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Original Article

Micturition defects and altered bladder function in the *klotho* mutant mouse model of aging

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Abstract: Introduction and objectives: Symptoms associated with detrusor underactivity (DU) or underactive bladder (UAB) can severely impact a person's quality of life, and growing old is the main etiological factor of DU and UAB. The gene *Klotho* has been associated with suppression of several aging phenotypes, and there is moderate *klotho* expression in the bladder. Given this, we hypothesized that the klotho gene is involved in regulation of bladder function. Thus, we examined a premature aging rodent genetic model with hypomorphic *klotho* expression for alterations in bladder function. Methods: *Klotho* mutant mice are established as a preclinical model of aging. Male and female klotho mice had micturition measured at weeks 4, 6, and 8 through metabolic cage and void spot assays. Histology was assessed at 4, 6, and 8 weeks. Lastly, bladder contraction was assessed using bladder strip tissue bath. All animals were gender- and age-matched with wild-type littermates for analysis. Results: Void spot and bladder contraction assays revealed that *klotho* mutant mice, similar to other aging models, have increased voiding frequency and decreased voiding volume per micturition event. The in vitro contractile response to electrical stimulation was weaker and muscarinic receptor subtype expression was reduced in the in *klotho* mutant mouse bladders. These data suggest that *klotho* mutant mouse bladders had impaired bladder function. Conclusions: *Klotho* mutant mice recapitulate many characteristics of an older dysfunctional bladder, including altered bladder function. Given the short time frame to bladder dysfunction and robustness of the model, this model will provide new insights to drive aging bladder research.

Keywords: Klotho, urinary dysfunction, aging bladder, micturition, voiding dysfunction, lower urinary tract symptoms (LUTS)

Introduction

Advancement in medical care and improved environmental conditions have increased the human lifespan [1]. In the United States alone, individuals over 65 years old will reach 71 million, or 20% of the population, by 2030 [2]. The incidence of lower urinary tract symptoms (LUTS) such as urgency, frequency, incontinence, and nocturia is increased in people over the age of 65 and grows with age [3]. LUTS are major contributing factors to a decreased quality of life, depression [1], and nursing home admissions [4]. Thus, there is a need to understand the development of LUTS in the context of aging to ultimately lead to improved prevention and treatment strategies.

Translating human aging bladder pathophysiology to an animal research model has been difficult as LUTS are often multifactorial with several physiological changes to the bladder [5]. These include decreased muscle tone, thinned bladder wall, decreased bladder capacity, and loss of nerves and muscarinic receptors in the bladder [2]. In addition, the risk of other comorbidities that can impact bladder function also occurs with aging such as neurological diseases, diabetes mellitus, and bladder outlet obstruction (BOO) [6]. While many of these models are sufficient for specific bladder diseases, none of them recapitulate aging of the human bladder entirely. Ideally, preclinical models would age naturally, though this can be cost and time prohibitive. A premature aging rodent mo-

del is attractive for aging bladder research as it could recapitulate most aspects of the aging model in a shorter timeframe and accelerate the discovery of possible therapeutics; however, this has not been previously investigated in the urinary bladder field.

One attractive accelerated aging model is the *αKlotho* mutant mouse model. The *αKlotho* (*KL*) gene has been suggested as a putative 'aging-suppressor' gene [7]. Klotho is conserved across mice and humans, with 98% homology between the two gene sequences [8]. In humans, serum klotho levels decrease with age and have been shown to be an independent predictor of mortality [9]. In mice, klotho deficiency has been found to cause accelerated aging, while its overexpression has an antiaging effect [10]. *Klotho* mutant mice develop normally until 3 weeks of age, after which they rapidly age and expire by 8-9 weeks [11]. These mice display multiple pathologies reminiscent of human aging including osteoporosis, arteriosclerosis, emphysema, hypokinesis, infertility, and ectopic calcification [11]. *Klotho* has strong expression in the distal tubule cells of the kidney, parathyroid glands, and choroid plexus of the brain, and moderate expression in the bladder [11]. Klotho has been linked to the suppression of the Wnt signaling pathway, inhibition of insulin-like growth factor (IGF) pathway, and the regulation of Ca^{2+}, phosphate, vitamin D_3, and oxidative stress [8]. Through the suppression of the Wnt-signaling pathway, klotho maintains stem cell function and stem cell quantity [12].

While the role of klotho in the bladder has not yet been explored [13], the age protective properties of klotho displayed in mice and its reported expression in the bladder suggests that klotho could play a role in maintaining a healthy bladder. Therefore, we hypothesize that *klotho* mutant mice recapitulate many pathophysiologies associated with aging, including age-associated functional bladder changes.

Here we demonstrate for the first time that *klotho* mutant mice could be used as a model for the aging bladder, as they display significant alterations in bladder function, including decreased void volumes, increased number of micturition events, and decreased response to electrical stimulation.

Materials and methods

Mouse strain/breeding

Drs. Makoto Kuro-o and Kazuhiro Shiizaki, Jichi University, Japan, provided us with two sets of *klotho* hypomorph breeders. Colonies were bred and maintained at Beaumont Research Institute. Experiments were conducted in accordance with National Institutes of Health Guidelines, and all protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Beaumont Health (IACUC # AL-17-03) and the University of Wisconsin-Madison. The ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines were followed to improve the communication of this research [14].

