement of various pathological phenotypes observed in *kl/kl* mice. The exogenous expression of *klotho* mRNA was detected in the liver at least 3 weeks after recombinant adenovirus treatment, but not in the brain and kidney where the endogenous *klotho* gene was abundantly expressed. Restricted expression of the *klotho* gene in the liver was still capable of ameliorating various phenotypes in *kl/kl* mice by compensating the function of Klotho protein expressed in the kidney, indicating that expression of Klotho protein in the kidney was dispensable. This evidence is supported by genetic rescue studies in which the exogenous *klotho* gene is not expressed in the kidney [1] and also supported by previous reports of parabiosis suggesting the presence of humoral factor [5]. These findings together confer a solid basis for the hypothesis that the pleiotrophic functions of the *klotho* gene product are mediated by systemic circulating factor(s) at least partly. The soluble factor(s) may be: (i) a functional part of Klotho protein secreted by proteolytic cleavage of the membrane form, (ii) some metabolites of Klotho protein, or (iii) some soluble factor(s) released by a signal cascade of the *klotho*.

Genetic rescue studies have demonstrated that all pathological phenotypes are improved by the continuous expression of the endogenous *klotho* gene. However, recovery of mutant phenotypes by adenovirus treatment is partial and insufficient and the effect is limited to a short duration. Gain in body weight at 1 week after recombinant adenovirus treatment and extension of life span were significant compared with specimens treated with a control adenovirus. However, body weight and life span are reduced compared with those in wild-type mice. As adenoviral vectors induce immune responses and expression of a transgene is transient, it is possible that the level and duration of expression of the Klotho protein is insufficient for persistent and long-term phenotypic recovery. In preliminary experiments, a higher dose of AxCAmKlo failed to produce an improved effect compared with the dose we used in this study (data not shown). Some improvements such as (i) transient modulation of the host immune system using CD40 ligand antibody or CTLA4Ig [19], (ii) the use of improved vectors, such as an adenoviral vector that deletes all viral genes [20], or an adeno-associated virus vector, (iii) the route of administration in the muscle, may give rise to longer and higher expression of the *klotho* gene.

The previous study by genetic rescue is not available for the examination of the recovery process after the onset of pathological phenotypes because the exogenous *klotho* gene begins to express during the embryonic stage, prior to the onset of pathological phenotypes and continuously expresses Klotho protein throughout the life span. However, this study allowed analysis of the recovery processes in which a single shot of adenoviral vector was intravenously injected into the *klotho* mutant at 4 or 5 weeks after birth where many of the pathological phenotypes had already become apparent [1]. In fact, in four mice that survived for more than 3 months, recovery from atrophy and maturation of the cells were observed in the genital organs and immune organs, and development of the subcutaneous fat layer was confirmed by histological analysis. In particular, the thymus, although almost negligible in *kl/kl* mice at 6 weeks of age, returned to normal size. The abnormal bone density

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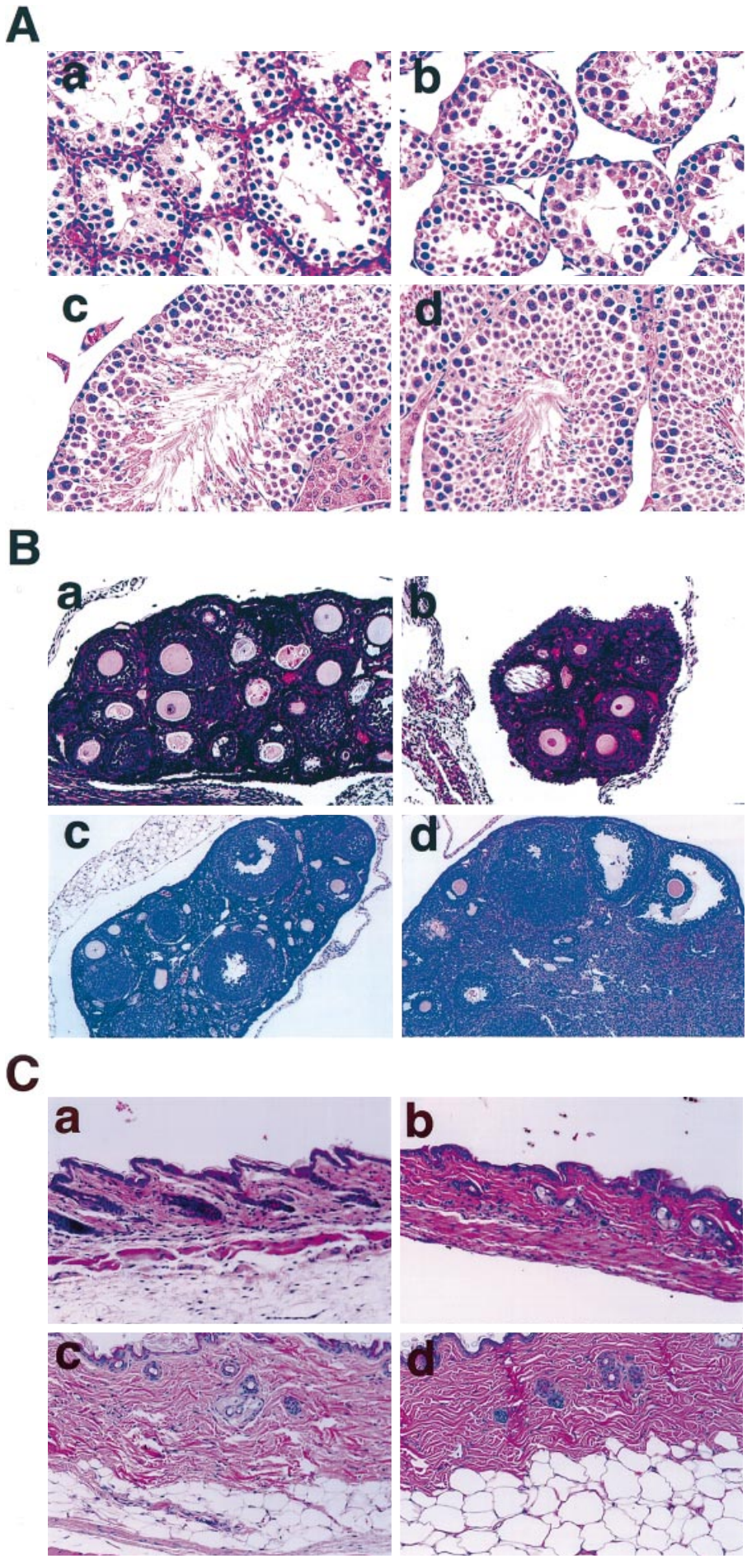

