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Premature aging-like phenotype in fibroblast growth factor 23 null mice is a vitamin D mediated process

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Abstract

Fibroblast growth factor 23 null mice (*Fgf-23*^{−/−}) have a short lifespan and show numerous biochemical and morphological features consistent with premature aging-like phenotypes, including kyphosis, severe muscle wasting, hypogonadism, osteopenia, emphysema, uncoordinated movement, T cell dysregulation, and atrophy of the intestinal villi, skin, thymus, and spleen. Furthermore, increased vitamin D activities in homozygous mutants are associated with severe atherosclerosis and widespread soft tissue calcifications; ablation of vitamin D activity from *Fgf-23*^{−/−} mice, by genetically deleting the *1α(OH)ase* gene, eliminates atherosclerosis and ectopic calcifications and significantly rescues premature aging-like features of *Fgf-23*^{−/−} mice, resulting in prolonged survival of *Fgf-23*^{−/−} *1α(OH)ase*^{−/−} double mutants. Our results indicate a novel role of Fgf-23 in developing premature aging-like features through regulating vitamin D homeostasis. Finally, our data support a new model of interactions among Fgf-23, vitamin D, and *klotho*, a gene described as being associated with premature aging process.

Keywords

rescue; *klotho*; phosphate

Aging is a complex biological process controlled by multiple genetic and environmental factors (1–4). Studies involving molecular mechanisms of human aging and its progression are challenging, as it takes decades to develop some of the age-related features. Since extensive subsets of age-associated phenotypes define human aging, the availability of animal models exhibiting multiple aging features are useful, not only to analyze the molecular mechanisms of age-related changes in various organs, but also for the in vivo screening of molecules that counteract age-associated syndromes including anti-oxidant agents and hormones (5,6). DNA damage through oxidative stress, among others, is thought to be an important contributing factor in aging, and has been extensively studied in animals (1,3,7–11). However, the potential role of humoral factor(s) regulating the aging process has not been studied in similar depth and detail. In this study, we show that genetic ablation of *Fgf-23* results in a syndrome that resembles premature aging.

FGF-23, a recently identified member of the FGF family, is involved in regulating phosphate homeostasis (12). FGF-23 is the only member of the FGF family that contains a proconvertase processing site. Cleavage of the intact 30 kDa-secreted protein results in two smaller fragments of ~18 kDa (amino fragment) and 12 kDa (carboxy fragment) of yet unknown function.

Although various studies have shown that FGF-23 can exert certain activities through binding to the known receptors for other members of the FGF family (13,14), the search for a novel receptor that specifically mediates the actions of FGF-23 is ongoing. Genetic modifications of FGF-23 in animals have shown phenotypes similar to those of human diseases. Transgenic mice overexpressing FGF-23 exhibit hypophosphatemia, reduced serum 1,25(OH)₂D₃ levels, and rickets (15–17), while *Fgf-23* null mice exhibit hyperphosphatemia, elevated serum 1,25(OH)₂D₃ levels with abnormal skeletogenesis (17,18). *FGF-23* transgenic mice mimic autosomal dominant hypophosphatemic rickets (ADHR), X-linked hypophosphatemia (XLH), and oncogenic osteomalacia (OOM) in humans, while the *Fgf-23* null mice mimic the features consistent with human familial tumoral calcinosis (FTC) (12,19,20).

In this study, using in vivo genetic manipulation approaches, we present a novel role of Fgf-23 in premature aging and show that the premature aging-like phenotype in *Fgf-23*^{-/-} mice is partly mediated through increased vitamin D activities.

MATERIALS AND METHODS

Experimental mice

We recently generated *Fgf-23* null mice (*Fgf-23*^{-/-}) (17) and *1α hydroxylase* gene knockout animals (21). We crossbred heterozygous *Fgf-23* and *1α(OH)ase* mutants to obtain compound heterozygous animals, which were then interbred to generate desired double homozygous mutants [*Fgf-23*^{-/-}/*1α(OH)ase*^{-/-}]. Routine PCR using genomic DNA, extracted from tail clips, was performed for genotyping of the various groups of mice (17,21). Mice were fed a standard diet ad libitum containing 0.7% calcium, 0.6% phosphorus, and 3.2 IU/g vitamin D₃ (PicoLab Mouse Diet 20–5058). Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were employed using protocols approved by the institution's subcommittee on animal care (IACUC).

Macroscopic phenotype

The total body weight of all mice was taken every 3–5 days starting at 2.5 wk of age until death. Survival of various groups of animals was recorded until death of control, *Fgf-23*^{-/-}, and double mutant *Fgf-23*^{-/-}/*1α(OH)ase*^{-/-} mice.

Biochemical measurements

Blood was obtained either by retro-orbital or tail bleeding of 3-, 6-, 9- and 11-wk-old wild-type, *Fgf-23*^{-/-}, and *Fgf-23*^{-/-}/*1α(OH)ase*^{-/-} littermates. Serum was isolated by centrifugation at 3000 g for 10 min and stored at -80°C. Serum phosphorus and serum calcium were determined by colorimetric measurements using the Stanbio Phosphorus Liqui-UV Test and Calcium (Arsenazo) LiquiColor Test, respectively. Total blood of 4-wk-old mice was used to determine routine hematological parameters such as cell counts.