Genotyping

1.2 µl of extracted DNA from tail biopsy was added to 5.0 µl 10 × LA PCR Buffer plus Mg^{2+} (*Takara*), 4.0 µl dNTP mix (*Takara*), 1.0 µl 10 µM common reverse primer, 1.2 µl 10 µM Klotho forward primer, 0.2 µl 10 µM wild-type forward primer [11], and 1.5 µl LA Taq (*Takara #RR002M*), into a 50 µl reaction. Genotyping PCR was performed at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 90 s, followed by an extension at 72°C for 10 min. SYBR Green I was added to products then run on a 1.2% agarose gel in TAE at 60 V for 1 h. Mutant (920 bp) and wild-type (458 bp) bands were visualized under UV light with the ChemiDoc XRS+ (*BIO-RAD)*.

Tissue collection and analysis

Mice aged week 4, 6, or 8 were anesthetized and monitored under 2-3% isoflurane. Mouse numbers used for experiments are indicated in Methods or Figure legends. Females were catheterized, and their bladders were instilled with 100 µl of 4% PFA. After 5 min bladder tissues were harvested and immersed in 4% PFA overnight at 4°C. Tissues were then dehydrated and embedded in paraffin blocks. Alternatively, female bladders were instilled with OCT, then removed and immediately frozen in excess OCT for sectioning. Male mice were unable to be catheterized so bladders and tissues were removed and immediately placed in Ca^{2+}-free dissection buffer (137 mM NaCl, 10 mM HEPES, 10 mM Glucose, 5.6 mM KCl, 1 mM $MgCl_2$) on ice for immediate use or frozen at

-80°C for downstream protein and RNA applications.

Mechanical separation of bladder

Bladders placed in Ca^{2+}-free dissection buffer were cleaned of exogenous tissues and cut open to reveal the urothelium. Three corners of the open bladder were pinned to a silica dissection plate. Using dissection tweezers, the urothelium was gently peeled away from the detrusor muscle at the remaining corner. Once separated, a pair of dissection scissors was used to carefully cut the connective tissue between the two layers to separate them. Once most of the separation was complete the pins were removed and the urothelium was completely removed from the detrusor and used for RNA extraction.

Creatinine determination

Whole blood was collected from 36 mice (12 of each genotype) at time of sacrifice, allowed to sit at room temperature for 30 min, and centrifuged at 1,500 × g for 10 min at 4°C. Serum layer was transferred to a new tube and stored at -80°C. Serum samples were thawed on ice and underwent HPLC analysis as previously described [15]. Column used: Kinetex 2.6 µm C18 100Å 100 × 4.6 mm (*Phenomenex cat #OOD-4462-E0*). Mobile phase: 10 mM Na octanesulfonic acid, pH 3.2: acetonitrile (95:5). Flow rate: 0.5 ml/min, injection volume: 10 µl, run time: 40 min, column oven temperature: 22°C, detection by UV at 236 nm. Reference standard: creatinine (0, 1, 2, 5, 10, 20, 50, 100 ng/ml).

Void spot assay

Void Spot Assays (VSA) were done as previously reported [16]. Briefly, at weeks 4, 6 and 8, a total of 176 mice were placed on filter paper (*BIO-RAD #1650921*) for 4 h with food but no water. Filter paper was dried overnight then scanned under UV light on ChemiDoc XRS+ (*BIO-RAD*) [17]. Void spots were counted and quantified by the ImageJ software [18]. Statistical analysis was performed in GraphPad Prism 6.

Analysis of voluntary voiding behavior in the metabolic cages

Mice were offered water containing 3% glucose and 0.125% saccharin to increase urine output two days prior to and during testing [19]. Mice (n = 14) were placed individually in metabolic cages (*Nalge Model 650-0322*) with floor grids optimized to minimize urine retention for 2 h. A waste plate was placed on an analytical balance (*Mettler Toledo New Classic MF, model MS 303S*) directly under the floor grid. Video records of activity and patterns of waste elimination were captured using Raspberry Pi module V2 cameras connected to Raspberry Pi processing units. Both Raspberry Pi units and balances were connected to a PC computer. Changes in weight sensed by balances were recorded and each weight change triggered a synchronized signal to the associated Raspberry Pi unit that created a 20 second recording of events during the corresponding weight change detected by the balance. Video recordings were subsequently reviewed to discriminate between fecal and urine voiding and to characterize the pattern of urination. Mice were returned to standard housing after completion of testing.

RNA extraction

Before beginning, all supplies were placed under a UV light for 15 min and wiped down with RNAse Zap (*Invitrogen*). 1 mL Trizol and 200 mg of 0.9-2.0 mm stainless steel beads (*Next Advance*) were added to Eppendorf Safe-Lock microcentrifuge tubes. Frozen bladders were added to tubes, and placed in Bullet Blender for 5 min at speed 10 at 4°C. The mixture was then moved to a biosafety cabinet and incubated at room temperature for 5 min. Next, 200 µl chloroform was added to tube containing bladder homogenate, incubated for 2 min at RT, then centrifuged at 12,000 × g at 4°C for 15 min. The top (aqueous) phase was carefully pipetted off then added to a new pre-labeled tube. 500 µl isopropanol was added and incubated for 10 min at RT, centrifuged at 12,000 × g at 4°C for 10 min. The supernatant was carefully removed and discarded while 1 mL 75% ethanol was added to gently re-suspend the pellet. The pellet was vortexed briefly then centrifuged at 7,500 × g at 4°C for 5 min. The supernatant was then discarded and the pellet allowed to air-dry for 5 min. Finally, the pellet was re-suspended in 20 µl of RNase-free H_2O and incubated at 60°C for 10 min at RT. All extracted RNA was assessed for quantity and quality using a Thermo-Fisher nanodrop before downstream use. RNA was placed at -80°C for long term storage or left on ice for immediate downstream applications.

