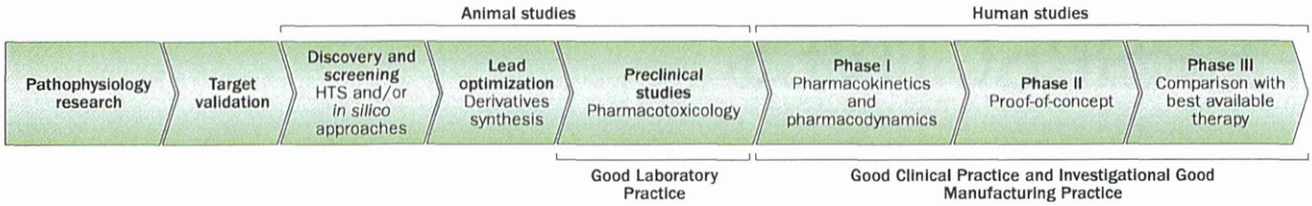


**Figure 1** | Procedures for drug discovery and clinical development. Research into disease pathophysiology enables identification and validation of target molecules. Numerous lead compounds are then identified using HTS or *in silico* approaches, such as structure based drug design, and a programme of structural optimization is launched. The physical and toxicological properties of the various compounds are tested in animals before selection of the candidate compound for clinical trials. Phase I trials focus on the pharmacokinetics and pharmacodynamics of the agent in humans, whereas efficacy is tested only in proof-of-concept phase II trials. Phase III trials are used to compare the efficacy of the new agent with that of the best available treatment. All procedures must be conducted according to pharmaceutical regulations (that is, Good Clinical Practice, Good Laboratory Practice and Good Manufacturing Practice). Abbreviation: HTS, high-throughput screening.

can be used mainly to investigate a variety of parameters such as pharmacokinetics and pharmacodynamics. On 11 June 2009, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use issued new guidance on 'exploratory clinical trials' and recommended the use of various validated biomarkers and molecular imaging technologies, such as positron emission tomography.[15] These techniques might enable early assessment of the distribution of drugs within the kidney and of their physiological and pharmacological effects. Trial participants can be patients from selected populations or healthy individuals, and the amount and type of nonclinical supporting data that is required is dependent on the extent of the proposed exposure to the new agent. Undoubtedly, use of biomarkers (Box 1) and new imaging methods (Box 2) would lower costs and, therefore, enable a greater number of promising compounds for the treatment of kidney diseases to be investigated than at present, increasing the likelihood of eventual approval.

## The role of academia

Currently, researchers in academia can undertake the entire research and development process for new drugs, from pathophysiological investigation to discovery of target molecules, identification of candidate compounds (using *in silico* approaches; Box 3), lead optimization, preclinical studies and exploratory clinical trials. They should select promising compounds that are active in human physiology and pharmacology, and provide the pharmaceutical industries with useful data for further full-scale development of novel drugs. Fortunately, academic researchers have access not only to basic science and technologies but also to various contracting research organizations. With proper efforts, universities might progress from target validation to identification of candidate compounds, Good Manufacturing Practice (GMP) synthesis and formulation, nonclinical Good Laboratory Practice (GLP) studies and phase I and phase II Good Clinical Practice (GCP) studies in humans. With government support, researchers have a solid infrastructure for drug discovery. However, a cost-effective drug-discovery framework is necessary to enable high-quality materials and data to be obtained within the context of limited budgets, labour and time.

### International research networks

In contrast to research in industry, which is not fundamentally based on open innovation and is closed in nature, drug development initiated in academic institutions benefits from many international research networks. If high-quality material (produced according to GMP guidelines) and GLP safety data are available, clinical trials might be conducted, in principle, through these international networks. Making materials and data available as open resources would facilitate this undertaking. Several initiatives have been launched to aid this process, including the Oxford University Structural Genomics Consortium, which aims to solve the structures of human proteins of medical relevance and place them into the public domain without restriction.[16]

Key to the goal of conducting clinical trials through international networks is the provision of internationally acceptable, high-quality nonclinical data packages and bulk investigational drugs for clinical trials. We are currently developing a new orally active, low-molecular-weight inhibitor of plasminogen activator inhibitor (PAI-1), which might offer a novel therapeutic strategy in renal and cardiovascular diseases.[17] In addition to antithrombotic action,[18,19] this drug stimulates regeneration of bone marrow[20] and blood vessels,[21] attenuates vascular senescence,[22] and has antifibrotic,[18,23] and anti-inflammatory[24] effects in experimental animals. This unapproved agent originates from a hit compound discovered through *in silico* techniques based on the structure of the human PAI-1 protein.[18] After structural optimization, which involved the synthesis of ~540 new lead compounds, a single compound was selected for clinical development. GMP synthesis and formulation and a panel of GLP toxicological studies have been completed and, in the spring of 2013, an investigator-driven phase I clinical trial in 32 healthy men was initiated in Japan.[25] We are now planning an investigator-driven Phase II trial to evaluate the effect of the drug on bone marrow proliferation in myeloablated

**Box 1** | Biomarkers

Biomarkers have the potential to facilitate drug discovery and clinical development in renal disease by enabling monitoring of kidney safety and evaluation of drug-induced nephrotoxicity.[44] Currently, diagnosis of nephrotoxicity is obtained using measurement of blood urea nitrogen or serum creatinine levels. However, these levels are nonspecific measures of renal function and are raised only after substantial deterioration has occurred. An urgent need exists to identify novel, early biomarkers of kidney damage for use in preclinical and clinical studies. Several potential biomarkers have been investigated; for example, urinary kidney injury molecule-1 has been identified as an early marker of proximal tubule injury in rats[45] and urinary type IV collagen has been suggested as a marker of glomerular damage in patients with glomerulonephritis.[46] The FDA and European Medicines Agency have approved a number of renal biomarkers for use in rodent drug-toxicity studies[47] but these have not yet been validated in humans.

**Box 2** | Molecular imaging

Molecular imaging techniques provide valuable data for drug discovery and clinical development.[48] Direct measurement of the effect of a drug in the human body should shorten the timeline for drug development and lower its costs. Molecular imaging probes, developed to target specific molecular pathways *in vivo*, enable visualization of the phenotypic expression of key molecular targets associated with disease processes. Using these probes, early biochemical and physiological abnormalities can be identified prior to the occurence of late structural changes that can be visualized using standard anatomic imaging techniques. Direct assessment of the sequential events involved in renal pathophysiology is difficult to obtain from analyses of blood or urine samples. Some of these events, such as renal tissue hypoxia, can now be assessed using molecular imaging techniques. For example, blood oxygen level-dependent MRI can be used to directly and quickly evaluate alterations in renal oxygen levels after an intervention.[49] Molecular imaging probes (such as 18F-fluoromisonidazoleand 18F-FRP-170) taken up by hypoxic cells should prove to be even more sophisticated tools.[50,51] Indeed, such probes have been used to detect ischaemic myocardium[52] and renal hypoxia in rat models.[53,54] Labelled probes might also be used to identify other disorders associated with kidney injury (such as, oxidative stress, inflammation and fibrosis) and, therefore, enable a faster process of drug discovery and approval in kidney diseases.

