Alpha-Klotho, a critical protein for lung health, is not expressed in normal lung

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Abstract

Alpha-Klotho (αKlotho), produced by the kidney and selected organs, is essential for tissue maintenance and protection. Homozygous αKlotho-deficiency leads to premature multi-organ degeneration and death; heterozygous insufficiency leads to apoptosis, oxidative stress, and increased injury susceptibility. There is inconsistent data in the literature regarding whether αKlotho is produced locally in the lung or derived from circulation. We probed murine and human lung by immunohistochemistry (IHC) and immunoblot (IB) using two monoclonal (anti-αKlotho Kl1 and Kl2 domains) and three other common commercial antibodies. Monoclonal anti-Kl1 and anti-Kl2 yielded no labeling in lung on IHC or IB; specific labeling was observed in kidney (positive control) and also murine lungs following tracheal delivery of αKlotho cDNA, demonstrating specificity and ability to detect artificial pulmonary expression. Other commercial antibodies labeled numerous lung structures (IHC) and multiple bands (IB) incompatible with known αKlotho mobility; labeling was not abolished by blocking with purified αKlotho or using lungs from hypomorphic αKlotho-deficient mice, indicating nonspecificity. Results highlight the need for rigorous validation of reagents. The lung lacks native αKlotho expression and derives full-length αKlotho from circulation; findings could explain susceptibility to lung injury in extrapulmonary pathology associated with reduced circulating αKlotho levels, for example, renal failure. Conversely, αKlotho may be artificially expressed in the lung, suggesting therapeutic opportunities.

KEYWORDS
human, immunoblot, immunohistochemistry, inhalational cDNA delivery, mice, monoclonal antibodies

1 INTRODUCTION

The lung interfaces with the exterior via an enormous surface area with constant exposure to pollutants, chemicals, biological toxins, fluctuating temperatures, allergens, microbial pathogens, and the highest oxygen tensions of any internal organ. The lung also receives the entire cardiac output bearing waste products from the periphery. In addition, lung

Jianning Zhang and Khoa Cao share equal contribution.

Abbreviations: IB, immunoblot; IHC, immunohistochemistry; PBS, phosphate-buffered saline; PLGA, poly-lactic-co-glycolic acid.
parenchyma experiences mechanical stress with each respiratory cycle and vascular distention with each cardiac cycle. Thus, the lung has high needs for cytoprotection and is generously bestowed with endogenous and blood-derived antioxidants.\(^1\)\(^-\)\(^4\) One essential cytoprotective protein is αKlotho, a member of the multifunctional Klotho gene family (α, β, and γ).\(^5\) Only αKlotho is secreted into body fluids (blood,\(^6\) cerebrospinal fluid,\(^7\) and urine\(^8\)), and is derived from the cleavage of transmembrane αKlotho by secretases.\(^9\)\(^-\)\(^11\) Transmembrane αKlotho is a co-receptor for the circulating mineral-regulating hormone fibroblast growth factor (FGF)\(^2\)\(^3\)\(^12\)\(^13\), while the released soluble αKlotho serves the antioxidative and cytoprotective functions in distal organs including the lung.\(^14\) Homozygous αKlotho hypomorphic (\(kl/kl\)) mice with negligible plasma αKlotho levels are small in body size and succumb to multi-organ failure at 2-3 months of age.\(^5\) Heterozygous \(kl/+\) mice (one normal allele) with ~50% of normal plasma αKlotho levels have normal lifespan, histology, and function in most organs,\(^5\) except that their lungs show age-exacerbated degenerative changes, air space enlargement, elevated compliance, increased apoptosis\(^15\)\(^,\)\(^16\) and oxidative DNA damage,\(^17\) highlighting the lung’s exquisite sensitivity to circulating αKlotho. Exogenous recombinant αKlotho protects the lung and cultured lung cells from oxidative stress.\(^17\)\(^,\)\(^18\) The highly enriched αKlotho content in human induced pluripotent stem cell secretome significantly contributes to protection of lung cells and lungs from hyperoxic injury.\(^19\) Multiple laboratories have shown direct or indirect αKlotho actions in the lung using in vitro systems.\(^20\)\(^-\)\(^31\) Cumulative literature unequivocally supports a pivotal cytoprotective role of αKlotho in the lung.

Circulating soluble αKlotho is derived mainly from the kidney.\(^32\)\(^,\)\(^33\) αKlotho mRNA and protein are abundantly expressed in distal and to a lesser extent proximal renal tubules.\(^32\)\(^,\)\(^34\) There is controversy concerning whether αKlotho present in the lung is produced by resident lung cells or derived from the circulation. Several lung cell lines show αKlotho mRNA expression by RT-PCR but none express αKlotho protein.\(^21\)\(^,\)\(^26\)\(^,\)\(^29\)\(^,\)\(^31\) On the other hand, Kuro-o and colleagues discovered that αKlotho could not detect αKlotho transcript in the intact lung,\(^5\) a finding independently reproduced by our group.\(^17\) Despite the absence of mRNA, αKlotho protein expression was reported in lungs and large airways by several groups using commercial antibodies.\(^23\)\(^,\)\(^26\)\(^,\)\(^30\) The discrepant in vitro and in vivo results, complicated by uncertain sensitivity and specificity of the various anti-αKlotho antibodies used in different studies, significantly impede progress in the understanding of αKlotho biology.