Table 1. qPCR primer list

Name	Sequence 5'→3'	Source
Kl Forward	TACGCAAAGTAGCCACAAAGG	[11]
Kl Reverse	AATTATGTGAATGAGGCTCTGAAAG	[11]
18S Forward	CCG CAG CTA GGA ATA ATG GA	Illumina
18S Reverse	CGG TCC AAG AAT TTC ACC TC	Illumina
M_1 Forward	TCCCTCACATCCTCCGAAGGTG	[31]
M_1 Reverse	CTTTCTTGGGCCTCTTGACTG	[31]
M_2 Forward	CTGGAGCACAACAAGATCCAGAAT	[31]
M_2 Reverse	CCCCCTGAACGCAGTTTTCAGT	[31]
M_3 Forward	GCAAGACCTCTGACACCAACT	[31]
M_3 Reverse	AGCAAACCTCTTAGCCAGCG	[31]
M_4 Forward	CGGCTACTGGCTTCTGCTACGTCAA	[31]
M_4 Reverse	CTGTGCCGATGTTCCGATACTGG	[31]

Reverse transcription

RNA was diluted to 100 ng/μl from initial concentration. 10 μl of diluted RNA was added to a PCR tube containing 2.0 μl 10 × Buffer, 2.0 μl 10 × Random Primers, 0.8 μl 25 × dNTPs, 4.2 μl H_2O, and 1.0 μl Reverse Transcriptase (*Applied Biosystems 4368814*). The reaction then cycled 10 min at 25°C, 2 h at 37°C, 5 min at 85°C. The resultant cDNA was then transferred to ice for use in qPCR.

qPCR

Klotho and muscarinic receptor 1-4 mRNA was quantified by qPCR. 10.0 μl of SYBR Select MasterMix, 0.6 μl of 10 μM Forward Primer, 0.6 μl of 10 μM Reverse Primer, and 7.8 μl dH_2O was added to each well of a 96-well MicroAmp Optical qPCR plate. Primer sequences are listed in **Table 1**. 1.0 μl of the appropriate cDNA was added to each well. Non-template control is dH_2O. The PCR reaction was run under the protocol: 50°C for 2 min, 95°C for 10 min, (95°C for 15 s, 62°C for 1 min) repeated 41 × on a QuantStudio 3 (*Applied Biosystems*). Triplicate expression values of each gene were set relative to the reference gene (18S ribosomoal RNA) via the ΔΔCt method. Data was then exported to Microsoft Excel for further analysis.

Chromogenic in situ hybridization (CISH) for Klotho detection

Klotho RNA was detected via *in situ* hybridization using the Mm-Kl probe (*ACD Bio #422081*) in the RNAScope system (*ACD Bio #322350*). Paraffin embedded tissue slides were deparaffinized by incubating at 61°C overnight then briefly in Xylene, followed by rehydration in 100% ethanol. Slide tissues underwent a 10 min peroxidase block and target retrieval in buffer for 20 min (supplied). Slides were rinsed in distilled H_2O followed by 100% ethanol then incubated at 40°C for 30 min with Protease Plus (supplied). Slides were washed in distilled H_2O then incubated with appropriate probe at 40°C for 2.5 h. Following probe incubation, the remaining hybridization steps were completed according to the RNAScope 2.5 HD Detection manufacturers protocol. Slides were then dehydrated through ethanol series and mounted with ProLong Gold. Slides were examined by bright-field microscopy.

Masson's trichrome staining

Bladder sections (4 μm) were stained with Masson's Trichrome (*ScyTek #TRM-2)* to detect the presence of fibrosis. Tissue slides were deparaffinized and rehydrated as described above. Modified Masson's Trichrome Stain Kit was performed per the manufacturer's instructions. Slides were then dehydrated through ethanol series and mounted with ProLong Gold. The amount of fibrosis (blue stain) was analyzed as previously described [16]. Briefly, blue staining was quantified as average percentage of fibrosis using ImageJ software color deconvolution. Each bladder section was measured in twelve regions of interest (ROI, 33 × 33 μm) from the detrusor region of the bladder (n = 4 per group) using threshold setting MaxEntropy.

Picrosirius red staining

Picrosirius red staining (PSR) was performed on bladder strip sections to detect the presence of collagen. Tissue slides were deparaffinized and rehydrated as described above. Tissue sections were then incubated with picrosirius red staining solution (0.5 g Direct Red 80 in 500 mL of saturated picric acid) for 1 h at 25°C. Slides were then washed 2x in 0.5% acetic acid in ddH_2O, dehydrated through ethanol series, and mounted with ProLong Gold. Slides were evaluated by fluorescence microscopy by exciting samples using a 561 nm laser detecting emissions between 635-685 nm. Image analysis was done as previously described [16]. Briefly, twelve 56 × 56 μm ROI were taken from the detrusor area of the bladder sections (n = 4 per group). Images were converted to 8-bit,

Table 2. Bladder mass/body weight ratio of 8-week-old mice (average ± SD)

Genotype	Body Mass (g)	Bladder Mass (mg)	Bladder/Body Ratio (%)	n
+/+	19.33 ± 0.3191	20.92 ± 0.6198	0.1062 ± 0.0031	43
Kl/+	19.29 ± 0.5254	21.77 ± 1.050	0.1094 ± 0.0035	19
Kl/Kl	8.664 ± 0.3128****	9.022 ± 0.4682****	0.1087 ± 0.0035	21

****P<0.001.

512 × 512 resolution image files and collagen presence was quantified as percentage of staining using the Li threshold setting in ImageJ software.