**Box 3** | *In silico* approaches

After validation of a target molecule using biochemical, cell-based and experimental animal approaches, industry conventionally launches high-throughput studies using large chemical libraries. Academic researchers, by contrast, often use less-costly *in silico* (computer-aided) approaches to identify candidate compounds. The availability of protein tertiary structure information enables target localization and efficient computational identification of candidate compounds, SBDD or fragment-based drug design.[55–57] Integration of detailed protein structural information, computational chemistry, medicinal chemistry and informatics enables virtual screening of new agents.[18,58] As well as drug discovery, design and optimization, SBDD is essential to elucidate the phamacological mechanisms of new agents.[59] This technique enabled the development of various drugs in current use in renal diseases, including direct renin inhibitors and tyrosine kinase inhibitors.

Abbreviation: SBDD, structure-based drug discovery.

patients undergoing chemotherapy and/or radiotherapy for haematological malignancies. If shown to be safe and efficacious in these patients, we plan to offer the drug to a large number of Japanese and foreign academic networks for clinical evaluation in thrombotic, inflammatory or fibrotic diseases. In addition, clinical development of the drug is underway in the USA in collaboration with Northwestern University, and a meeting has been organized with the FDA to discuss the feasibility of using our Japanese GLP data and GMP materials in US trials.

Many academic researchers, unfortunately, are unfamiliar with the latest regulations on pharmaceutical practices and novel, efficient strategies for preclinical and clinical drug development. Scientists must collaborate with regulatory authorities for progress—to build-up experience and results. New drug development might then extend globally beyond its current reach. Initiatives to help facilitate communication between scientists and regulatory authorities include the International Society of Nephrology (ISN) Nexus symposium, 'New era of drug discovery and clinical trials in kidney disease', which will be held in Bergamo, Italy, in April 2014.[26] In the future, major pharmaceutical companies are likely to remain at the centre of drug discovery in developed countries, whereas in developing countries academia might be able to drive development in accordance with national health policies.

## Remaining challenges

### Noncommunicable diseases

Drug development remains challenging even for large pharmaceutical companies. Aging populations and changes in disease patterns are important issues in developing countries as well as in the developed world. The WHO has focused its efforts on non-communicable diseases (NCDs) in developing countries and currently recognizes cancer, cardiovascular disease, diabetes and chronic respiratory disease as key NCDs.[27] These diseases account for 60% of deaths worldwide, and 80% of deaths in low and middle-income countries.[27] High-quality international clinical trials leading to the eventual approval of novel, effective therapies should help to solve these problems in developing and developed countries.

### Orphan diseases

At present, the problem of orphan diseases (that is, rare diseases for which industry sees little financial incentive to develop and market new curative or preventative therapies, such as Alport syndrome) is not necessarily addressed by large pharmaceutical companies.[28] Academia should undertake research in this area, conduct the first-in-human studies that are necessary to attract the attention of industry and, therefore, provide a real synergy between academia and pharmaceutical companies. Regulatory systems, such as the FDA Fast Track Process, are now in place to increase the speed of drug development and expedite the availability of drugs to treat serious diseases and fill unmet medical needs (that is, providing a therapy where none exists or providing a therapy that might be potentially better than the available therapy). The aim of such systems is to make new drugs available to patients as rapidly as possible.[29]

### Healthcare in developing countries

As the economic situations and the promotion of science and technology in developing countries improves, the gap between the developed and developing world is narrowing. Governments, regulatory agencies, industry and academia in both developed and developing countries should urgently collaborate to address specific, scientific and clinical problems, including kidney disease and NCDs. Currently, major pharmaceutical companies mainly target markets in developed countries, such as the USA, European countries and Japan.[30] However, developing countries are experiencing an increasing demand for advanced healthcare and medicines as a result of high levels of economic growth.[30] This demand, coupled with improved medical technology and expanded healthcare systems, has resulted in rapid expansion of pharmaceutical markets in these countries.[31]

Dialysis for patients with ESRD is a good example of an unmet medical need currently faced by developing countries. The strategies implemented to address this need will undoubtedly differ from those used in the developed world, in which haemodialysis is the main form of renal replacement therapy (this modality is used by ~90% of Japanese patients on dialysis and ~70% of patients on dialysis worldwide).[32] In contrast to developed countries, in which a gradual increase in the number of patients requiring dialysis has occured, the number of untreated patients who require dialysis in developing countries is increasing rapidly.[32] As the cost of haemodialysis-centered healthcare is prohibitive, the

governments of several developing countries are now exploring alternative options, such as peritoneal dialysis.[33] As this modality is expected to become a standard renal replacement therapy for patients with ESRD in developing countries, the development of new therapies to treat complications associated with long-term peritoneal dialysis, such as ultrafiltration failure as a result of peritoneal sclerosis, will be required.

International academic societies have an important role in promoting drug and clinical development. For example, the ISN has formed an Advisory Committee for Clinical Trials and Studies to support and raise standards in investigator-driven clinical trials in developing countries, and has initiated a number of international clinical research projects to address emerging medical problems in these countries, including altitude polycythaemia in Latin America, Mesoamerican nephropathy in Central America and haemolytic uraemic syndrome caused by *Shigella* infection in India.

### Use of surrogate end points

In clinical trials, direct assessment of robust, definitive end points, such as patient survival, mortality and morbidity, is often practically and financially inefficient for both the sponsor industry and for the patients. Use of surrogate end points might provide a solution to this problem. By definition, a surrogate end point predicts and captures the effect of a drug on a true clinical end point.[34] Surrogate end points are useful when they are similar to but more efficient to measure than the hard clinical end point of interest. For example, in some diseases, efficacy can be represented by progression-free survival durations rather than overall survival durations. However, surrogate end points might be difficult to validate and, without practical guidelines, the choice of appropriate surrogate end point remains challenging.

The issue of surrogate end points is particularly important in the setting of kidney disease, which requires large trials with substantial human and financial resources. Currently, end points for progression of kidney disease in clinical trials include doubling of serum creatinine levels, a large decline in glomerular filtration rate (GFR), or progression to ESRD, all of which occur late in the course of CKD. Use of these well-accepted end points might, therefore, result in exclusion of patients with earlier stages of kidney disease from clinical trials, although early treatment might prove more efficacious and cost-effective in this population than in patients with more-severe disease.

Alternatively, use of small changes in estimated (e)GFR as a surrogate end point for progression of kidney disease might increase the number of patients who reach end points in clinical trials and, therefore, enable a reduction in the number of patients or length of follow-up required to demonstrate a statistically significant effect. A *post hoc* analysis of the RENAAL and IDNT trials, which evaluated the efficacy of the angiotensin receptor blockers alosartan and irbesartan in a total of 3,228 adult patients with type 2 diabetes mellitus and nephropathy, demonstrated that use of declines in eGFR that represent increases in serum creatinine levels of <100% (that is, less than doubling) as clinical trial end points might not improve statistical power, particularly if the drugs exert acute effects on GFR (as do angiotensin receptor blockers).[35] However, different conclusions might be reached in different populations using different drugs.