To resolve the above discrepancies and clarify the source of the documented αKlotho actions in the lung, we probed normal murine and human lungs, lungs from hypomorphic αKlotho-deficient (\(kl/kl\)) mice, and lungs from wild-type mice following inhalational delivery of αKlotho cDNA, using two validated monoclonal antibodies against the KI1 and K12 domains of αKlotho protein, and three other frequently used commercial antibodies. Our aims were twofold: a) To determine the sensitivity and specificity of αKlotho protein detection by immunoprecipitation (IP), immunoblot (IB), and immunohistochemistry (IHC) using commonly available antibodies, and b) To definitively prove whether αKlotho protein is endogenously expressed in the lung.

## METHODS

### 2.1 Animals

All experimental protocols were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. Wild-type and hypomorphic αKlotho-deficient (\(kl/kl\)) mice (129/Sv background)\(^5\)\(^,\)\(^35\) were bred in our laboratory. Human lung and kidney tissues were obtained from the Lung Tissue Research Consortium of the National Heart, Lung and Blood Institute.

The mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg), xylazine (10 mg/kg), and acepromazine (2 mg/kg) killed by exsanguination, and the organs perfused with phosphate-buffered saline (PBS). One kidney was frozen in liquid nitrogen; the other was fixed in 4% paraformaldehyde. The right lung was removed and snap-frozen in liquid nitrogen for immunoblotting. The left lung was fixed by tracheal instillation of 4% paraformaldehyde at a constant airway pressure (25 cmH\(_2\)O).

### 2.2 Antibodies and reagents

Rat monoclonal antibodies (Antibody 1: clone-KM2076, anti-αKlotho K11 domain; Antibody 2: clone-KM2119, anti-αKlotho K12 domain) were generously gifted by Dr Makoto Kuro-o (Jichi Medical University, Tochigi, Japan);\(^36\) these are also available commercially (KO603 and KO604, respectively, TransGenic Inc, Fukuoka, Japan). The other commercial antibodies were: Antibody 3: Rat anti-mouse αKlotho monoclonal MAB1819 (R&D Systems); Antibody 4: Rabbit polyclonal anti-mouse αKlotho ab203576 (Abcam); Antibody 5: Rat monoclonal anti-mouse αKlotho sc74205 (Santa Cruz, Dallas TX). For IP, a synthetic anti-Klotho antibody sb48 (also termed sb106) was used.\(^6\)

Recombinant αKlotho protein containing the ectodomain of mouse αKlotho (amino acid number 31-982) with C-terminal V5 and 6xHis tags was generated and purified in our laboratory in mammalian cells as described previously.\(^37\)

### 2.3 IP and IB

Total lung or kidney lysate was prepared as previously described.\(^37\) Thirty micrograms of protein of lysate was obtained from the Lung Tissue Research Consortium of the National Heart, Lung and Blood Institute. The mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg), xylazine (10 mg/kg), and acepromazine (2 mg/kg) killed by exsanguination, and the organs perfused with phosphate-buffered saline (PBS). One kidney was frozen in liquid nitrogen; the other was fixed in 4% paraformaldehyde. The right lung was removed and snap-frozen in liquid nitrogen for immunoblotting. The left lung was fixed by tracheal instillation of 4% paraformaldehyde at a constant airway pressure (25 cmH\(_2\)O).
solubilized in Laemmli's sample buffer and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. After transferring to polyvinylidene difluoride membranes, proteins were immunoblotted with different primary antibodies and β-actin for loading control. Signal was visualized using the enhanced chemiluminescence (ECL) kit (Perkin-Elmer LAS, Inc).

2.4 | IHC

For epitope retrieval, paraformaldehyde-fixed paraffin-embedded tissue sections were pretreated with 0.01 mol/L citrate buffer (pH 6.0) in a microwave oven for 14 minutes, including a boiling period of 1.5 minutes to enhance antigen retrieval. Tissue sections were washed with PBS (15 minutes), followed by 0.1% TritonX-100 (10 minutes), incubated with a blocking solution (PBS, 3% BSA, 10% donkey serum; 40 minutes), then reacted with the primary antibody or neutralized primary antibodies (4°C overnight). Neutralization of primary antibody was achieved by incubation with purified mouse αklotho (molar ratio of αKlotho protein: antibody 4:1, 22°C × 1 hour). Peptides encompassing the known epitopes for Antibody 1 (peptide FRDTEALR in Kl1 region) and Antibody 2 (peptide LEVQEMTD in Kl2 region) were also used for blocking. After washing with PBS (3 × 20 minutes), sections were incubated with Alexa Fluor 555-coupled donkey anti-rat IgG antibody (Invitrogen, Carlsbad, CA, USA) × 1 hour, counterstained with Alexa Fluor 488-phalloidin (Invitrogen) for filamentous actin and DAPI Fluoromount-G (SouthernBiotech, Birmingham, AL, USA) for nuclei, and examined with a Zeiss LSM880 microscope.