In vitro studies of bladder contractility

Contractile function of the bladder wall was evaluated as previously described [20]. Mice were euthanized, and bladders rapidly collected. Bladders were cut into 2 strips, longitudinally, and placed between platinum-coated electrodes in 15 ml tissue baths containing oxygenated modified Krebs solution (pH 7.4; NaCl, 137.7; NaH_2PO_4, 1.4; KCl 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1; $NaHCO_3$, 16.3; and glucose, 7.8; all concentrations in mM) maintained at 37°C. One end of each strip was secured to a fixed metal rod and the other was attached to a force displacement transducers (*Grass FT-03, Grass Instruments, West Warwick, RI*), and stretched gently with an tension of 9.8 mN (+/+) and 4.9 mN (*Kl/Kl*). Tissues were allowed to equilibrate for 1 h. Concentration-response curves of carbachol (CCh; 0.01 to 100 μM) were constructed. Response to electrical field stimulation (EFS) were generated using a Grass S88 stimulator (10 V, 0.5 ms duration, pulse train 5 s, at an increasing frequency of 1-50 Hz with 3 min interval). At the end of the experiment, tissues were exposed to 137.7 mM KCl (but no NaCl) Kreb's solution and the contractile responses to carbachol and EFS were normalized to KCl-induced responses in each tissue preparation.

Statistical analysis

Data are presented as arithmetic means ± SD. The data from multiple groups were analyzed using two-way ANOVA followed by Bonferroni post hoc comparisons (GraphPad Prism, San Diego, CA). Unpaired Student's t-tests were used as appropriate. *p* values <0.05 were considered significant.

Results

Klotho mRNA is abundantly expressed in the urothelium and lamina propria with decreased expression in the detrusor of murine urinary bladder

The bladders of *klotho* mutant mice (*Kl/Kl*) were significantly smaller in size and weight (9.022 ± 0.4682 mg) than wildtype (+/+; 20.92 ± 0.6198 mg) and heterozygous (*Kl/+*; 21.77 ± 1.050 mg) littermates. However, the average body weight of *klotho* mutant mice (8.664 ± 0.3128 g) was also significantly less when compared to wild-type (19.33 ± 0.3191 g) and heterozygous (19.29 ± 0.5254 g) littermates, consistent with what has been previously reported [11]. The decrease in both body and bladder weights of *klotho* mutant mice resulted in a bladder/body ratio that is similar across all genotypes (+/+ 0.1062 ± 0.0031; *Kl/+*: 0.1094 ± 0.0035; *Kl/Kl*: 0.1087 ± 0.0035) (**Table 2**).

Presence of *klotho* mRNA was determined in wild-type mice and confirmed absent in *klotho* mutant mouse bladders (**Figure 1**). *Klotho* mRNA levels were measured in whole bladders of wild-type, heterozygous, and *klotho* mutant mice. When compared to wild-type, heterozygous mice expressed about 50% *klotho* mRNA, while *klotho* mutant bladders did not express *klotho* mRNA (**Figure 1A**). The spleen does not express klotho [11] and was used as a negative control. Mechanical separation and subsequent qPCR analysis indicated that the majority of *klotho* mRNA was present in the urothelium and lamina propria, with a lower amount in the detrusor muscle (**Figure 1B**). In addition, through RNA probe hybridization with chromogenic detection in mouse bladders we identified high expression of *klotho* mRNA in the urothelium and lamina propria and moderate expression throughout the detrusor muscle (**Figure 1C**). As expected, no *klotho* mRNA was detected in *klotho* mutant bladder urothelium, lamina propria, or detrusor (**Figure 1D**).

Klotho mutant mice have increased micturition frequency and decreased voided volume

To measure the effects of the absence of klotho in the bladder, mice underwent micturition

