Published in final edited form as:
Ageing Res Rev. 2007 May ; 6(1): 73–79. doi:10.1016/j.arr.2007.02.002.

Premature aging in *klotho* mutant mice: cause or consequence?

Beate Lanske[*] and **M. Shawkat Razzaque**[$]
Department of Developmental Biology, Harvard School of Dental Medicine, Research and Educational Building, 190 Longwood Ave, Boston, MA 02115, USA

Abstract

Suitable mammalian models for aging with wide range of age-associated pathology are desirable to study molecular mechanisms of human aging. Recent studies have identified that *fibroblast growth factor 23* (*Fgf-23)* null mice and *klotho* hypomorphs could generate multiple premature aging-like features, including shortened lifespan, infertility, kyphosis, atherosclerosis, extensive soft tissue calcifications, skin atrophy, muscle wasting, T-cell dysregulation, pulmonary emphysema, osteoporosis/osteopenia, abnormal mineral ion metabolism, and impaired vitamin-D homeostasis. The strikingly similar *in vivo* phenotypes of two separate genetically altered mouse lines implicate that the premature aging-like features may be partly regulated through a common signaling pathway involving both Fgf-23 and klotho; such speculation is experimentally supported by the observation that Fgf-23 requires klotho as a cofactor to exert its functions. Despite about 2,000 fold higher serum levels of Fgf-23 in *klotho* mutants (compared to wild-type animals), these mice show physical, biochemical and morphological features similar to *Fgf-23* null mice, but not as *Fgf-23* transgenic mice; these observations suggest that widely encountered premature aging-like features in *klotho* mutant mice are due to the inability of Fgf-23 to exert its bioactivities in absence of klotho. The results of recent studies showing klotho as a cofactor in Fgf-23 signaling consequently explains that the premature aging-like features in *klotho* deficient mice is not a primary cause, rather a consequence of lacking Fgf-23 activity. These understandings will help us to redefine the role of klotho as an aging factor.

Keywords

FGF-23; Kotho; Vitamin-D; Calcification; Premature aging

Phosphate homeostasis

Maintaining phosphate homeostasis is of crucial biological importance, as it regulates fundamental cellular functions and skeletal mineralization; it is also an important component of nucleic acids, biologically active signaling proteins, coenzymes, and lipid bilayer of the cell membranes. Ingested phosphate is mostly absorbed in the small intestine and is either incorporated in cells in organic forms, deposited as a component of bone mineral, or eliminated by the kidney; the rate of renal reabsorption and/or excretion is determined by the specific needs of the body.

Roughly 70% of the phosphate is absorbed in the duodenum and jejunum, through a sodium-dependent active transport, a process stimulated by 1,25-dihydroxyvitamin D_3 [1,25 $(OH)_2D_3$]; besides parathyroid hormone (PTH) and low-phosphate diets can also stimulate

Corresponding authors: Tel. (617) 432 5748, fax (617) 432 5767.
[*]beate_lanske@hsdm.harvard.edu (B. Lanske)
[$]mrazzaque@hms.harvard.edu (M.S. Razzaque)

intestinal absorption of phosphate by exerting effects on vitamin-D. For instance, low extracellular phosphate could stimulate renal activity of 1α-hydroxylase [1α(OH)ase] to increase the synthesis of $1,25(OH)_2D_3$. Kidney is the most important organ that helps in maintaining phosphate homeostasis by controlling urinary phosphate excretion to keep the physiologic balance. About 60–70% of renal reabsorption of phosphate occurs in the proximal tubules via a sodium gradient-dependent process (Gaasbeek and Meinders, 2005; Magagnin et al., 1993; Tenenhouse, 2005). The sodium/phosphate co-transporters (NaPi2a, and NaPi2c), located on the apical brush border membrane of the proximal tubules, contribute to about 85% of the reabsorption. Abundance of NaPi cotransporter proteins determines the rate of active phosphate transport; increased levels of PTH and/or a high-phosphate diet cause an endocytic internalization of the NaPi transporters, and the resultant effect being less reabsorption and an increase in urinary phosphate wasting. In contrary, low levels of PTH and/or a low-phosphate diet cause insertion of the NaPi transporters into the brush border membrane, and thereby increase phosphate uptake (Tenenhouse, 2005; Traebert et al., 2000). The PTH and vitamin-D activities, however, cannot completely explain the complex regulation of the phosphate homeostasis, and the search for phosphatonin (factor responsible for inhibition of phosphate reabsorption) that regulates renal phosphate wasting has led to the identification of fibroblast growth factor 23 (FGF-23) (ADHR_Consortium, 2000; Shimada et al., 2001).