The identification of specific patient populations in which use of surrogate end points rather than hard end points might be appropriate, remains important for drug development. The validity of appropriate surrogate end points should, therefore, be established scientifically and statistically, hopefully as a result of adequate collaboration between regulatory agencies, academia and industry.

### Assesment of benefits and risks

The risks and benefits of new treatments should be assessed in patients. For example, the prognosis of patients diagnosed with pancreatic cancer remains poor. As the survival of patients with this disease is low, a large sample size with survival as the clinical end point is required to demonstrate a drug benefit. Such an evaluation of efficacy ignores a possible improvement in patient quality of life. An industry sponsor, therefore, proposed use of a clinically relevant end point of quality of life assessed using weight, pain killer usage, maintenance of disease status and pain. A clinical trial of gemcitabine in 126 patients with advanced pancreatic cancer that used this end point showed that treatment resulted in a better quality of life and longer survival.[36] The sensitivity of the study might have depended on any component of the chosen end point but statistical reviewers at the FDA, conducting sensitivity analyses, confirmed that each component provided benefit and the data based on the newly defined clinical benefit variable were robust.

Despite their limitations, use of surrogate end points might be necessary to expedite drug approval with an attendant increase in patient benefits. Rigourous application of a definitive end point might sometimes result in depriving a subgroup population (for example patients with early stages of kidney disease) of a useful treatment. The example of gemcitabine illustrates that the sponsor might not have developed the drug if only a robust end point had been considered. Surrogate markers should, therefore, occasionally be taken into consideration for drug approval in view of the potential benefits for patients.

The respective roles of academia and industry sponsors in the assessment of the risks and benefits of new drugs should be clearly delineated. Collaboration between academia, industry and regulatory agencies is necessary to efficiently and effectively maximize the benefits and minimize the risks of new agents.

## The Japanese framework

To stimulate closer collaboration between academia, regulatory authorities and experts, the Japanese government and regulatory authority introduced initiatives to accelerate registration of innovative drugs, medical devices and cellular and tissue-based products. Although these initiatives have not yet yielded reliable results, they are expected to boost collaborations between academia and regulatory agencies in Japan.

### Consultation

The wealth of basic research conducted by academia in Japan is recognized worldwide. However, the country lags behind other developed nations, including the USA and countries in the European Union, in the translation of basic research into practical products such as new drugs or medical devices. Many obstacles, particularly budget shortfalls, insufficient knowledge of regulatory systems, and poor development strategies result in attrition of potential therapies. Researchers involved in the approval of innovative drugs and medical devices should, therefore, be fully aware of the regulatory requirements stipulated in the Japanese Pharmaceutical Affairs Law.[37]

In July 2011, the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan launched a consultation system called Pharmaceutical Affairs Consultation on Research and Development Strategy

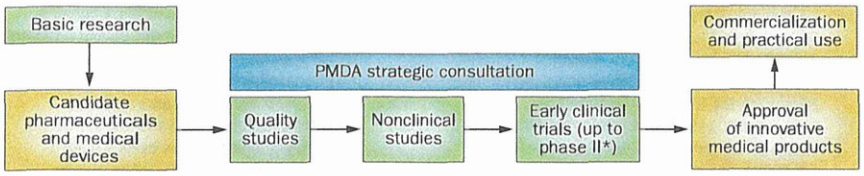


**Figure 2** | The PMDA Pharmaceutical Affairs Consultation on Research and Development Strategy. This Japanese system was launched by the PMDA to foster the creation of innovative medical products by academia and venture businesses by providing guidance on the tests needed in the early development stages (that is, quality and toxicity studies of biologics, cell-based and tissue-based products) and the design of the clinical trials (that is, end points and sample sizes) required to enable commercialization of these products. *Further studies are handled by the conventional PMDA consultation system. Abbreviation: PMDA, Pharmaceuticals and Medical Devices Agency. Permission obtained from PMDA.

(Figure 2).[38] This system offers guidance and advice to academic researchers on the design of nonclinical and early-stage clinical studies, which conform to pharmaceutical regulations, and ultimately determine the approval of submissions. Applicants are strongly recommended to take part in a free introductory consultation, which explains the procedures of the consultation system and the Japanese pharmaceutical system. Those researchers who are already familiar with these procedures can omit the introductory consultation and address relevant issues identified in a free pre-consultation. Finally, scientific discussions are conducted in a face-to-face consultation session. As of the end of December 2013, PMDA had conducted 600 introductory consultations, 669 pre-consultations, and 158 face-to-face consultations.[39] This assistance is expected to provide new, safe and effective strategies for drug development, eventually leading to the approval of innovative products.

### The Science Board

In May 2012, the Science Board was launched by the PMDA with the aim of strengthening its scientific foundation. The Board is connected to PMDA through the Office of Review Innovation (Figure 3). Through discussions with PMDA review officers, the Board intends to establish evaluation methods for state-of-the-art technologies at all stages, from basic technology to developmental support, application review, and post-marketing authorization.[40] The Science Board includes experts from academia, most of whom are leaders in their field who are involved in the development of medical products. To foster transparency, no individual products are discussed by the Board and statements on possible conflicts of interest of Board members are open to the public. Meetings of the Board are closed to the public because agenda items and data might be confidential but, with the exception of confidential information, all materials and meeting minutes are released on the PMDA website.[41]

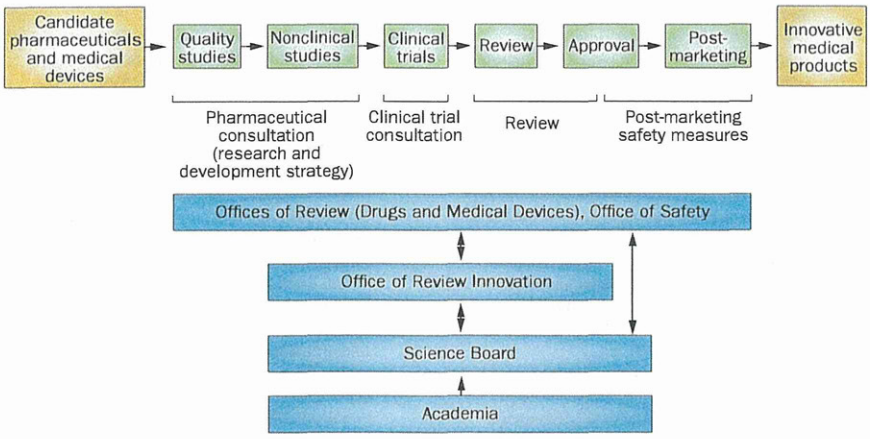


**Figure 3** | The role of the Science Board in the regulation of drug discovery in Japan. Through discussions with the Office of Review Innovation of the PMDA, the Science Board aims to establish evaluation methods for state-of-the-art technologies at all stages, from basic technology to post-marketing authorization safety guidelines. Board members are external experts from academia. Abbreviation: PMDA, Pharmaceuticals and Medical Devices Agency. Permission obtained from PMDA.