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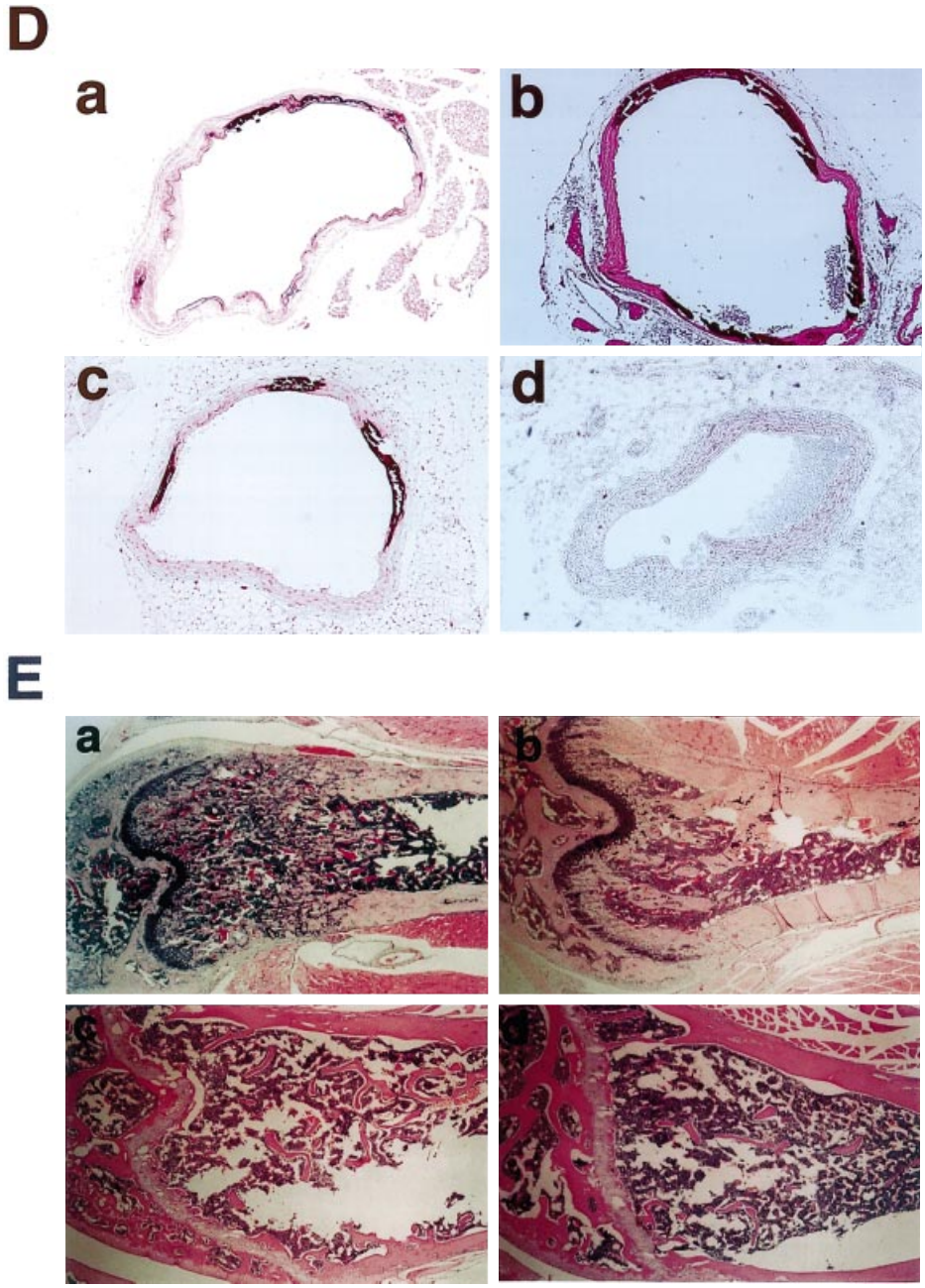