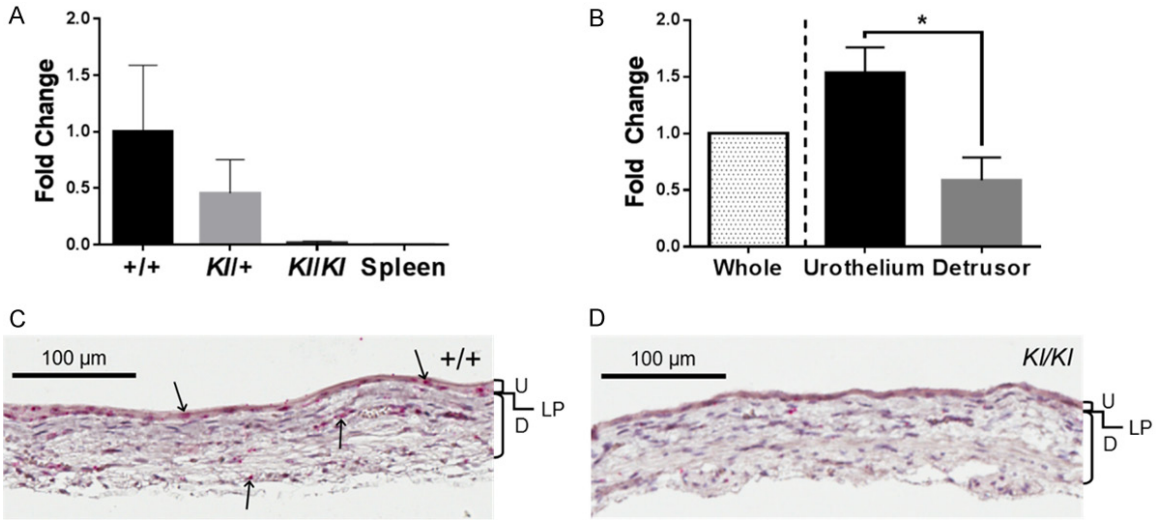

Figure 1. *Klotho* RNA is absent in the *klotho* mutant bladder and localizes mainly to wild-type mouse bladder urothelium. A. Whole bladder *klotho* mRNA expression was evaluated by qPCR in wild-type (+/+), heterozygous (*Kl*/+), and *klotho* mutant (*Kl*/*Kl*) bladders. B. To determine localization of *klotho* mRNA, bladders were mechanically separated into urothelium and detrusor muscle and *klotho* abundance was determined by qPCR (**P<.05*). C. *Klotho* mRNA detection by chromogenic in situ hybridization in wild-type bladder was then used to confirm qPCR findings by identifying mRNA in wild-type bladder sections. D. The absence of *klotho* mRNA in *klotho* mutant bladders was also confirmed by chromogenic in situ hybridization. (U = urothelium; LP = lamina propria; D = detrusor).

studies using two complimentary approaches. Micturition was measured by metabolic cages to measure voiding behavior in real time and by VSA, which uses filter paper to evaluate voiding patterns. Micturition patterns are altered in *klotho* mutant mice, both male and female, when compared to wild-type littermates (**Figure 2A-F**). Voiding patterns were examined at 6 weeks of age in male mice using metabolic cages. The average voided volume per void of *klotho* mutant mice was significantly decreased compared to wild-type littermates (+/+: 146.6 ± 90.3 µl; *Kl/Kl*: 33.4 ± 19.6 µl; n = 7 for each genotype, P<0.01) while the total number of voids was significantly increased (+/+: 2.6 ± 1.4; *Kl/Kl*: 15.3 ± 8.8; P<0.01) (**Figure 2A**). Voiding behavior was then analyzed by VSA in both genders at 4, 6, and 8 weeks of age. *Klotho* mutant mice had increased micturition frequency as evidenced by an increase in the number of urine spots by VSA starting at 4 weeks of age. This phenotype became progressively more significant compared to wild-type littermates with increasing age (**Figure 2B**, **2C**). A decrease in urine volume per voiding event was also observed in *klotho* mutant mice compared to wild-type littermates (**Figure 2B**, **2D**). This was observed in both genders. The primary void spot volume, defined as the largest urine spot, in both male and female *klotho* mutant mice was significantly less than the wild-type mice (**Figure 2E**). Lastly, the primary void area as a percentage of total voided area was significantly less in *klotho* mutant mice than wild-type littermate mice (**Figure 2F**).

To determine if any possible kidney function defects were impacting the micturition pattern, serum creatinine was measured. Elevated serum creatinine levels can indicate impaired kidney function. There was no difference in serum creatinine levels of *klotho* mutant mice (0.625 ± 0.093 ng/mL) compared to wild-type littermates (0.554 ± 0.043 ng/mL) and heterozygous littermates (0.437 ± 0.036 ng/mL) (**Table 3**).

Klotho mutant bladders have impaired response to electrical field stimulation in the absence of fibrosis

We then sought to determine if micturition alterations in *klotho* mutant mice could be due to differences in neurogenic or myogenic pathways. One frequent cause of bladder stiffening and altered micturition patters in rodent models is the presence of fibrosis in the detrusor muscle. We examined wild-type and *klotho* mutant mouse bladders for fibrosis by Masson's trichrome stain to visualize muscle (red) and fibrotic (blue) tissues. No difference was observed in detrusor fibrosis between wild-type and *klotho* mutant bladders (15.02% ± 2.66% vs