The Science Board is made up of four subcommittees: the Pharmaceuticals & Bio-Products subcommittees discuss issues related to biomarkers, the Medical Devices subcommittee is concerned with the scope of generic medical devices and the development of combination products, and the Cellular & Tissue-Based Products subcommittee discusses how to ensure the quality and safety of cell-based and tissue-based products, particularly with regard to tumourigenicity. Each subcommittee also addresses subjects in its area of expertise, put forward by the PMDA and experts on the Science Board. PMDA reviewers and experts, fully informed on the new technologies, discuss opinions from relevant PMDA offices on evaluation methods and provide appropriate consultation, advice and reviews during the application process. If needed, the subcommittees also invite outside experts to discuss the issue at hand. Eventually, a list of discussion topics is reported to PMDA by the Science Board.

### Exchange of human resources

MHLW launched 21 new collaborative projects in the 2012 fiscal year and a further three projects in the 2013 fiscal year.[42] These projects involve PMDA, the National Institute of Health Sciences and academia. PMDA implements exchanges of human resources with universities and research institutions under the Initiative for Accelerating Regulatory Science Concerning Innovative Drugs, Medical Devices, and Regenerative Medicine, which was launched by MHLW on 1 October 2012.[43] This initiative should satisfy regulatory requirements to facilitate the research and development of innovative drugs, medical devices and biologics. Synergies between those activities are expected to make a global contribution by bringing innovative products to market.

## Conclusions

The tedious and expensive path to the approval of new drugs and medical devices curbs innovation in developed and developing countries. Fortunately, new technologies for drug discovery and clinical development are now available (including *in silico* approaches and molecular imaging techniques) and drug efficacy can be documented in investigator-driven clinical trials. Evaluation of the efficacy of a new drug currently relies on difficult to reach hard end

points, such as overall survival, mortality and or morbidity. Use of surrogate end points might enable a quicker evaluation of efficacy but these have not been validated in kidney diseases. Regulatory agencies are keenly aware of the current problems in drug development and have shown readiness to adapt their requirements. For example, the Japanese regulatory authorities provide a novel, thoroughly delineated framework which promotes collaboration with academia. In the future, regulatory agencies, industry and academia must collaborate closely to enable new drug development to extend globally beyond its current reach.

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**Author contributions**

All authors wrote the article. T.M., T.A., H.H., K.W., F.Y. and M.T. researched the data and made substantial contributions to discussions of the content. D.E.V., T.K., Y.O. and C.v.Y.d.S. reviewed and/or edited the manuscript before submission.

# PAI-1–regulated extracellular proteolysis governs senescence and survival in *Klotho* mice

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**Cellular senescence restricts the proliferative capacity of cells and is accompanied by the production of several proteins, collectively termed the "senescence-messaging secretome" (SMS). As senescent cells accumulate in tissue, local effects of the SMS have been hypothesized to disrupt tissue regenerative capacity. *Klotho* functions as an aging-suppressor gene, and *Klotho*-deficient (*kl/kl*) mice exhibit an accelerated aging-like phenotype that includes a truncated lifespan, arteriosclerosis, and emphysema. Because plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor (SERPIN), is elevated in *kl/kl* mice and is a critical determinant of replicative senescence in vitro, we hypothesized that a reduction in extracellular proteolytic activity contributes to the accelerated aging-like phenotype of *kl/kl* mice. Here we show that PAI-1 deficiency retards the development of senescence and protects organ structure and function while prolonging the lifespan of *kl/kl* mice. These findings indicate that a SERPIN-regulated cell-nonautonomous proteolytic cascade is a critical determinant of senescence in vivo.**

FGF23 | IGFBP3 | IL-6 | TM5441

Advanced age contributes to the development of frailty and disease in humans, but the fundamental mechanisms that drive physiological aging are incompletely understood (1, 2). Cellular senescence, which halts the proliferative capacity of cells, is associated with the manifestation of the senescence-associated secretory phenotype (3) and the production and secretion of a distinct set of proteins (2, 4), including insulin-like growth factor-binding proteins (IGFBPs), interleukins (ILs), transforming growth factor type β (TGF-β), and plasminogen activator inhibitor-1 (PAI-1) (5), collectively termed the "senescence-messaging secretome" (SMS) (6). In addition to this pattern of protein production and secretion, senescent cells display a distinctive morphology, and can be identified by increased expression of senescence-associated β-galactosidase (7). The tumor suppressor and proapoptotic protein p53 plays a central role in inducing replicative senescence by regulating the transcription of genes involved in cell cycle arrest and apoptosis, including the cyclin-dependent kinase inhibitors $p16^{Ink4a}$ and p21 (8). Senescence can be triggered by a number of factors, including DNA damage (9), oncogene induction (10), and oxidative stress (11). Although the relationship between cellular senescence and physiological aging remains an area of intense investigation, it is becoming increasingly evident that the two processes are fundamentally linked. Senescent cells accumulate in aging tissues and have been hypothesized to disrupt tissue regeneration, which may reflect cell-nonautonomous effects of the SMS (6).

In the last decade, numerous examples of genetically modified mice with phenotypes reminiscent of human aging have been described and investigated. These include the $BubR1^{H/H}$ progeroid (12) and *Klotho*-deficient (*kl/kl*) mice (13). $BubR1^{H/H}$ progeroid mice exhibit an age-dependent increase in the expression levels of PAI-1 in numerous locations, including white adipose tissue, skeletal muscle, and the eye (12). $BubR1^{H/H}$ mice have a shortened average lifespan (24 wk) and develop various aging-like phenotypic abnormalities, including sarcopenia, cataracts, arterial stiffening, and impaired wound healing (14). Klotho functions as an aging-suppressor protein by impeding the development of senescence in vitro and in vivo through inhibition of the Wnt (15), TGF-β (16), and IGF1 signaling pathways (17). Thus, *kl/kl* mice exhibit a rapidly progressive phenotype after weaning that includes a truncated lifespan (8–12 wk), renal sclerosis, arteriosclerosis, emphysema, and osteoporosis (13). Membrane-bound Klotho forms a heterodimer with fibroblast growth factor (FGF) receptors (FGFRs) generating a high-affinity receptor for FGF23. Signals transduced by FGF23 via the Klotho–FGFR complex inhibit 1,25-$(OH)_2$ vitamin $D_3$ and parathyroid hormone synthesis and promote renal phosphate excretion. *kl/kl* mice exhibit a remarkable increase in plasma levels of FGF23, as well as significant increases in serum levels of calcium, phosphate, vitamin $D_3$, and creatinine (13). Interestingly, *kl/kl* mice also have an age-dependent increase in plasma PAI-1 levels as well as increased PAI-1 expression in a number of tissues including kidney, aorta, and heart (18). Because PAI-1 is necessary and sufficient to induce replicative senescence in vitro downstream of p53 (19) and is markedly increased in *kl/kl* mice, we hypothesized that PAI-1 is a critical determinant of the phenotypic abnormalities developed by *kl/kl* mice. Here we examined the impact of PAI-1 on senescence and physiological aging in vivo by breeding *kl/kl* and PAI-1–deficient ($pai\text{-}1^{-/-}$) mice to generate *kl/kl* mice with partial ($kl/klpai\text{-}1^{+/-}$) or complete ($kl/klpai\text{-}1^{-/-}$) PAI-1 deficiency.