2.5 | Pulmonary αKlotho cDNA delivery

To demonstrate our ability to detect αKlotho expression in the lung, adult 129/Sv mice (5 mo old, Charles River, Wilmington, MA, USA) were anesthetized (ketamine 50 mg/kg, xylazine 5 mg/kg, i.p.) and intubated. Heart rate and oxygen saturation were monitored (Kent Scientific Torrington, CT, USA). Full-length mouse αKlotho with a C-terminal FLAG tag inserted before the stop codon were cloned into expression vector (pEF1/myc-His[A], Life Technologies). αKlotho cDNA or the vector (control) was encapsulated within poly-lactic-co-glycolic acid (PLGA) nanoparticles, kindly prepared by Kytai Nguyen's laboratory (University of Texas Arlington) following established methods.38,39 Nanoparticles (0.2 mg) were suspended in sterile saline (50 µL), sonicated for 2 minutes (300VT ultrasonic homogenizer, BioLogics, Manassas, VA, USA), aerosolized and delivered into the trachea (MicroSprayer Model IA-1C, High Pressure Syringe FMJ-250, Penn-Century, Wyndmoor, PA, USA). A total of nine mice were used. Four mice received αKlotho-containing nanoparticles; two each were studied 4 and 7 days later. Five mice received vector-containing nanoparticles; two were studied after 4 days later and three were studied 7 days later. Mice were deeply anesthetized (ketamine 100 mg/kg, xylazine 10 mg/kg, acepromazine 2 mg/kg, i.p.) and killed by exsanguination. The right lung was removed and snap-frozen in liquid nitrogen. The left lung was fixed by tracheal instillation of 4% paraformaldehyde at a constant airway pressure (25 cmH2O). αKlotho expression in the lungs was probed by IHC and immunoblotting as described above.

3 | RESULTS

3.1 | IB and IP

Mouse lung lysate was immunoblotted with each of the five antibodies. Labeling specificity was tested by preincubation with purified recombinant αKlotho. Kidney lysate served as positive control. The monoclonal Antibodies 1 and 2 did not show labeling with lung lysate regardless of the exposure times while Antibodies 3, 4, and 5 labeled multiple bands that are not compatible with αKlotho's electrophoretic mobility (a broad band approximately 130 kD; Figure 1A). Moreover, labeling by Antibodies 3-5 was not blocked by incubation with purified αKlotho protein (Figure 1A). To test if αKlotho is expressed in limited regions or the signal may be “diluted” in whole lung lysates, we isolated peripheral and central lung regions for IB by Antibody 1 and again observed no specific staining even though strong labeling was present in the kidney lysate on the same blot (Figure 1B). To test if αKlotho expression is age-dependent, we performed IB on lungs from neonatal mice (day P5) with Antibody 1 and observed no staining in the lung either (data not shown). To ensure that low protein abundance is not the reason for the lack of labeling by Antibody 1, lysate from the entire mouse lung was immunoprecipitated with asynthetic anti-Klotho antibody sb48 (previously termed sb106) known to perform well with the lung and kidney lanes for long (lung) and short (kidney) exposure, and we did not observe any signal from the lung (data not shown). The monoclonal Antibodies 3, 4, and 5 yielded inconsistent and uninterpretable results in kidney and lung.

3.2 | IHC

Several published reports described αKlotho expression in lung parenchyma by IHC and very intense
**Figure 1** Immunoprecipitation-immunoblot and for αKlotho in murine lung and A549 lung epithelial cells. Studies were performed with five antibodies. **Antibody 1:** Rat monoclonal Anti-αKlotho Kl1 (KM2076). **Antibody 2:** Rat monoclonal Anti-αKlotho Kl2 (KM2119). **Antibody 3:** R&D Cat# MAB1819; **Antibody 4:** Abcam Cat# ab203576; **Antibody 5:** Santa Cruz Cat# sc74205. Three wild-type mice were used. (A), Representative immunoblot of lung lysate and A549 lung epithelial cell line probed with each antibody and blockade with purified recombinant αKlotho. Kidney lysate served as positive control. (B), Representative immunoblot of αKlotho in samples taken from different regions of the lung and probed using Antibody 1. Kidney lysate served as positive control and the heart as negative control. (C), Representative immunoprecipitation (IP)-immunoblot (IB) of αKlotho immunoprecipitated from lung lysate of two animals using a well validated synthetic anti-αKlotho antibody sb48 (synonymous with sb106)⁶, and blotted with Antibodies 1 to 5. In (B) and (C), both a short and a long exposure are shown (left and right panels, respectively). Kidney lysate served as positive control.
labeling particularly in the airways using various commercial antibodies. As in the case of IB and IP (Figure 1), no αKlotho labeling was detected in wild-type mouse lung by IHC using Antibody 1 or 2, contradicting the widespread labeling observed with Antibody 3, 4, or 5 (Figure 2A, upper row). Use of standard antigen retrieval techniques could not bring out staining by Antibody 1 (not shown). Preincubation of the antibodies with purified recombinant αKlotho did not abolish labeling by commercial Antibody 3, 4, or 5 except for minor diminution of labeling by Antibody 3 (Figure 2A, lower row), indicating that these are likely nonspecific binding.

Further testimony on the specificity, or lack thereof, of these antibodies was provided by the IHC results in the kl/kl hypomorphs that express negligible αKlotho. As in wild-type mice, there was no staining in the lung of kl/kl mice using Antibody 1 or 2, suggesting again that the positive signal