Fibroblast growth factor 23

FGF-23 is a 30 kDa-secreted protein that is processed by a pro-convertase type enzyme into two smaller fragments of approximately 18 kDa (amino fragment) and 12 kDa (carboxy fragment); the exact biological significance of these fragments is not clear, and of intense focus of current research. In contrast to the phosphaturic effects (renal urinary phosphate wasting) of the full-length synthetic FGF-23 protein, the intraperitoneal administration of a synthetic carboxyl terminal fragment of FGF-23 (aa 180–251) or an N-terminal fragment of FGF-23 (25–179) to mice did not produce any such phosphaturic effects (Shimada et al., 2002). Whether *in vivo* differential processing of the FGF-23 fragments could have diverse biological affects needs to be further investigated. Since the canonical FGFR binding domain is absent in carboxyl terminal fragments of FGF-23, any *in vivo* response by this fragment would suggest the existence of a novel receptor, in addition to known classic receptors for FGFs.

Recent genetically modified animal studies have provided insights into the role of FGF-23 in regulating phosphate homeostasis. Transgenic mice over-expressing FGF-23 exhibit hypophosphatemia, with no significant changes in serum levels of calcium (Bai et al., 2004; Larsson et al., 2004; Shimada et al., 2004b), while an opposing effect of high serum levels of phosphate, and increased vitamin D activities are documented in *Fgf-23* null mice (Shimada et al., 2004a; Sitara et al., 2004). More importantly, the phenotype of *Fgf-23* null animals mimics patients with familial tumoral calcinosis (FTC), an autosomal recessive disorder characterized by ectopic calcifications and elevated serum levels of phosphate due to inactivating mutations in the *FGF-23* gene (Benet-Pages et al., 2005; Frishberg et al., 2006). Conversely, the phenotype of *FGF-23* transgenic animals mimics patients with autosomal dominant hypophosphatemic rickets (ADHR) carrying mutations in the *FGF-23* gene that lie within 3 nucleotides of each other in the proprotein convertase cleavage site (ADHR_Consortium, 2000); these mutations prevent proteolytic cleavage of the FGF-23 protein, with net effect being phosphate wasting in the affected patients, perhaps due to enhanced biologic activities of FGF-23. These genetically altered mouse models have clear clinical relevance and provide the *in vivo* tool to study, in depth, the biology of FGF-23. Available information supports the notion that FGF-23 is the master molecule to regulate phosphate homeostasis.

However, how and where FGF-23 binds to its receptor is of intense focus of research, and preliminary observations suggest that FGF-23 could exert its bioactivities through binding with the known receptors of the FGF family (Yu et al., 2005); recent studies have provided convincing evidence that klotho acts as a cofactor in FGF-23 and receptor binding to induce subsequent intracellular signaling (Kuro-o, 2006; Urakawa et al., 2006).

Klotho

The klotho gene encodes a single-pass transmembrane protein. The extracellular domain of Klotho protein consists of two homologous domains that share sequence homology to the β-glucosidase of bacteria and plants. The klotho gene has selective expression in such tissues as kidney (in the distal convoluted tubules) and brain (in the choroid plexus) (Li et al., 2004). Polymorphisms in the human *KLOTHO* gene are suggested to correlate with the occurrence of age-related pathologies, including osteoporosis, and coronary artery diseases, and predicted to influence overall survival (Arking et al., 2002). Experimental studies have shown that mice lacking *klotho* activities produce phenotypes resembling human aging that include muscle and skin atrophy, osteopenia, vascular and soft tissue calcification, pulmonary emphysema, and the resultant effect being short life span (Kuro-o, 2001; Nabeshima, 2002); *klotho* mutant mice also show biochemical abnormalities that include high serum levels of phosphate, and increased vitamin-D activities. Interestingly, physical, biochemical and morphological features documented in *klotho* mutant mice completely resemble the features documented in *Fgf-23* null mice, raising the possibility of close functional *in vivo* interactions between these two molecules.