## Results

**PAI-1 Deficiency Prolongs the Survival of *kl/kl* Mice.** We systematically monitored the effect of PAI-1 deficiency on the growth, vigor, and survival of littermate *kl/kl* ($n = 26$), $kl/klpai\text{-}1^{+/-}$ ($n = 39$), $kl/klpai\text{-}1^{-/-}$ ($n = 25$), and WT ($n = 16$) mice (Fig. 1) all in the

### Significance

Plasminogen activator inhibitor-1 (PAI-1) is an essential mediator of cellular senescence in vitro and is one of the biochemical fingerprints of senescence in vivo. *Klotho*-deficient (*kl/kl*) mice display a complex phenotype reminiscent of human aging and exhibit age-dependent increases in PAI-1 in tissues and in plasma. Thus, we hypothesized that PAI-1 contributes to the aging-like phenotype of *kl/kl* mice. We observed that either genetic deficiency or pharmacological inhibition of PAI-1 in *kl/kl* mice was associated with reduced evidence of senescence, preserved organ structure and function, and a fourfold increase in median lifespan. These findings indicate that PAI-1 is a critical mediator of senescence in vivo and defines a novel target for the prevention and treatment of age-related disorders in man.

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The authors declare no conflict of interest.

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same genetic background (75% C57BL/6J, 25% C3J). We observed that either partial or complete absence of PAI-1 prolonged the survival of *kl/kl* mice. Log-rank analysis indicated that the survival curves for the WT, *kl/kl*, *kl/klpai-1*$^{+/-}$, and *kl/klpai-1*$^{-/-}$ mice differed significantly ($P < 0.0001$). The median survival of *kl/kl* mice was 58 d, and this value increased with PAI-1 deficiency: 2.8-fold (163 d) in *kl/klpai-1*$^{+/-}$ mice and 4.2-fold (246 d) in *kl/klpai-1*$^{-/-}$ mice. Whereas all of the *kl/kl* mice died within 120 d, 65% of *kl/klpai-1*$^{+/-}$ and 82% of *kl/klpai-1*$^{-/-}$ mice were alive beyond 120 d. Although median survival indicates a dose–response relationship between genotype and mortality ($P = 0.0002$ by log-rank test for trend), the mean lifespan increased similarly in *kl/klpai-1*$^{+/-}$ (250 ± 169 d) and *kl/klpai-1*$^{-/-}$ (254 ± 123 d) mice (mean ± SD), corresponding to 4.2- and 4.5-fold increases, respectively. Furthermore, we achieved a similar prolongation of lifespan in *kl/kl* mice ($n = 11$) (Fig. 1*B*) by the administration of an orally active small-molecule PAI-1 antagonist, TM5441, whose pharmacokinetic properties, toxicity, and specificity have been described recently (20). In contrast with the inconsistent effects based on sex of a low phosphate diet on survival in *kl/kl* mice (21), both males and females appear to benefit from complete PAI-1 deficiency. However, survival of *kl/klpai-1*$^{+/-}$ females ($n = 19$) was not as long as that of males ($n = 20$) of the same genotype (median survival 121 d vs. 315 d, mean lifespan 208 ± 151 d vs. 285 ± 182, respectively; $P = 0.16$). Nevertheless, *kl/klpai-1*$^{+/-}$ females do live longer than *kl/kl* females ($n = 14$) (median survival 121 d vs. 58 d, mean lifespan 208 ± 151 d vs. 57 ± 18, respectively; $P = 0.0004$) (Fig. S1). This improvement in survival was also associated with evidence of increased overall vigor and health, as *kl/klpai-1*$^{-/-}$ mice exhibited near-normal weight gain over time (Fig. 1 *A* and *C*) and spontaneous physical activity (Fig. 1*D*).

**PAI-1 Deficiency Normalizes Senescence and Telomere Length in *kl/kl* Mice.** To characterize the extent of senescence in *kl/kl* mice and how PAI-1 deficiency affects it, plasma levels of the SMS factors IGFBP-3 and IL-6, and telomere length were measured in liver, aorta, tail, and kidney tissue. We observed that *kl/kl* mice had increased levels of IGFBP-3 (Fig. 2*A*) compared with WT mice ($P = 0.02$). With partial ($P = 0.03$ vs. *kl/kl* mice) or complete PAI-1 deficiency ($P = 0.02$ vs. *kl/kl* mice), IGFBP-3 levels did not significantly differ from those seen in WT mice. Similarly, we found that compared with levels in WT mice, *kl/kl* mice had a 13-fold increase ($P = 0.02$) in plasma levels of proinflammatory cytokine IL-6, which functions in the acquisition of the senescent phenotype in vitro (Fig. 2*B*). Compared with *kl/kl* mice, IL-6 levels were reduced by 79% in *kl/klpai-1*$^{+/-}$ ($P = 0.03$) and 83% in *kl/klpai-1*$^{-/-}$ ($P = 0.0001$) mice. These observations suggest that the elevated PAI-1 levels in *kl/kl* mice are a dominant factor in contributing to increases in plasma IGFBP-3 and IL-6 and further augment the senescent phenotype in these mice.

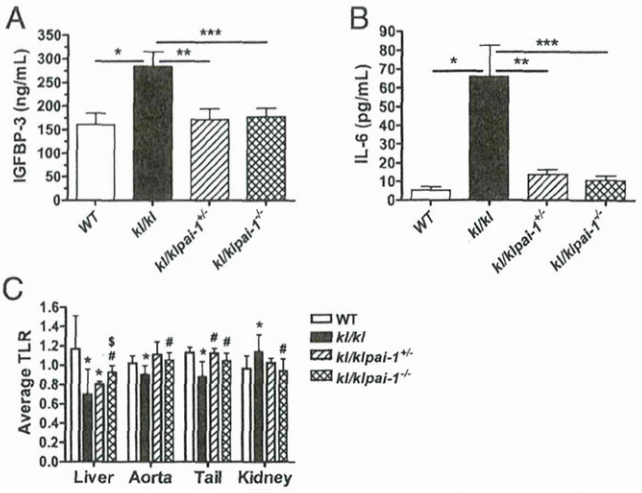


**Fig. 2.** Effect of PAI-1 deficiency on plasma levels of SMS factors and ATLR in various tissues from age-matched littermate *Klotho* mice. (*A*) Determination of IGFBP-3 levels in plasma samples ($n = 6$ per group). *$P = 0.02$, **$P = 0.03$, and ***$P = 0.02$. (*B*) Quantitation of circulating IL-6 levels ($n = 6$ to 14). *$P = 0.02$, **$P = 0.03$, and ***$P = 0.0001$. (*C*) Quantitation of ATLR by qRT-PCR in liver, aorta, tail, and kidney tissue ($n = 6$ to 14). *$P < 0.05$ compared with WT, #$P < 0.05$ compared with *kl/kl*, and $$P < 0.05$ compared with *kl/klpai-1*$^{+/-}$. Data are plotted as mean ± SD.