**FIGURE 2** Immunohistochemistry for αKlotho expression in the lung and kidney of wild-type and kl/kl hypomorphic αKlotho-deficient mice. Studies were performed with five antibodies. Antibody 1: Rat monoclonal Anti-αKlotho K1 (KM2076). Antibody 2: Rat monoclonal Anti-αKlotho K2 (KM2119). Antibody 3: R&D Cat# MAB1819; Antibody 4: Abcam Cat# ab203576; Antibody 5: Santa Cruz Cat# sc74205. Three wild-type and 2 kl/kl mice were used. (A), Representative images of lungs from wild-type mice probed with the five antibodies (upper row) and blockade with purified recombinant αKlotho (lower row). (B), Representative images of lungs probed with the five antibodies in wild-type (upper row) and kl/kl (lower row) mice. (C), Kidney sections served as control for αKlotho expression probed by IHC using five antibodies in wild-type (upper two rows) and homozygous hypomorphic kl/kl (lower 2 rows) mice, with and without blockade by incubation with purified recombinant αKlotho. Bar = 100 μm
observed in these lungs using Antibody 3, 4, or 5 is nonspecific (Figure 2B). Furthermore, the positive labeling of kidney in wild-type mice using Antibody 1 or 2 was completely blocked by recombinant αKlotho, attesting to their specificity, whereas the positive labeling using Antibody 3, 4, or 5 was not blocked (Figure 2C, upper two rows). If αKlotho is genuinely expressed in the lung, αKlotho labeling should be decreased in kl/kl mice as its 5′-flanking region is disrupted. Indeed, a lack of αKlotho labeling in kidney sections from kl/kl mice was demonstrated by IHC using Antibodies 1 or 2. In contrast, widespread labeling was still observed using Antibodies 3, 4, and 5 that were not blocked by recombinant αKlotho (Figure 2C, lower 2 rows).

To exclude the possibility that peculiarities of the lung precludes Antibody 1 or 2 from reacting with αKlotho and that our results may represent false negatives, we examined a situation when αKlotho protein is artificially expressed in murine lung by tracheal delivery of nanoparticles bearing αKlotho cDNA (Figure 3). We previously validated this inhalational approach showing successful pulmonary cDNA delivery and sustained expression and bioactivity of the erythropoietin receptor.38 At 4 and 7 days following αKlotho cDNA delivery, αKlotho protein expression was clearly detected by IB using Antibody 1 or 2, showing the characteristic 130 kD band while no 130 kD signal was observed in the lungs of vector-treated animals (Figure 3A). Similarly, in animals treated with αKlotho cDNA, intense positive αKlotho labeling in lung tissue was detected by IHC using Antibodies 1 or 2 (Figure 3B). αKlotho protein expression in lung cells may be artificially induced and specifically detected using Antibodies 1 and 2.
We extended these studies from murine to human lung and kidney tissue, by probing with each of the five antibodies and blocking by incubation with purified αKlotho protein (Figure 4). In adult human lung (upper 2 rows), no labeling was observed with Antibody 1 or 2, whereas nonspecific staining was observed using Antibody 3, 4, or 5 that was not blocked. In human kidney (lower 2 rows), specific labeling was observed using Antibody 1 or 2 that was blocked by incubation with purified αKlotho protein, whereas the positive labeling by Antibody 3, 4, or 5 was not blocked. These results show that native αKlotho expression was similarly present in both murine and human kidneys and similarly absent in murine and human lungs.
4 | DISCUSSION

4.1 | Summary of the findings

We present novel data disproving the notion of native αKlotho protein expression in murine and human lung by meticulously ensuring that the monoclonal Antibodies 1 (anti-K11, KM2076) and 2 (anti-K12, KM2119) are sensitive and specific in detecting αKlotho natively expressed in the kidney and artificially expressed in the lung. We further demonstrate the nonspecificity of several commercial anti-αKlotho antibodies commonly used in previously published studies that reported positive αKlotho expression in lung tissue. While Antibodies 1 and 2 yielded no staining on IHC, the other antibodies labeled numerous lung structures. While IB using Antibodies 1 and 2 was negative in murine lung, the other antibodies produced multiple bands incompatible with the electrophoretic mobility of full-length αKlotho that were not blocked by preincubation with purified recombinant αKlotho protein or using lung tissue from kl/kl hypomorphic mice, arguing against the specificity of Antibodies 3, 4, and 5. In contrast, labeling with Antibody 1 or 2 in the kidney, which is known to express abundant αKlotho, was completely abrogated by purified αKlotho protein, indicating specificity. We conclude that the lung normally does not express full-length αKlotho; the unequivocal findings of αKlotho-mediated in vivo cytoprotection in the lung is derived from circulating αKlotho produced mainly by the kidney or exogenous

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FIGURE 4  Immunohistochemistry of αKlotho expression in human lung and kidney. Paraformaldehyde-fixed human lung and kidney sections from two subjects were probed with five antibodies; specificity was tested by preincubation with purified recombinant αKlotho. Antibody 1: Rat monoclonal Anti-αKlotho K11 (KM2076). Antibody 2: Rat monoclonal Anti-αKlotho K12 (KM2119). Antibody 3: R&D Cat# MAB1819; Antibody 4: Abcam Cat# ab203576; Antibody 5: Santa Cruz Cat# sc74205. Representative images are shown. Bar = 100 µm
soures including the delivery of conditioned media of human induced pluripotent stem cells that are enriched in αKlotho content. Nevertheless, pulmonary αKlotho protein may be experimentally expressed by targeted delivery of αKlotho cDNA to the lung and specifically detected using commercially available monoclonal anti-Ki1 and anti-KI2 antibodies. We cannot rule out the possibility that αKlotho taken up by the lung from the circulation can be processed in lung cells to smaller fragments. These results highlight the need for rigorous validation of each reagent used in αKlotho research to avoid reaching erroneous conclusions.