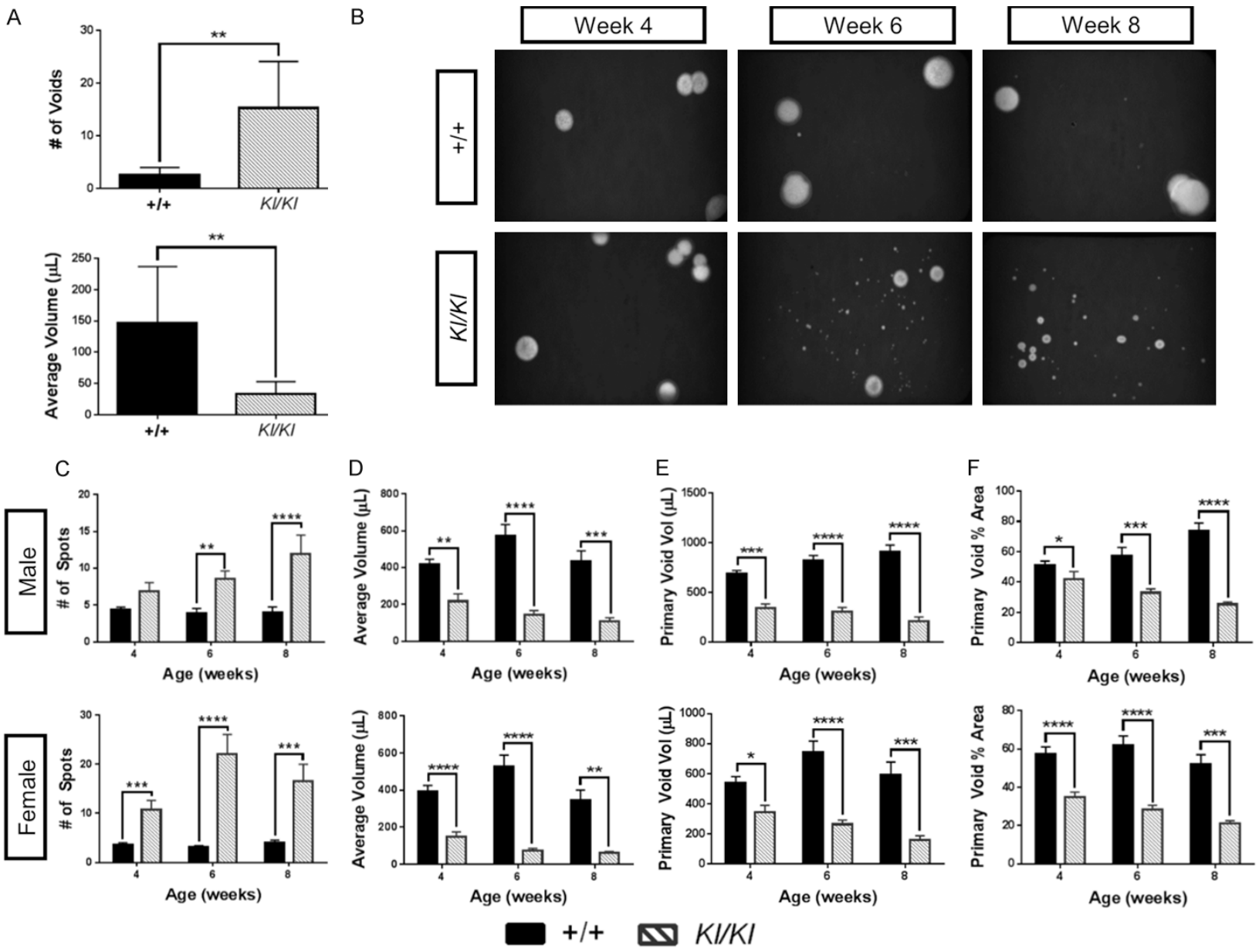
A
# of Voids
Average Volume (µL)
+/+
KI/KI
B
Week 4
Week 6
Week 8
+/+
KI/KI
C
D
E
F
Male
Female
# of Spots
Average Volume (µL)
Primary Void Vol (µL)
Primary Void % Area
Age (weeks)
+/+
KI/KI

Figure 2. *Klotho* mutant mice display decreased volumes of urine with an increase in void frequency. (A) Micturition average volume and frequency was measured by metabolic cages to determine voiding behavior in real time (n = 7 of each genotype). (B) Additionally, void spot assays on filter paper were performed on wild-type and *klotho* mutant mice for 4 hours at weeks 4, 6, and 8 to measure void frequency and volume. Analysis of void spot assays generated by male and female wild-type and *klotho* mutant mice were analyzed to quantify the (C) the number of void spots, (D) average volume per void, (E) volume of primary void, and (F) percent primary void volume of total voided volume. (**P<.05; **P<.01*; ****P<.005*; *****P<.001*).

Table 3. Serum creatinine measurements of 8-week-old mice (average ± SD)

Genotype	Serum Creatinine (ng/mL)	n
+/+	0.554 ± 0.043	12
Kl/+	0.437 ± 0.036	12
Kl/Kl	0.625 ± 0.093	12

No significant difference was determined by Student's t-test.

19.44% ± 4.32%) (**Figure 3A**). To further quantify collagens present in wild-type vs *klotho* mutant bladder we performed picrosirius red staining, in which collagen is stained red. There was no difference observed in the amount of fibrosis detected in the wild-type compared to the *klotho* mutant bladders (75.4% ± 16.4% vs 81.3% ± 10.0%) (**Figure 3A**). With little evidence of alterations in muscle tissue between wild type and *klotho* mutant bladders, we next sought to explore if there is a neurogenic impact on *klotho* mutant mice.

Bladders from *klotho* mutant or wild-type mice were isolated and their contractile responses were measured by detrusor strip stimulation assay (n = 11-14). To determine the effects of neuronal stimulation of the smooth muscle, electrical field stimulation (EFS) was used to selectively activate nerves within the tissue. EFS was administered from 1-50 Hz, tissue contractility was measured, and EFS response was normalized to potassium chloride (KCl)-induced contraction. Bladders of *klotho* mutant mice showed a decrease in response to EFS compared to wild-type at 10 Hz (72.1 ± 7.2 vs 56.6 ± 5.4), and showed a significantly reduced response at 20 Hz (125.6 ± 5.7 vs 101.6 ± 6.4) and 50 Hz (149.6 ± 5.5 vs 131.1 ± 6.0) (**Figure 3B**).