Although elevated plasma levels of SMS components may reflect systemic senescence, they are nonspecific in nature and do not provide precise identification of which tissues are actually senescent. To address this limitation, telomere length was determined in several different tissues. Liver, aorta, and tail tissue samples from *kl/kl* mice displayed moderate but significant decreases in the average telomere length ratio (ATLR), whereas renal tissue had 16% longer ATLRs compared with those of WT animals (Fig. 2*C*). In contrast, ATLRs of liver and tail tissues from *kl/klpai-1*$^{+/-}$ and liver, aorta, and tail, tissues from *kl/klpai-1*$^{-/-}$ mice were significantly longer than those of *kl/kl* mice. These findings indicate that PAI-1 deficiency provides partial protection of telomere integrity in numerous tissues. The anomalous

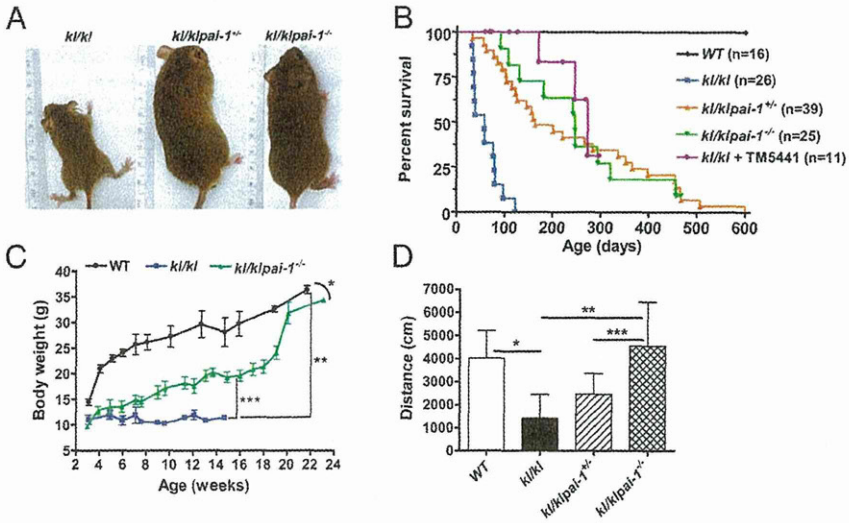


**Fig. 1.** Effects of PAI-1 deficiency in *Klotho* mice. (*A*) Size and appearance of 8-wk-old littermate mice. (*B*) Survival curve. Log-rank analysis showed that the survival curves for the WT, *kl/kl*, *kl/klpai-1*$^{+/-}$, and *kl/klpai-1*$^{-/-}$ mice differed significantly ($P < 0.0001$). (*C*) Bodyweight measurements starting from 3 wk of age. *$P = 0.002$, **$P = 0.0001$, and ***$P = 0.0003$. (*D*) Open field physical activity measurements recorded as distance traveled in 20 min in age-matched animals. *$P = 0.027$, **$P = 0.018$, and ***$P = 0.036$. Data are plotted as mean ± SD.

preservation of telomere length in renal tissue from *kl/kl* mice may reflect the lack of turnover in renal cells and the early induction of replicative senescence in the kidneys of *kl/kl* mice (22).

Because the kidneys are one of the most severely compromised organs in *kl/kl* mice, we also examined kidneys for biomarkers of senescence, including $p16^{Ink4a}$ and p21. We detected strong immunostaining for $p16^{Ink4a}$ localized in the nuclei of tubules in kidney tissues from *kl/kl* mice (Fig. 3), but not for p21. In contrast, kidney sections from *kl/klpai-1*$^{-/-}$ mice had only minimal evidence of $p16^{Ink4a}$ accumulation (Fig. 3). Quantitative real-time PCR (qRT-PCR) analysis showed that the relative expression of $p16^{Ink4a}$ in kidneys from *kl/kl* mice is 3.2-fold higher than that in WT mice ($P = 0.001$) (Fig. 3*E*). In *kl/klpai-1*$^{+/-}$ and *kl/klpai-1*$^{-/-}$ mice, $p16^{Ink4a}$ expression was reduced by 80% ($P = 0.04$) and 92% ($P = 0.0001$) compared with the *kl/kl* mice, respectively, and in *kl/klpai-1*$^{-/-}$ mice by 78% compared with the levels seen in WT animals ($P = 0.0001$).

**Effect of PAI-1 Deficiency on the Biochemical Hallmarks of *kl/kl* Mice.** In an effort to explain the effects of PAI-1 deficiency on the *kl/kl* phenotype, we measured plasma levels of factors that are biochemical hallmarks of *kl/kl* mice, including FGF23, vitamin $D_3$, calcium, phosphate, creatinine, and PAI-1 (Table 1). As expected, *kl/kl* mice displayed a more than 1,200-fold increase in FGF23 levels [$2.8 \times 10^5 \pm 1.4 \times 10^5$ pg/mL vs. $225 \pm 65$ pg/mL in WT mice ($P = 0.004$)], reflecting the loss of functioning receptors for FGF23. Both *kl/klpai-1*$^{+/-}$ and *kl/klpai-1*$^{-/-}$ mice exhibited a nearly 98% reduction in plasma FGF23 levels compared with *kl/kl* mice [$3.4 \times 10^3 \pm 2.1 \times 10^3$ pg/mL ($P < 0.0001$) and $3.9 \times 10^3 \pm 0.9 \times 10^3$ pg/mL ($P = 0.0001$), respectively]. Similarly, vitamin $D_3$ levels were reduced in *kl/klpai-1*$^{+/-}$ ($P = 0.032$) and *kl/klpai-1*$^{-/-}$ ($P = 0.0003$) mice compared with the levels in *kl/kl* mice. Interestingly, partial or complete PAI-1 deficiency had only a marginal impact on serum levels of calcium, phosphate, and creatinine in *kl/kl* mice. As expected, PAI-1 antigen was not detectable in plasma from *kl/klpai-1*$^{-/-}$ mice, and levels in *kl/klpai-1*$^{+/-}$ animals were reduced by nearly 50% compared with those from *kl/kl* mice ($P < 0.05$). In addition, PAI-1 expression levels were reduced in tissues from *kl/klpai-1*$^{+/-}$ mice compared with those of *kl/kl* mice (Fig. S2).