4.2 Organ-specific αKlotho expression

In addition to the kidney, αKlotho is endogenously expressed in the parathyroid gland, brain, breast, gonads, and sino-atrial node. Some organs do not express αKlotho but clearly derive benefits from circulating αKlotho. For example, the myocardium does not express αKlotho but clearly derive benefits from circulating αKlotho. Circulating αKlotho protects cells, αKlotho activates the Nrf2 network of antioxidant factor regulation, ion transport, mineral metabolism, proteins to alleviate injury. Circulating αKlotho protects pulmonary complications independent of the severity of kidney injury. By inference, age-related decline in renal αKlotho synthesis may also heighten susceptibility to lung injury in the elderly.

4.4 αKlotho detection

Endogenous αKlotho transcripts have been detected in cultured lung cells transfected with αKlotho cDNA; yet none of the lung cell lines express αKlotho at baseline. Li et al performed IHC using an unspecified polyclonal anti-αKlotho antibody, and reported co-localization with alveolar macrophages and decreased staining in lungs of smokers and patients with chronic obstructive pulmonary disease (COPD); however, control specimens showed high background intensity, and specificity of staining was not established. Gao et al performed IHC and IB with commercial antibodies reporting intense αKlotho staining in the lungs of healthy nonsmokers compared to markedly reduced staining in smokers and COPD patients, whereas αKlotho staining intensity was similar in ozone-exposed mice compared to air-exposed controls. Also using commercial anti-αKlotho antibodies, αKlotho staining was detected by IHC and IB in bronchial epithelium of cystic fibrosis patients. However, in that study the two detected bands (~65 and ~80 kD) were below the expected size of full-length/secreted αKlotho (~130 kD) and no control IHC was shown. Furthermore, the detected bands are inconsistent with the single band (130 kD) shown in another study by the same group using the same antibody that probed airway epithelia from COPD patients. Usuda et al using monoclonal anti-KI1 (KM2076) detected αKlotho expression by IHC in 33% of resected lung cancer specimens, and suggested that expression predicts good outcome. There was no description or data validating antibody specificity in the above studies.

Both transmembrane and secreted full–length αKlotho are glycoproteins migrating around ~130 kD. A 65-70 kD band reported in the literature likely represents fragments of αKlotho.
containing the KI1 domain. Compatible with our findings in Klotho protein is the fact that we were unable to detect Klotho mRNA in normal murine lungs. In the Protein Atlas database from the Human Protein Atlas, Klotho mRNA is reported to be present at very low levels. The small discrepancy between findings in these two studies is likely due to different amounts of tissue, PCR primers, conditions, and cycle numbers. As the earlier reported presence of αKlotho mRNA expression by RT-PCR in alveolar macrophages could not be confirmed by protein expression, it may be that partially processed transcripts are primed and amplified by the highly sensitive RT-PCR but not translated, whereas the existence of a short αKlotho protein translated from alternatively spliced transcript published in earlier studies may represent illegitimate splicing as has been reported for other genes. There is good evidence that αKlotho mRNA may be transcribed but not translated into protein. Mencke et al provided convincing data that the so-called “spliced Klotho mRNA” is actually destined for degradation by nonsense-mediated mRNA decay and not translated into protein. RT-PCR is very sensitive and could amplify non-specific targets especially when a high cycle number is used. Thus, it is risky to draw conclusions based solely on the presence of αKlotho mRNA without corroboration by the corresponding protein expression.

Our previous study demonstrated the sensitivity and specificity of anti-KI1 (KM2076) for detecting serum αKlotho by IP and IB. Multiple synthetic anti-αKlotho antibodies, for example, sb106 (now called sb48) have been developed for use in immunoprecipitation and on unfixed cells; however, synthetic antibodies only detect native nondenatured αKlotho and cannot detect denatured proteins by IHC and IB even in the kidney with its abundant αKlotho expression. The ability of sb48 (sb106) to label transfected cells may be due to the highly abundant overexpression but a major reason is explained by lack of fixation. In contrast, KM2076 and KM2019 are well proven to label denatured Klotho. Enzyme-linked immunosorbent assays (ELISA) are known to exhibit suboptimal specificity and sensitivity in detecting αKlotho in serum and cannot detect αKlotho in tissue lysates. To date, no laboratory including ours has been able to detect native αKlotho by IP followed by mass spectrometry using validated antibodies (including KM2076 and KM2019) from any tissue including the kidney.

4.5 Significance of absent endogenous αKlotho expression in the lung

Our finding of the absence of native αKlotho production in the normal lung carries significant physiological impact. Karl Popper logically emphasized that no amount of positive experimental outcomes can absolutely confirm a scientific theory, but a single reproducible counterproof is decisive in showing the theory to be incorrect. Not only is a negative finding just “as true” as a positive one, it actually possesses greater power in supporting conclusions. By resolving the controversy regarding the source of αKlotho in the lung, these results permit accurate data interpretation of studies designed to elucidate the organ-specific mechanisms of action of this essential protein.