FGF-23, klotho and receptor interactions

Recent studies have shown that both the full length and the extracellular domain of klotho protein are able to bind to various FGF receptors (FGFRs) (Kuro-o, 2006); signal-transducing FGFRs contain an extracellular ligand-binding domain and an intracellular tyrosine kinase domain. In general, in order for FGF to bind to and activate the FGFR system, heparin sulphate proteoglycans or heparin-like molecules are required (Amaya et al., 1991; Mohammadi et al., 2005a; Mohammadi et al., 2005b; Ornitz et al., 1992) FGF-23 is a recently identified member of the FGF family, and is significantly distinct from other FGFs in that it contains a pro-convertase processing site. Whether FGF-23 also needs heparin-like molecules to activate the FGFR system is an intense area of research (Yu et al., 2005), but it is becoming increasingly clear that FGF-23 needs klotho as a cofactor to induce its receptor activities to exert biological effects. Although the extracellular domain of klotho does not directly bind to FGF-23, it enhances FGF-23 binding to its receptor complex with much higher affinity than to FGFR alone (Kuro-o, 2006). Furthermore, FGF-23, in presence of klotho could activate downstream signaling events, as determined by phosphorylation of FGF receptor substrate-2a, extracellular signal-regulated kinase (ERK) and early growth response element-1 (Egr-1) (Kuro-o, 2006; Urakawa et al., 2006). These observations, though preliminary, are validated by two separate groups of investigators (Kuro-o, 2006; Nabeshima, 2006; Urakawa et al., 2006), indicating that klotho indeed acts as a cofactor in FGF23-FGFR interaction and subsequent signaling. One of the likely scenarios is that Klotho regulates FGF-23 signaling through interacting with glycosaminoglycans, a notion that needs experimental validation (Razzaque and Lanske, 2006; Yu et al., 2005).

Why *klotho* mutants show similar phenotypes as *Fgf-23* mutants?

Since both FGF-23 and klotho appear to be in the same signaling cascade, it is not surprising to find out strikingly similar phenotypes in *Fgf-23* null and *klotho* mutant mice; these observations imply the fact that premature aging-like phenotypes in both these genetically altered mouse models are the consequence of the disruption of a common signaling pathway

(Razzaque and Lanske, 2006). It is however, interesting to note that despite extremely high serum levels of Fgf-23 (about 2,000 fold higher) in *klotho* mutants, Fgf-23 is unable to exert its phosphaturic effects in these mice. Both human diseases with increased FGF-23 activity, and experimental studies with over-expression of FGF-23 in transgenic animals have convincingly demonstrated that FGF-23 is a potent phosphaturic factor that induces renal phosphate wasting (ADHR_Consortium, 2000; Bai et al., 2004). However, the lack of phosphaturic activity despite extremely high levels of Fgf-23, signifies that Fgf-23 is unable to exert its physiological functions in the absence of *klotho*. Hence, it is obvious that the strikingly similar physical, biochemical and morphological phenotypes in the *Fgf-23* null mice and *klotho* mutant mice, that include shortened lifespan, emphysema, infertility (Fig-1), kyphosis, atherosclerosis, extensive soft tissue calcifications, skin atrophy, muscles wasting, T-cell dysregulation, pulmonary emphysema, osteoporosis/osteopenia, abnormal mineral ion metabolism, and impaired vitamin-D homeostasis are due to either absence of Fgf-23 (*Fgf-23* null mice) or inability of Fgf-23 to exert its function (*klotho* mutant mice). It is, therefore, reasonable to suggest that the premature aging-like phenotypes in the *klotho* mutants are mostly caused by the inability of Fgf-23 to exert its bioactivities.