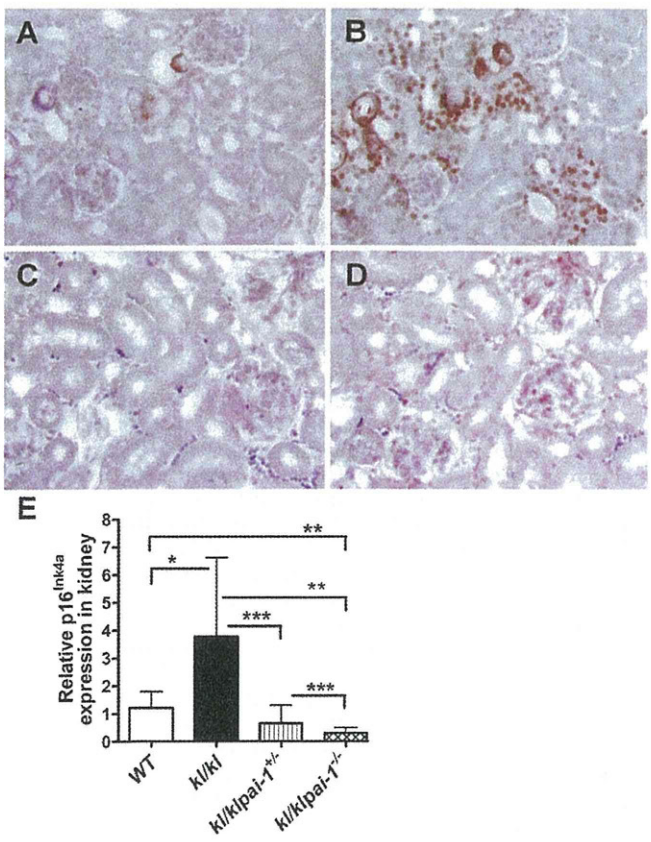


**Fig. 3.** Effect of PAI-1 deficiency on renal $p16^{Ink4a}$ expression in *Klotho*-deficient mice. (*A* and *C*) Control immunostaining of kidney sections from *kl/kl* and *kl/klpai-1*$^{-/-}$ mice, respectively, in the absence of the anti-$p16^{Ink4a}$ antibody. (*B* and *D*) Immunodetection of $p16^{Ink4a}$-positive cells in kidney sections from *kl/kl* and *kl/klpai-1*$^{-/-}$ mice, respectively. (*E*) Quantitation of relative $p16^{Ink4a}$ expression in the kidney. qRT-PCR analysis was performed on total RNA samples purified from WT ($n = 8$), *kl/kl* ($n = 6$), *kl/klpai-1*$^{+/-}$ ($n = 4$), and *kl/klpai-1*$^{-/-}$ ($n = 8$) kidneys. *$P = 0.001$, **$P = 0.0001$, and ***$P = 0.04$. Data are plotted as mean ± SD. (Magnification: *A–D*, 60×.)

**PAI-1 Deficiency Preserves Organ Structure in *kl/kl* Mice.** As reported previously, *kl/kl* mice develop emphysema that is characterized by a progressive, age-dependent enlargement of air spaces and associated alveolar destruction (Fig. 4) (23). Histological analysis of lung tissues from *kl/klpai-1*$^{+/-}$ and *kl/klpai-1*$^{-/-}$ (Fig. 4) mice showed that PAI-1 deficiency primarily prevents alveolar enlargement. Consistent with the preservation of pulmonary structural integrity, pulmonary function was also maintained with PAI-1 deficiency. We found that *kl/kl* mice had a 40% decrease in $PaO_2$ levels ($P = 0.018$) in arterial blood samples. Arterial oxygenation normalized with partial ($P = 0.05$) and complete ($P = 0.02$) PAI-1 deficiency in *kl/kl* mice (Fig. 4*E*). These results indicate that PAI-1 is an important contributor to the emphysematous changes observed in *kl/kl* mice.

Finally, we analyzed mice for evidence of ectopic calcification, which has been reported to increase with age in *kl/kl* mice. Whereas the age-matched WT littermate mice had no detectable calcification, we observed prominent calcium deposits in the kidneys of *kl/kl* mice (Fig. 5 *A* and *B*) ($P = 0.002$). However, analysis of kidneys from *kl/klpai-1*$^{+/-}$ (Fig. 5*C*) and *kl/klpai-1*$^{-/-}$ (Fig. 5*D*) mice showed that ectopic calcification areas were significantly reduced by 41% ($P = 0.03$) and 96% ($P < 0.0001$), respectively. To test the effect of PAI-1 deficiency on the impaired osteogenic signaling observed in *kl/kl* mice (24, 25), we measured the serum levels of aldosterone and alkaline phosphatase (ALP) activity (Table 1). Although aldosterone levels were significantly higher in *kl/kl* mice than that of WT animals, it was not altered significantly in *kl/klpai-1*$^{-/-}$ mice. Furthermore, we did not observe any difference in ALP activity among the mice groups studied here. This observation indicates that partial or complete loss of PAI-1 expression protects against age-induced ectopic calcification in *kl/kl* mice without altering serum levels of phosphate, calcium, ALP and aldosterone.

## Discussion

PAI-1 is expressed in senescent cells and tissues, and is recognized as a primary component of the SMS. Most mammalian models of aging investigated thus far exhibit evidence of increased PAI-1 expression (12, 14). Furthermore, genetic or therapeutic interventions that prolong survival or reduce senescence in tissues in vivo are coincidentally associated with reductions in PAI-1. To our knowledge, this in vivo study is the first to investigate systematically the role of PAI-1 not only in the development of senescence, but also in the aging-like pathology of a mammal. The results from this study suggest that the onset of physiological aging can be delayed by modulating PAI-1, which subsequently prevents the nuclear accumulation of senescence marker $p16^{Ink4a}$ and maintains the structural and functional integrity of vital organs. Furthermore, the ability of a small-molecule PAI-1 antagonist to augment survival to a similar extent in *kl/kl* mice indicates that the observed effects are likely cell-nonautonomous. The protective effects of partial or complete PAI-1 deficiency are in agreement with previous work from our laboratory indicating that transgenic overexpression of

**Table 1. Effects of PAI-1 deficiency on blood levels of the biochemical hallmarks of *klotho* mice**

| Circulating factors assayed | WT $n = 4$ to 5 | *kl/kl* $n = 6$ to 9 | *kl/klpai-1*$^{+/-}$ $n = 5$ to 6 | *kl/klpai-1*$^{-/-}$ $n = 6$ to 10 |
|---|---|---|---|---|
| Phosphate, mg/dL | 7.7 ± 1.6 | 14.0 ± 2.8* | 10.8 ± 2.6* | 12.6 ± 2.7* |
| Calcium, mg/dL | 7.9 ± 0.6 | 10.4 ± 0.9* | 10.1 ± 1.2* | 10.6 ± 0.8* |
| Creatinine, mg/dL | 0.22 ± 0.11 | 0.31 ± 0.33 | 0.21 ± 0.11 | 0.18 ± 0.06 |
| PAI-1, ng/mL | 1.8 ± 0.4 | 45.2 ± 4.8* | 24.3 ± 2.7* | 0.0 |
| FGF23, pg/mL | 225 ± 65 | 295,657 ± 139,709* | 3,924 ± 1,316*,# | 3,795 ± 870*,# |
| Vitamin $D_3$, pg/mL | ND | 1359 ± 145 | 698 ± 419# | 314 ± 244# |
| Aldosterone, pg/mL | 102 ± 20 | 160 ± 55* | ND | 223 ± 96* |
| ALP, U/mL | 20 ± 7 | 21 ± 6 | 17 ± 7 | 19 ± 5 |

ND, not determined.
*$P < 0.05$ compared with WT.
#$P < 0.05$ compared with *kl/kl*.