Like the myocardium, the lung depends heavily on circulating αKlotho for maintenance and protection, rather than being equipped with its own αKlotho protein expression. The lack of native local αKlotho production does not diminish the biological importance of αKlotho in lung homeostasis or contradict the cumulative literature overwhelmingly supporting αKlotho as essential for lung health. Given the continuous physico-chemical insults imposed on the lung, it is not surprising that local endogenous cytoprotective mechanisms need to be supplemented by circulating factors such as kidney-derived αKlotho to neutralize the toxicity of blood-borne whole-body waste products traversing the lung. As renal αKlotho production declines with age and disease, an imbalance between pulmonary toxin delivery and cytoprotective capacity is created that predisposes to lung degeneration and dysfunction. Local or systemic diseases, for example, diabetes mellitus and cardiovascular disorders, that cause renal impairment further reduce αKlotho synthesis and accelerate widespread age-exacerbated organ degeneration in the lung. Primary acute or chronic lung disease may secondarily impair renal function and reduce circulating αKlotho level, thereby aggravating lung degeneration in a vicious cycle. Thus, circulating αKlotho delivery to the lung is a plausible mechanism of pulmonary-renal crosstalk and explains an important aspect of reciprocal interdependence between these two organs.

4.6 Conclusions

We provide unequivocal proof that a) αKlotho protein is not normally expressed in murine or human lung tissue, although expression may be artificially induced; b) therefore, the biological actions of αKlotho on the lung is normally derived from circulating αKlotho and c) the reported αKlotho detection by several commonly used commercial antibodies are nonspecific artefacts. These results resolve a major controversy of pulmonary αKlotho expression, and promote valid methodology and accurate data interpretation for future studies in this emergent field. It is absolutely critical to advance the current understanding of the role of αKlotho in pulmonary physiology and pathobiology; however, validated reagents must be used and these are accessible to all investigators. In addition, the validity of any new reagent for elucidating αKlotho expression must be rigorously established to avoid reaching erroneous
conclusions. This caveat broadly applies to the study of αKlotho biology in extrapulmonary organs as in the lung. In terms of physiological significance, the dependence of the lung on extrapulmonary source of αKlotho for health maintenance and cytoprotection heightens the susceptibility to lung injury from either direct insult or secondary development of acute respiratory distress syndrome in renal failure or other extrapulmonary diseases associated with reduced circulating αKlotho levels. Whether there is local pulmonary expression of αKlotho in pathological states remains to be investigated. Finally, the ability to artificially express αKlotho in the lung via inhalational cDNA delivery holds promise for noninvasive translational interventions designed to fortify αKlotho-mediated cytoprotection in the lung.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Jianning Zhang, Khoa Cao, Liping Li and Johanne V. Pastor performed experiments, and analyzed and interpreted the data. Orson W. Moe and Connie CW Hsia conceived and designed the project, supervised the performance of experiments and analyzed the data. Orson W. Moe and Connie CW Hsia conceived and performed experiments, and analyzed and interpreted the data. Orson W. Moe and Connie CW Hsia conceived and performed experiments, and analyzed and interpreted the data.

REFERENCES

1. Hsia C, Ravikumar P, Ye J. Acute lung injury complicating acute kidney injury: A model of endogenous alphaKlotho deficiency and distant organ dysfunction. Bone. 2017;100(Special Issue: Kidney and Bone):100-109.
2. Poljsak B, Fink R. The protective role of antioxidants in the defence against ROS/RNS-mediated environmental pollution. Oxid Med Cell Longev. 2014;2014:671539.
3. Valavanidis A, Vlachogianni T, Fiotakis K, Loridas S. Pulmonary oxidative stress, inflammation and cancer: respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms. Int J Environ Res Public Health. 2013;10:3886-3907.
4. Araneda OF, Tuesta M. Lung oxidative damage by hypoxia. Oxid Med Cell Longev. 2012;2012:856918.
5. Kuro-o M, Matsumura Y, Aizawa H, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature. 1997;390:45-51.
6. Barker SL, Pastor J, Carranza D, et al. The demonstration of alphaKlotho deficiency in human chronic kidney disease with a novel synthetic antibody. Nephrol Dial Transplant. 2015;30:223-233.
7. Imura A, Iwano A, Tohyama O, et al. Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. FEBS Lett. 2004;565:143-147.
8. Hu MC, Shi M, Zhang J, Quinones H, Kuro-o M, Moe OW. Klotho deficiency is an early biomarker of renal ischemia-reperfusion injury and its replacement is protective. Kidney Int. 2010;78:1240-1251.
9. Chen CD, Tung TY, Liang J, et al. Identification of cleavage sites leading to the shed form of the anti-aging protein klotho. Biochemistry. 2014;53:5579-5587.
10. Chen CD, Podvin S, Gillespie E, Leeman SE, Abraham CR. Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. Proc Natl Acad Sci USA. 2007;104:19796-19801.
11. Bloch L, Sineshchekova O, Reichenbach D, et al. Klotho is a substrate for alpha-, beta- and gamma-secretase. FEBS Lett. 2009;583:3221-3224.
12. Urakawa I, Yamazaki Y, Shimada T, et al. Klotho converts canonical FGF receptor into a specific receptor for FGF23. Nature. 2006;444:770-774.
13. Kurosu H, Ogawa Y, Miyoshi M, et al. Regulation of fibroblast growth factor-23 signaling by klotho. J Biol Chem. 2006;281:6120-6123.
14. Hu MC, Shizaki K, Kuro-o M, Moe OW. Fibroblast growth factor 23 and Klotho: physiology and pathophysiology of an endocrine network of mineral metabolism. Annu Rev Physiol. 2013;75:503-533.
15. Suga T, Kurabayashi M, Sando Y, et al. Disruption of the klotho gene causes pulmonary emphysema in mice. Defect in maintenance of pulmonary integrity during postnatal life. Am J Respir Cell Mol Biol. 2000;22:26-33.
16. Ishii M, Yamaguchi Y, Yamamoto H, Hanaoka Y, Ouchi Y. Airspace enlargement with airway cell apoptosis in klotho mice: a model of aging lung. J Gerontol A Biol Sci Med Sci. 2008;63:1289-1298.
17. Ravikumar P, Ye J, Zhang J, et al. alpha-Klotho protects against oxidative damage in pulmonary epithelia. Am J Physiol Lung Cell Mol Physiol. 2014;307:L566-L575.
18. Ravikumar P, Li L, Ye J, et al. alphaKlotho deficiency in acute kidney injury contributes to lung damage. J Appl Physiol (1985). 2016;120:723-732.
19. Gazdhar A, Ravikumar P, Pastor J, et al. Alpha-Klotho enrichment in induced pluripotent stem cell secretome contributes to antioxidative protection in acute lung injury. Stem Cells. 2018;36:616-625.
20. Chen B, Wang X, Zhao W, Wu J. Klotho inhibits growth and promotes apoptosis in human lung cancer cell line A549. J Exp Clin Cancer Res. 2010;29:99.
21. Chen B, Ma X, Liu S, Zhao W, Wu J. Inhibition of lung cancer cells growth, motility and induction of apoptosis by Klotho, a novel secreted Wnt antagonist, in a dose-dependent manner. *Cancer Biol Ther.* 2012;13:1221-1228.