Effects of *Fgf-23* or *klotho* ablation on vitamin-D homeostasis in mice

Both *Fgf-23* null and *klotho* mutant mice have shown to have increased renal expression of the *1α(OH)ase* gene, accompanied by elevated serum levels of active vitamin-D, $1,25(OH)_2D_3$ (Shimada et al., 2004a; Sitara et al., 2004; Sitara et al., 2006; Tsujikawa et al., 2003); a significant rescue of premature aging-like features has been achieved by either reducing vitamin-D activities or genetically ablating vitamin-D activities from *Fgf-23* null and *klotho* mutant mice (Razzaque and Lanske, 2006; Tsujikawa et al., 2003). Reducing vitamin-D activities in *klotho* ablated mice by feeding a vitamin-D deficient diet has resulted, not only in disappearance of ectopic calcifications, but also in gain of fertility, and most importantly prolonged survival; these observations suggest that the premature aging-like features in *klotho* mutant mice are downstream events resulting from increased activity of vitamin-D (Tsujikawa et al., 2003). In the same line, when vitamin-D activities were genetically ablated from *Fgf-23* null mice by deleting the *1α(OH)ase* gene (*Fgf-23*$^{-/-}$/*1α(OH)ase*$^{-/-}$ compound mutants), most of the premature aging-like features in *Fgf-23* null mice were rescued (Razzaque and Lanske, 2006; Razzaque et al., 2006; Razzaque et al., 2005); the phenotype of *Fgf-23*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double mutants resulted in the disappearance of ectopic calcifications from heart, kidney, and lung (Fig-2); moreover, the generalized atrophic changes in skin, intestine and other organs of *Fgf-23* null mice were rescued in *Fgf-23*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double mutants, and the resultant effect being increased overall survival of vitamin-D ablated *Fgf-23* null mice. It is, therefore, reasonable to conclude that most of the premature aging-like features in *Fgf-23* null and *klotho* mutant mice are due to hypervitaminosis-D which is most likely the consequence of lack of activity of its counter regulatory hormone, i.e., Fgf-23 (Liu et al., 2006; Razzaque and Lanske, 2006; Razzaque et al., 2006; Saito et al., 2005).

Is klotho an anti-aging factor?

The possible anti-aging effects of klotho were initially suggested from the observation that *klotho* hypomorph mice develop numerous premature aging-like features; recent studies, however, have clearly shown that premature aging-like phenotypes in *klotho* ablated mice are caused by abnormal mineral ion homeostasis due to altered regulation of Fgf-23 and vitamin-D. FGF-23 is a counter regulatory molecule of vitamin-D, and loss of Fgf-23 activity results in hypervitaminosis-D in both *klotho* mutants and *Fgf-23* null mice; most of the premature aging-like phenotypes in these mouse strains are caused by the hyperactivity of vitamin-D, as suppression of vitamin-D activities from these mouse models, not only reduces the premature aging-like features, but also extends life span (Razzaque and Lanske, 2006; Razzaque et al.,

2006; Tsujikawa et al., 2003). It will be, therefore, interesting to explore the molecular mechanisms of extended survival of *klotho* over-expressing mice.

Reduced oxidative stress and repressing intracellular signals of insulin and IGF-1 have been suggested to be related to increased survival of *klotho* transgenic mice (Kurosu et al., 2005). Such speculation is partly based on the observation that compound *klotho* hypomorphs with insulin receptor substrate-1 deleted mice (*kl/IRS-1*$^{+/-}$) could extend life-span, and survived as long as 120 to 130 days after birth (Kurosu et al., 2005). Of relevance, the *klotho* hypomorphs survive less than 100 days. In the view of the fact that the survival of *klotho* hypomorphs with reduced vitamin-D activity is actually much longer (more than 180 days without producing any obvious premature aging-like features) (Tsujikawa et al., 2003) than *kl/IRS-1*$^{+/-}$ mice (Kurosu et al., 2005), the oxidative stress and suppression of insulin signaling cascade, therefore, appear to play a minor role in producing the premature aging-like phenotypes in *klotho*-deficient mice. Although oxidant stress and IGF-1 activity are known factors that influence the mammalian aging process (Adamo and Farrar, 2006; Edwards et al., 2003; Sonntag et al., 2005; Zha et al., 2006), the involvement of klotho in such regulation requires carefully designed studies.