PAI-1 is sufficient to induce several aging-like phenotypic abnormalities, including age-dependent spontaneous coronary thrombosis, systemic amyloid deposition, and hair loss (26).

The normalization of FGF23 and vitamin $D_3$ levels in partial or complete PAI-1 knockout models strongly indicates that PAI-1 directly influences FGF23 signaling in *kl/kl* mice. Recent observations demonstrate that FGF23 is highly sensitive to cleavage by the serine protease furin, which is rapidly inhibited by PAI-1 (27, 28). In addition to FGF23, *kl/kl* mice have augmented expression of other furin substrates, including IGF1, TGF-β, MMP2, and MMP9 (29, 30). PAI-1 is known to regulate the proteolytic activation and/or clearance of many of these proteins. The precise identity and function of other proteases that are inhibited by PAI-1 and that contribute to the *Klotho* phenotype merits further investigation.

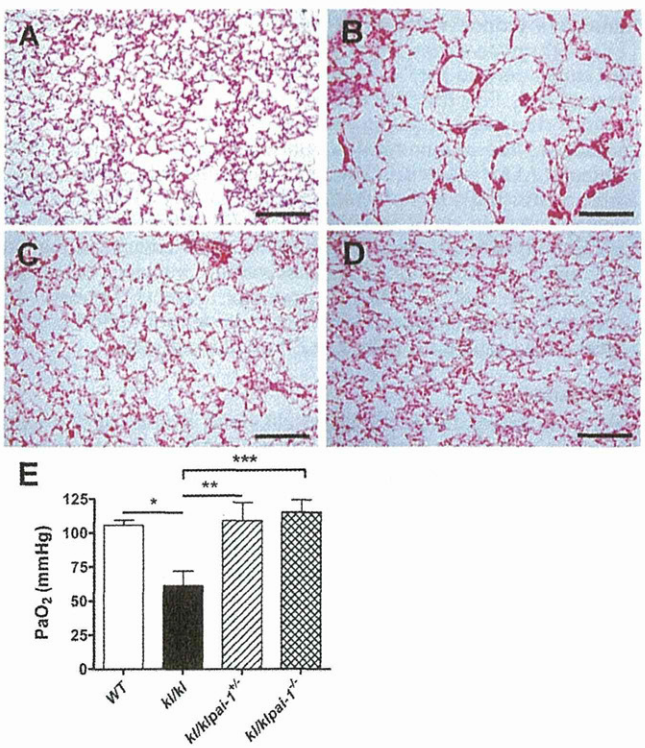


**Fig. 4.** Effects of PAI-1 deficiency on lung morphology and function. Masson's trichrome staining of lung sections in (*A*) WT, (*B*) *kl/kl*, (*C*) *kl/klpai-1*$^{+/-}$, and (*D*) *kl/klpai-1*$^{-/-}$ mice. (Scale bars: 150 μm.) (*E*) Partial pressure of oxygen ($PaO_2$) measurements in arterial blood ($n = 4$ for each group). *$P = 0.018$, **$P = 0.05$, and ***$P = 0.02$. Data are plotted as mean ± SD.

Because FGF23 signaling is impaired in *kl/kl* mice, the negative feedback inhibition on vitamin $D_3$ synthesis is dysfunctional. High vitamin $D_3$ and phosphate levels in *kl/kl* mice likely stimulate FGF23 synthesis in bone continuously, whereas the elevated PAI-1 levels reduce the proteolytic clearance of FGF23. Together, these combined effects on production, signaling, and metabolism likely explain the >1,200-fold increase in plasma FGF23 levels observed in *kl/kl* mice. Recent reports indicate that dietary deficiency of phosphate, zinc, and calcium significantly improves the lifespan of klotho mice (21, 25, 31). Although we did not detect any significant changes in the levels of phosphate and calcium in *kl/klpai-1*$^{-/-}$ mice, we observed that PAI-1 deficiency significantly prolongs the survival of *kl/kl* mice indicating that, in addition to the mineral homeostasis, PAI-1–regulated extracellular proteolysis strongly influences the aging phenotype.

Our findings also reveal a previously unrecognized role of PAI-1 in modulating the effects of FGF23. The increased plasma levels of PAI-1 in *kl/kl* mice are not surprising, but deserve some mechanistic explanation. Numerous factors likely contribute to the *Klotho* phenotype, and prominent on that list are the effects of TGF-β (16), Wnt (15), and IGF1 (17, 32). Importantly, all three of these factors can directly induce PAI-1 expression. As the phenotype matures, PAI-1 production is likely further augmented by the effects of progressive hypoxemia, the induction of the p53 pathway, aldosterone excess (25), and elevated levels of other components of the SMS, including IL-6, interferons, TGF-β, and IGFBPs.

Discovery of the *Klotho* gene has shed light into the molecular mechanisms of tissue calcification. The elevated phosphate and calcium levels in *kl/kl* mice certainly contribute to the pattern of ectopic calcification (33, 34). Recently, hyperaldosteronism was reported to be the major inducer of the osteog enic signaling, which was partially reversed by spironolactone without normalizing plasma levels of vitamin $D_3$, FGF23, calcium, and phosphate (24, 25). Furthermore, Lim et al. reported that FGF23 treatment reduced the aldosterone-induced expression of osteogenic factors in vitro (35). In addition, PAI-1 deficiency drastically reduced FGF23 levels and calcification without altering aldosterone and ALP levels in *kl/kl* mice (Table 1). These findings suggest that PAI-1, which is regulated by aldosterone (36), plays an unexpected but pivotal role in tissue calcification. The reduced plasma levels of vitamin $D_3$ and prevention of tissue calcification in *kl/klpai-1*$^{-/-}$ mice require the restoration of Klotho-independent FGF23 signaling. The present study strongly indicates that PAI-1 plays a direct role in the regulation of FGF23 signaling in *kl/kl* mice.

The reduced IGFBP-3 levels in *kl/kl* mice provide an important mechanistic insight into the protective effects of PAI-1 deficiency on the *Klotho* phenotype. It was recently demonstrated that the PAI-1–IGFBP-3 cascade promotes stress-induced senescence in human breast fibroblasts (37). Expression levels of IGFBP-3 are increased in response to senescence-inducing stimuli. However, the proteolytic metabolism of IGFBP-3 by tissue-type plasminogen activator (t-PA) prevents the induction of cellular