Klotho mutant bladders maintain response to carbachol but have decreased expression of muscarinic receptor subtypes

Next, the tissue response to increasing concentrations of cholinergic agonist carbachol (CCh) was measured. CCh activates the neurotransmitter receptors in the bladder and can evaluate post-junctional neuronal response. No significant differences were seen with CCh-induced contractions in *klotho* mutant compared to wild-type bladders, except in response to 30 μM CCh (120.6 ± 4.3 vs 133.6 ± 3.5) (**Figure 4A**). The implications of the biological significance of this difference is unclear as CCh levels of 30 μM are higher than any naturally occurring, biological comparison of acetylcholine release.

Given that the *klotho* mutant bladders had a decreased contractility response to EFS but not CCh, suggests that there may be a pre-junctional defect in the bladders of *klotho* mutant mice. Therefore, we investigated if the neuromuscular junction receptors were altered. Muscarinic receptors (M_1-M_4) are known to be less abundant in the aging bladder and play a vital role in bladder contraction [2]. Muscarinic receptors located on the urothelium allow for direct contractions by M_3 and inhibition of the relaxation signal (M_2) [21]; M_1 and M_4 are located on the pre-junction neurons in the bladder where they facilitate and inhibit the release of neurotransmitters, respectively [21]. Due to their role in the contractile process, alterations in muscarinic receptors can result in voiding dysfunction. In the bladders of *klotho* mutant mice, the mRNA for all muscarinic receptor subtypes (M_1-M_4) was found to be significantly decreased by about half compared to wild-type bladders (**Figure 4B**).

Discussion

Aging bladder research is challenging as there are many associated comorbidities that can be difficult to mimic in rodent models. None of the existing models recapitulate aging of the human bladder entirely. Using the hypomorphic *klotho* mutant mouse as a premature aging model is attractive in that it mimics most aspects of aging in a short timeframe.

Here, we show that the *klotho* mutant mice have increased voiding frequency and decreased

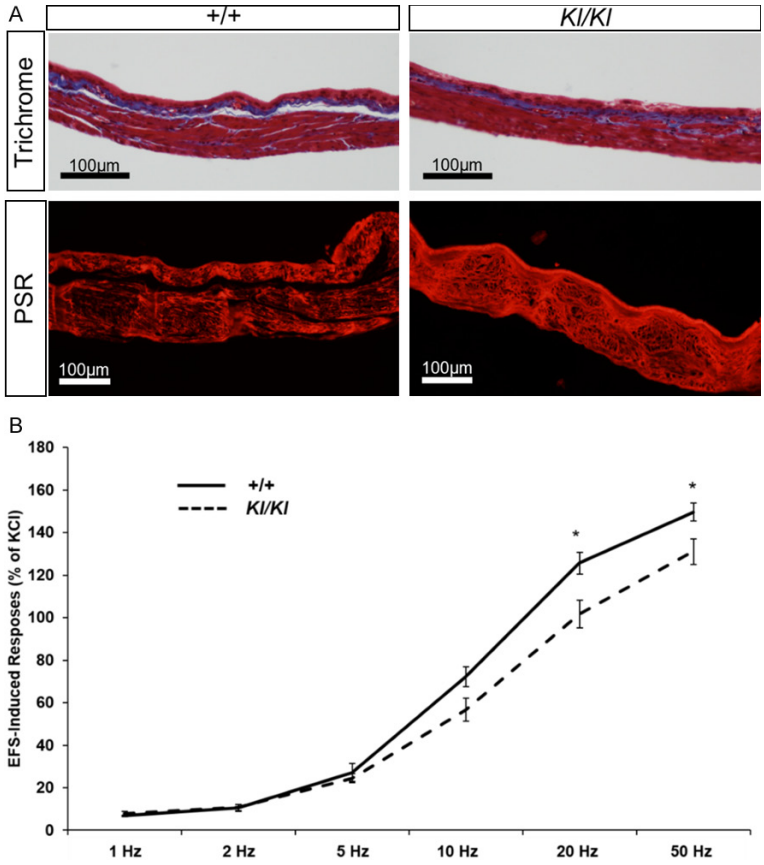

Figure 3. *Klotho* mutant bladders have impaired response to electrical field stimulation in the absence of fibrosis. A. Masson's Trichrome staining was performed to visualize the detrusor muscle (red) and fibrosis (blue) present in bladder tissue sections of wild-type and *klotho* mutant bladders. Picrosirius red staining (bottom) was also performed to visualize collagen (red) presence in the bladders of wild-type and *klotho* mutant mice. B. Bladder strip assays were performed on *klotho* mutant and wild-type mice (n = 11-14) to measure neutrally-evoked bladder contractions. Bladder strips were stimulated by electrical field stimulation from 1-50 Hz and contractile response was measured and normalized to the KCl (137.7 mM) response. (*$P<0.05$).

voiding volume per micturition event, reminiscent of an aging bladder model [22]. While the bladders of *klotho* mutant mice are significantly smaller than wild-type littermates, relative to body weight, the bladder to body mass ratio is the same. The decreased bladder size could account for some of the smaller volume micturition events; however, if the bladder is functioning properly, the voiding patters would closely resemble that of wild-type mice. The aging bladder phenotype progressed as the *klotho* mutant mice grew older with the number of micturition events increasing and average void volume decreasing between 4 and 8 weeks. Therefore, we do not believe the bladder size of *klotho* mutant mice impacts the observed increase in void spots or decreased volume per micturition event.