Again summarizing the available experimental studies, it appears that suppression/elimination of vitamin-D activities from *klotho* mutant mice, and *Fgf-23* null mice could rescue most, if not all, of the premature aging-like phenotypes from these genetically altered mouse lines (Razzaque and Lanske, 2006; Tsujikawa et al., 2003); that include but are not limited to the prevention in the occurrence of atherosclerosis, ectopic calcifications in various soft tissues, osteopenia, skin atrophy, emphysema, and hypogonadism, and the resultant effect being extended survival in both the mutants. Furthermore, altered glucose/insulin homeostasis observed in *klotho* deficient and *Fgf-23* null mice can be markedly improved by reducing vitamin-D activities from these mutants, suggesting that altered glucose/insulin homeostasis in *klotho*-mutant and *Fgf-23* null mice is indeed a secondary effect caused by the increased vitamin-D activities in these mice (Hesse et al., 2006; Tsujikawa et al., 2003). It has, therefore, become increasingly clear that premature aging-like features in *klotho* mutants are caused by the inability of Fgf-23 to exert its effects, that lead to increased activity of its counter regulatory hormone, $1,25(OH)_2D_3$ (Liu et al., 2006), resulting in altered mineral ion homeostasis to produce most of the premature aging-like features.

Concluding remarks

In this brief article, based on recent studies, we have provided relevant data to explain why we believe that premature aging-like features of *klotho* mutant mice, a widely known model for aging research, is actually due to the inability of Fgf-23 to exert its function in these mutant mice. Since, klotho acts as a cofactor to propagate Fgf-23 signaling, in *klotho* mutant mice, despite significantly high levels of Fgf-23, it is unable to exert its function, and therefore exhibits phenotypes that resemble the ones found in *Fgf-23* null mice; moreover, lack of Fgf-23 activity in *klotho* mutant mice eliminates the physiologic counter regulation of Fgf-23 and vitamin-D, resulting in hypervitaminosis-D in these mice to induce premature aging-like features in *klotho* mutants. We, therefore, speculate that FGF-23, by affecting the activities of vitamin-D might play a major role in the aging process that include but are not limited to senile osteoporosis and vascular calcifications. Finally, in this article, we wanted to highlight two important functional aspects of klotho: **1)** is premature aging in *klotho* mutant mice a primary cause or a secondary consequence? The existing observations clearly suggest that the widespread premature aging-like features of *klotho* mutant mice are the consequence of resistance to Fgf-23 activity, leading to the vitamin-D hyperactivity. That raises another important question, **2)** how klotho might act as an anti-aging molecule, if the premature aging-like features are not the primary cause of its deficiency in *klotho* mutant mice? Further

controlled *in vivo* studies will explain these complicated, yet clinically important issues of the exact role of klotho in aging.