Figure 5. Histological analysis of *kl/kl* mice which showed improved survival by AxCAmKlo infusion. Histological analysis of *kl/kl* mice with hematoxylin–eosin (A–C, E) and von Kossa (D) staining. A: sectioned testes. Untreated *kl/kl* mice (a, 4 weeks old; b, 7 weeks old). Adenovirus-treated *kl/kl* mice (c, 27 weeks old) and wild-type littermate (d). Sperm can be observed (c, d). Magnification: ×20. B: sectioned ovary. Untreated *kl/kl* mice (a, 4 weeks old; b, 10 weeks old). Calcification can be recognized (b). Adenovirus-treated *kl/kl* mice (c, 15 weeks old) and wild-type littermate (d, 15 weeks old). Mature secondary follicles can be recognized (c, d). Magnification: ×10 (a, b) or ×5 (c, d). C: sectioned skin. Untreated *kl/kl* mice (a, 4 weeks old; b, 7 weeks old). Adenovirus-treated *kl/kl* mice (c, 27 weeks old) and wild-type littermate (d, 27 weeks old). The subcutaneous fat and its layer can be detected (c, d). Magnification: ×10. D: sectioned aorta. Untreated *kl/kl* mice (a, 4 weeks old; b, 10 weeks old). Adenovirus-treated *kl/kl* mice (c, 27 weeks old) and wild-type littermate (d, 27 weeks old). von Kossa staining in which calcium is stained black. Magnification: ×5. E: trabecular bones in the distal ends of the femora. Sections of the distal ends of the femora of control *kl/kl* mice (a, 10 weeks old). Adenovirus-treated *kl/kl* mice (c, 27 weeks old) and wild-type littermate (b, 10 weeks old; d, 27 weeks old). Ten weeks after birth, the number of trabecular bones in *kl/kl* mice is abnormally high compared to that of the wild-type. 27 weeks after infection with the virus, no significant difference in the number of trabecular bones was observed. Magnification: ×40

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of *kl/kl* mice was improved in some, but not all, areas following the recombinant adenovirus infusion. In ectopic calcification, although calcification was reduced in the aorta of *kl/kl* mice infused with the recombinant adenovirus, some degree of calcification still remained. Furthermore, to confirm that tissues in the *klotho* mutant retain the potential to recover, impaired ovaries prepared from *klotho* mutants at 4 to 5 weeks of age were transplanted into wild-type mice in which ovariectomy had been previously performed and the growth and maturation of the transplanted ovaries and fertility were analyzed. The *kl/kl* ovaries began to grow soon after transplantation and finally matured follicles could be detected which became fertile within 1 month (Y.N. and Y.N., unpublished results). Although the recovery of phenotypes was limited, many of the pathological abnormalities were improved by the induced expression of the Klotho protein after the onset of pathological phenotypes, suggesting that organs and tissues keep the potential to grow and the ability to get healthy again even in *klotho*-negative circumstances when *klotho* function is recovered within a potential critical period. To relate the different effects of a recombinant adenovirus AxCAmKlo expressing the *klotho* gene to survival of *kl/kl* mice, further analysis will be required to examine the differences, such as (i) the amount of Klotho protein, (ii) antibody production and potential immune response against the *klotho* transgene, and (iii) liver histology between long-term surviving mice and animals with a mildly extended life span.

The tail vein application of adenoviral vector AxCAmKlo restricts expression of the Klotho protein to the liver and therefore can only be considered as a tool in the possible understanding of some function(s) of the *klotho* pathway. However, the recombinant adenovirus constructed in this study will provide an important tool in understanding the molecular function of Klotho protein and hopefully be applied to the development of therapy in various age-related diseases, including arteriosclerosis, osteoporosis and emphysema.

Acknowledgements

We thank I. Saito for supplying the adenovirus AxCALacZ, the cosmid pAxCAwt and *Eco*T22I-digested Ad5dlX, J. Miyazaki for the CAG promoter, T. Matsuzaki and T. Kojima for maintaining the mice, Y. Nagoshi for help with mouse genotyping, Y. Shimizu for technical assistance, and Y. Kikuchi and H. Nakano for their encouragement.

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