The observed micturition changes in klotho mutant mice could result from an underlying anatomical, muscular, or neurological defect. No loss of muscle tissue or fibrosis in the form of excessive collagen deposition was detected in the *klotho* mutant bladders. While we cannot rule out altered composition of the extracellular matrix in the absence of klotho, the small bladder size was the only notable histological difference between *klotho* mutant and wild type mice. Stimulation of bladder strips in vitro using CCh, a muscarinic agonist, did not yield altered bladder contractility in klotho mutant mice, however the bladder strips did have a reduced contractile response to EFS. This suggests that there is no post-junctional defect in the klotho mutant bladders, but instead the defect could be pre-junctional.

Muscarinic receptors of the bladder are responsible for the contraction and relaxation of the muscle and when dysregulated can result in bladder dysfunction [21]. There are four muscarinic receptors that play a part in bladder contraction and relaxation: M_1 and M_4 located on the distal efferent nerve endings and M_2 and M_3 located post-junctional, on the detrusor. qPCR analysis demonstrated a loss of all muscarinic receptors in the bladder in the absence of klotho but the effects on the function of the muscarinic receptors has not yet been investigated. A decrease in muscarinic receptor abundance has been previously reported in the bladder of aged rats [23] and in human aging bladders [24]. Pre-junctional M_1 is responsible for contractions of the detrusor muscle by increasing the release of acetylcholine and noradrenaline [25] and its downregulation can in part be attribut-

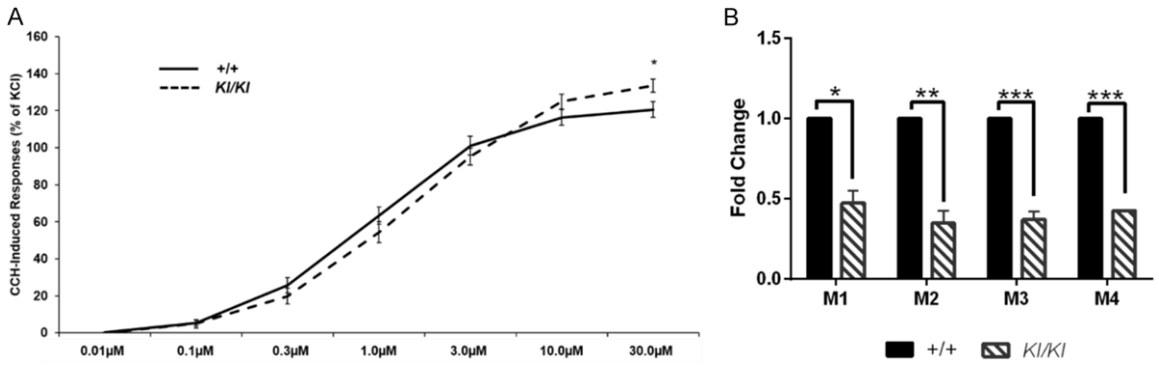

Figure 4. Klotho mutant bladders maintain response to carbachol but have decreased expression of muscarinic receptors. A. Bladder strip assays were performed on *klotho* mutant and wild-type mice (n = 11-14) through stimulation by increasing concentrations of CCh. Contractile response was measured and normalized to the KCl (137.7 mM) response. B. qPCR analysis of muscarinic receptor subtypes 1-4 mRNA was performed on whole bladder lysates from wild-type and *klotho* mutant mice. (**P<.05; **P<.01; ***P<.005*).

ed to inactivation of the upstream JAK2/STAT3 pathway observed in *klotho* mutant mice [26]. Alternatively, M_3 receptors, which rely on extracellular calcium to contract the detrusor muscle [27], could be dysregulated by altered extracellular calcium levels due to the absence of klotho.

There are several limitations to this study. First, klotho is known to play an important role in kidney function [28], so we cannot completely rule out that observed bladder changes are not due to alterations in kidney function. However, serum creatinine, a common measure of kidney function, was normal in *klotho* mutant mice. Future experiments could tease out a more direct role of klotho in the bladder by generating a bladder specific knock-out mouse model. Another limitation is that cystometry could not be performed due to the small size of *klotho* mutant bladders; therefore, all physiology experimentation was done ex vivo.

This study is the first to describe the bladder phenotype of *klotho* mutant mice. We have shown *klotho* mutant mice have increased voiding frequency and decreased voiding volume per micturition event, reminiscent of an aging bladder model. The contraction response to EFS was weaker in the *klotho* mutant mice and muscarinic receptor levels were decreased in *klotho* mutant bladders. These data suggest that these mice have impaired bladder function in part due to a neural prejunctional defect. *Klotho* mutant mice display characteristics of an aging, dysfunctional bladder, including altered bladder function and neural defects. Given the short life span of *klotho* mutant mice, aging bladder dysfunction presents quickly and robustly, allowing this model to provide new insights to drive aging bladder research quicker than using a traditional aging bladder model. Klotho administration via a recombinant adenoviral vector into *klotho* mutant mice resulted in a significant increase in lifespan [29]. Thus, targeting Klotho production or providing exogenous klotho could be used as a possible therapeutic method to rejuvenate the bladder. In addition, as soluble Klotho is detectable in the urine [30] klotho levels could be used as a biomarker for early detection of an aging bladder, allowing for early therapeutic intervention in people that are developing bladder dysfunction. This ability could allow for early therapeutic intervention, reducing the number of individuals admitted to nursing homes due to incontinence.

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Disclosure of conflict of interest

None.

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