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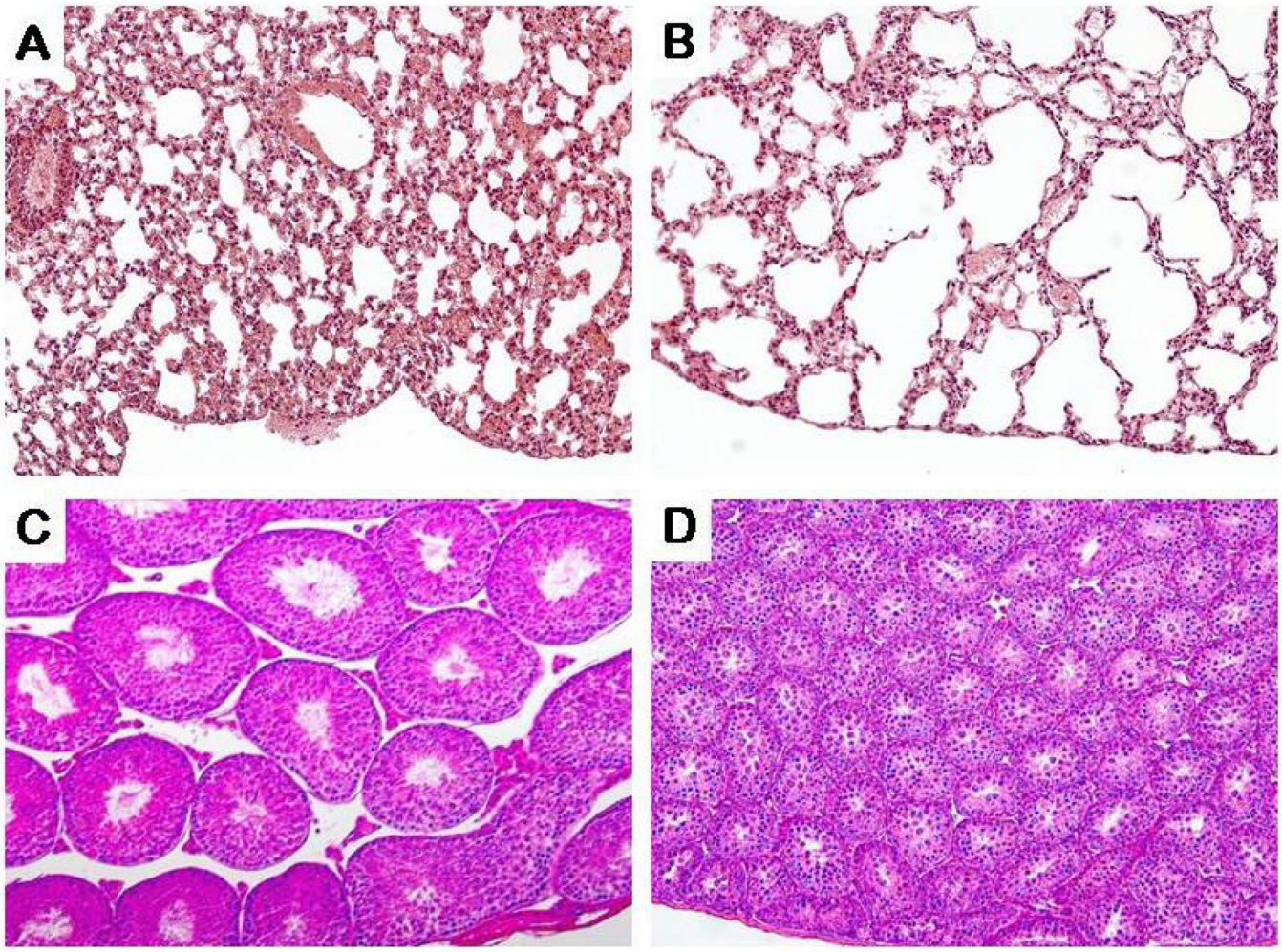

Figure-1. Pulmonary emphysema and testicular atrophy in *Fgf-23* null mice

Histological appearances of control (**A**) and *Fgf-23* null mice (**B**) lungs, showing dilated alveolar spaces, resembling emphysema in *Fgf-23* null mice (**B**). Histological features of testis, obtained from control (**C**) and *Fgf-23* null mice (**D**). Note that *in vivo* genomic ablation of the *Fgf-23* gene has resulted in severe atrophic changes in the testis, and the resultant effect being infertility. The histological images are taken from 6 weeks old mice. (Magnification: **A** and **B** ×20; **C** and **D** ×10).