22. Wang Y, Chen L, Huang G, et al. Klotho sensitizes human lung cancer cell line to cisplatin via PI3k/Akt pathway. *PLoS ONE.* 2013;8:e57391.

23. Gao W, Yuan C, Zhang J, et al. Klotho expression is reduced in COPD airway epithelial cells: effects on inflammation and oxidant injury. *Clin Sci (Lond).* 2015;129:1011-1023.

24. Shin IS, Shin HK, Kim JC, Lee MY. Role of Klotho, an antiaging protein, in pulmonary fibrosis. *Arch Toxicol.* 2015;89:785-795.

25. Li L, Wang Y, Gao W, et al. Klotho reduction in alveolar macrophages contributes to cigarette smoke extract-induced inflammation in chronic obstructive pulmonary disease. *J Biol Chem.* 2015;290:27890-27900.

26. Ibi T, Usuda J, Inoue T, Sato A, Takeghara K. Klotho expression is correlated to molecules associated with epithelial-mesenchymal transition in lung squamous cell carcinoma. *Oncof Lett.* 2017;14:5526-5532.

27. Kim SJ, Cheresh P, Eren M, et al. Klotho, an antiaging molecule, attenuates oxidant-induced alveolar epithelial cell mtDNA damage and apoptosis. *Am J Physiol Lung Cell Mol Physiol.* 2017;313:L16-L26.

28. Li L, Zhang M, Zhang L, Cheng Y, Tu X, Lu Z. Klotho regulates cigarette smoke-induced autophagy: implication in pathogenesis of COPD. *Lung.* 2017;195:295-301.

29. Chen B, Liang Y, Chen L, et al. Overexpression of Klotho inhibits HELF fibroblasts SARS-related protumoral effects on non-small cell lung cancer cells. *J Cancer.* 2018;9:1247-1258.

30. Krick S, Grabner A, Baumlin N, et al. Fibroblast growth factor 23 and Klotho contribute to airway inflammation. *Eur Respir J.* 2018;52:1800236.

31. Blake DJ, Reese CM, Garcia M, Dahlmann EA, Dean A. Soluble extracellular Klotho decreases sensitivity to cigarette smoke induced cell death in human lung epithelial cells. *Toxicol In Vitro.* 2015;29:1647-1652.

32. Hu MC, Shi M, Zhang J, et al. Renal production, uptake, and handling of circulating alphaKlotho. *J Am Soc Nephrol.* 2016;27:79-90.

33. Lindberg K, Amin R, Moe OW, et al. The kidney is the principal organ mediating klotho effects. *J Am Soc Nephrol.* 2014;25:2169-2175.

34. Hu MC, Shi M, Zhang J, et al. Klotho: a novel phosphaturic protein, in pulmonary fibrosis. *Arch Toxicol.* 2015;89:785-795.

35. Liu L, Liu X, Zhang Y, et al. High phosphate-induced downregulation of PPARgamma contributes to CKD-associated vascular calcification. *J Mol Cell Cardiol.* 2018;114:264-275.

36. Nakahara T, Kawai-Kowase K, Matsui H, et al. Fibroblast growth factor 23 inhibits osteostatic gene expression and induces osteoprotogerin in vascular smooth muscle cells. *Atherosclerosis.* 2016;253:102-110.

37. Mencke R, Harms G, Mirković K, et al. Membrane-bound Klotho is not expressed endogenously in healthy or uraemic human vascular tissue. *Cardiovasc Res.* 2015;108:220-231.

38. Yamada S, Giachelli CM. Vascular calcification in CKD-MBD: roles for phosphate, FGF23, and Klotho. *Bone.* 2017;100:87-93.

39. Chang JR, Guo J, Wang Y, et al. Intermedin1-53 attenuates vascular calcification in rats with chronic kidney disease by upregulation of alpha-Klotho. *Kidney Int.* 2016;89:586-600.

40. Hu MC, Shi M, Zhang J, et al. Klotho deficiency causes vascular calcification in chronic kidney disease. *J Am Soc Nephrol.* 2011;22:124-136.