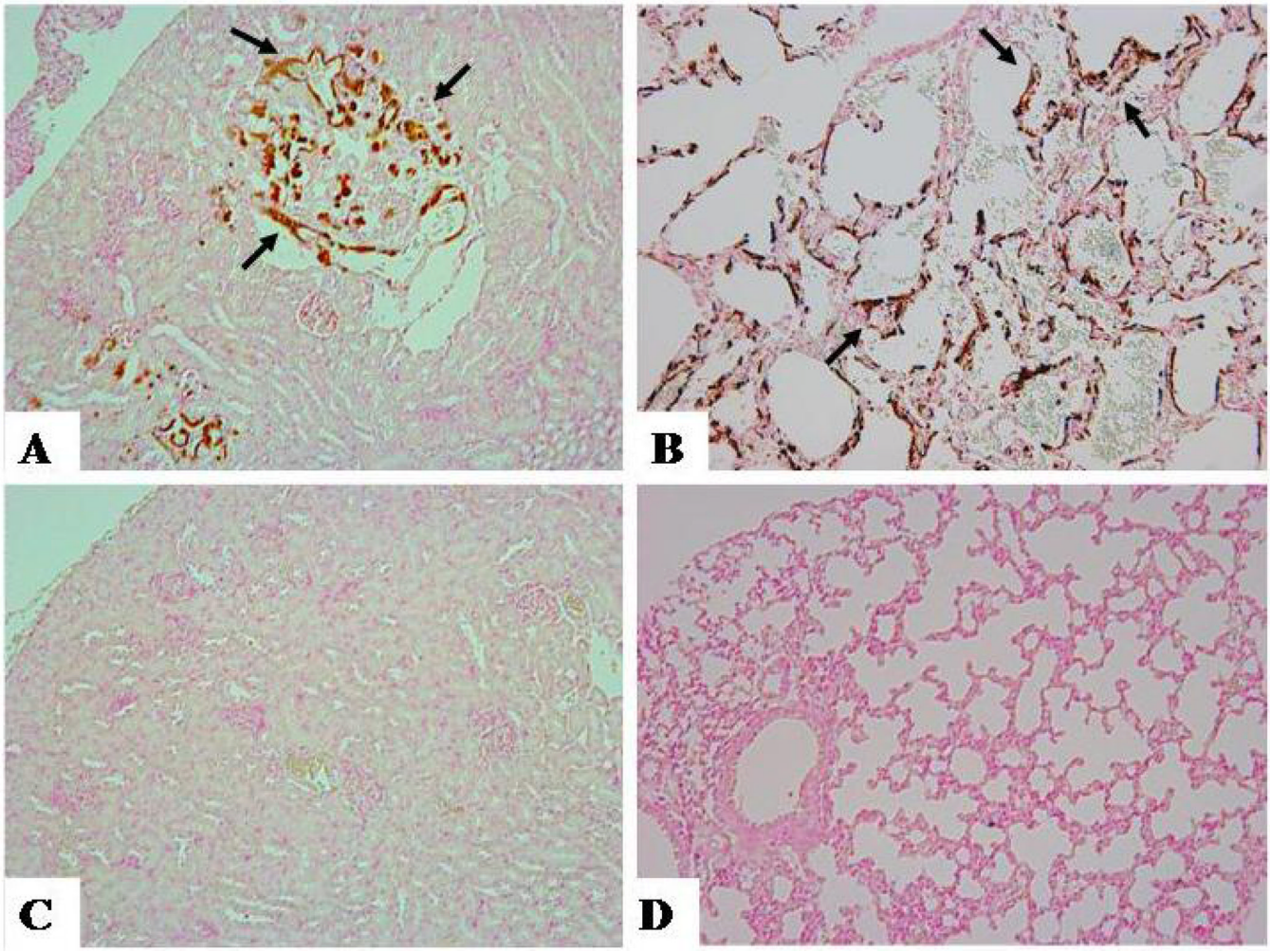

Figure-2. Soft tissue calcification

von Kossa staining on paraffin sections of the kidney (**A, C**) and lung (**B**, **D**), showing widespread renal (**A**) and pulmonary (**B**) calcifications (arrows) in *Fgf-23* null mice. Note that *in vivo* ablation of the *1α-hydroxylase* gene from *Fgf-23* null mice has eliminated renal (**C**) and pulmonary (**D**) calcifications from double null mice ($Fgf\text{-}23^{-/-}/1\alpha(OH)ase^{-/-}$). The histological images are taken from 6 weeks old mice. (Magnification: ×10).