41. Hu MC, Kuro-o M, Moe OW. alphaKlotho and vascular calcification: an evolving paradigm. *Curr Opin Nephrop Hypertens.* 2014;23:331-339.

42. Yamamoto M, Clark JD, Pastor JV, et al. Regulation of oxidative stress by the anti-aging hormone klotho. *J Biol Chem.* 2005;280:38029-38034.

43. Hu MC, Kuro-o M, Moe OW. Renal and extrarenal actions of Klotho. *Semin Nephrol.* 2013;33:118-129.

44. Cha SK, Hu MC, Kurosu H, Kuro-o M, Moe O, Huang CL. Regulation of renal outer medullary potassium channel and renal K(+) excretion by Klotho. *Mol Pharmacol.* 2009;76:38-46.

45. Semba RD, Moghekar AR, Hu J, et al. Expression of klotho mRNA and protein in rat brain parenchyma from early postnatal development into adulthood. *Brain Res.* 2013;1527:1-14.

46. Wolf I, Levanon-Cohen S, Bose S, et al. Klotho: a tumor suppressor and a modulator of the IGF-1 and FGF pathways in human breast cancer. *Oncogene.* 2008;27:7094-7105.

47. Li SA, Watanabe M, Yamada H, Nagai A, Kinuta M, Takei K. Immunohistochemical localization of Klotho protein in brain, kidney, and reproductive organs of mice. *Cell Struct Funct.* 2004;29:91-99.

48. Takeshita K, Fujimori T, Kurotaki Y, et al. Sinoatrial node dysfunction and early unexpected death of mice with a defect of klotho gene expression. *Circulation.* 2004;109:1776-1782.

49. Hu MC, Shi M, Cho HJ, et al. Klotho and phosphate are modulators of pathologic uremic cardiac remodeling. *J Am Soc Nephrol.* 2015;26:1290-1302.

50. Cheng L, Zhang L, Yang J, Hao L. Activation of peroxisome proliferator-activated receptor gamma inhibits vascular calcification by upregulating Klotho. *Exp Ther Med.* 2017;13:467-474.

51. Liu L, Liu Y, Zhang Y, et al. High phosphate-induced downregulation of PPARgamma contributes to CKD-associated vascular calcification. *J Mol Cell Cardiol.* 2018;114:264-275.
59. Tang X, Wang Y, Fan Z, et al. Klotho: a tumor suppressor and modulator of the Wnt/beta-catenin pathway in human hepatocellular carcinoma. Lab Invest. 2016;96:197-205.

60. Shi M, Flores B, Gillings N, et al. alphaKlotho mitigates progression of AKI to CKD through activation of autophagy. J Am Soc Nephrol. 2016;27:2331-2345.

61. Kusaba T, Okigaki M, Matui A, et al. Klotho is associated with VEGF receptor-2 and the transient receptor potential canonical-1 Ca2+ channel to maintain endothelial integrity. Proc Natl Acad Sci USA. 2010;107:19308-19313.

62. Kuro-o M. Klotho as a regulator of oxidative stress and senescence. Biol Chem. 2008;389:233-241.

63. Hu MC, Shi M, Cho HJ, et al. The erythropoietin receptor is a downstream effector of Klotho-induced cytoprotection. Kidney Int. 2013;84:468-481.

64. Nagai R, Saito Y, Ohyama Y, et al. Endothelial dysfunction in the klotho mouse and downregulation of klotho gene expression in various animal models of vascular and metabolic diseases. Cell Mol Life Sci. 2000;57:738-746.

65. Saito Y, Nakamura T, Ohyama Y, et al. In vivo klotho gene delivery protects against endothelial dysfunction in multiple risk factor syndrome. Biochem Biophys Res Commun. 2000;276:767-772.

66. Krick S, Baumlin N, Aller SP, et al. Klotho inhibits interleukin-8 secretion from cystic fibrosis airway epithelia. Sci Rep. 2017;7:14388.

67. Usuda J, Ichinose S, Ishizumi T, et al. Klotho predicts good clinical outcome in patients with limited-disease small cell lung cancer who received surgery. Lung Cancer. 2011;74:332-337.

68. Han X, Li L, Yang J, King G, Xiao Z, Quarles LD. Counter-regulatory paracrine actions of FGF-23 and 1,25(OH)2 D in macrophages. FEBS Lett. 2016;590:53-67.

69. The Human Protein Atlas. https://www.proteinatlas.org/ENSG00000133116-KL/tissue/lung.

70. Cooper DN, Berg LP, Kakkar VV, Reiss J. Ectopic (illegitimate) transcription: new possibilities for the analysis and diagnosis of human genetic disease. Ann Med. 1994;26:9-14.

71. Wimmer K, Eckart M, Rehder H, Fonatsch C. Illegitimate splicing of the NF1 gene in healthy individuals mimics mutation-induced splicing alterations in NF1 patients. Hum Genet. 2000;106:311-313.

72. Mencke R, Harms G, Moser J, et al. Human alternative Klotho mRNA is a nonsense-mediated mRNA decay target inefficiently spliced in renal disease. JCI Insight. 2017;2(20):e94375. https://doi.org/10.1172/jci.insight.94375

73. Neyra JA, Moe OW, Pastor J, et al. Performance of soluble Klotho assays in clinical samples of kidney disease. Clin Kidney J. 2019;1:1-10. https://doi.org/10.1093/ckj/sfz085

74. Popper K. The logic of scientific discovery. Abingdon-on-Thames: Routledge; 1959.

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