Skeletal analyses

Skeletal changes in *Fgf-23*^{-/-} mice and their control littermates were analyzed by X-ray, quantitative CT (pQCT) and PIXImus measurements. Alizarin red S staining of total body skeletons, routine histology, and von Kossa staining were executed as described in our earlier studies (22).

Histological analyses

Assorted fixatives as 4% paraformaldehyde, 10% buffered formalin, 95% ethanol, and Carnoy's solution were used to fix the tissues before embedding in paraffin. Four to six micrometer paraffin sections of diverse tissues were produced on a Microm HM 360 microtome (Microm, Walldorf, Germany) and subsequently mounted on SuperFrost Plus slides. Sections were routinely stained with hematoxylin and eosin, periodic acid-schiff (PAS), periodic acid-Schiff methenamine silver (PAM), Masson's trichrome, von Kossa, and Congo red. Histological examination was carried out by light microscopy.

Immunohistochemical analyses

Immunohistochemical staining was performed as described previously (23). Briefly, tissue sections were deparaffinized with xylene, rinsed thoroughly with 95% ethanol, and then soaked in 0.3% hydrogen peroxide in methanol for 15 min at room temperature to inactivate endogenous peroxidase activity. The tissue sections were incubated with 10% goat serum for 30 min, before the addition of the primary antibody against α -smooth muscle actin (α -SMA) with a dilution of 1:100 (Dako, Glostrup, Denmark). Slides were washed with phosphate buffered saline and processed further using histofine SAB-PO Kit (Nichirei, Tokyo, Japan), according to the instructions provided by the manufacturer, and then reacted with 3,3'-diaminobenzidine and H_2O_2 .

Proliferation and apoptosis

To evaluate the extent of proliferation in the different groups of mice, intraperitoneal injection of BrdU (100 μ g/g body wt.) was performed 2 h before death. Tissues were fixed routinely in 10% buffered formalin and processed for paraffin embedding. Sections were stained for BrdU by incubating at 4°C overnight using the mouse anti-BrdU antibody and were visualized with a BrdU-labeling kit (Zymed Laboratories). The extent of apoptosis was determined by TUNEL assays using a commercial kit (Roche Diagnostics), according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA isolated from kidneys of various group of mice was used to detect relative expression of *1 α (OH)lase* and *klotho* mRNA, as described earlier (24). Real-time PCR was performed in duplicate, and all the reactions were controlled by standards (nontemplate control and standard positive control). The quantity of mRNA was calculated by normalizing the C_T (threshold cycle value) of *1 α (OH)lase* or *klotho* to the C_T of the housekeeping gene *GAPDH* of the same RNA sample, according to the following formula: the average *GAPDH* C_T value (each multiplex PCR is done in duplicate) was subtracted from the average *1 α (OH)lase* or *klotho* C_T value, this result represent the ΔC_T . This ΔC_T is specific and can be compared with the ΔC_T of a control calibration sample. The subtraction of control ΔC_T from the ΔC_T of *1 α (OH)lase* or *klotho* samples is referred as $\Delta\Delta C_T$. The relative expression of *1 α (OH)lase* or *klotho* (in comparison to control littermates) in renal tissues isolated from *Fgf-23*^{-/-} mice and *Fgf-23/1 α (OH)lase* was determined by $2^{-\Delta\Delta C_T}$. The sequences of the primers used were as follows: *1 α (OH)lase* (forward 5'-ACAAGGACACCTAAACCGAACAC-3'; reverse 5'-AGCTACTGA CTGGTCCTATCACAGAA-3'), *klotho* (forward 5'-TGGCTTTCCTCCTTTACCTG-3'; reverse 5'-GCCGACACTGGG TTTTGT-3'), and *GAPDH* (forward 5'-ACTGAGGACCAG GTTGTC-3'; reverse 5'-TGCTGTAGC CGTATTCATTG-3').

In vitro T cell proliferation assay

T cell proliferation was examined after stimulation of cells with concanavalin A (Con A) or phytohemagglutinin (PHA-P) as a mitogen, using routinely established protocol (25). Briefly,

T cells isolated from thymus, lymph nodes, and spleen of control and *Fgf-23*^{-/-} mice at 6 wk of age were cultured in vitro. The medium used was RPMI-1640 with 10% fetal calf serum, 2.5 mM HEPES buffer, penicillin/streptomycin 100 U/100 µg/ml, and 5 × 10⁻⁵ M 2-mercaptoethanol. Cultures were incubated at 37° in an atmosphere of 5% CO₂. Cells were cultured in 96-well culture plates, each well containing 2.5 × 10⁻⁵ viable cells in 200 µl of medium. Wells were set up in quadruplicate, four wells with no stimulus, four wells with Con A at 5 µg/ml final concentration, and four wells with PHA-P at 10 µg/ml final concentration, for each animal. After 48 h of culture, -0.5 µCi of ³H thymidine in 25 µl of medium was added to each well, and the plates were harvested onto fiber glass filter 24 h later. Radioactive uptake (cpm) was counted by scintillation counter. The results were expressed as Δcpm, which represents the difference of counts of sample with Con A to the corresponding control counts obtained without Con A.

RESULTS

Premature aging-like phenotypes of the *Fgf-23*^{-/-} mouse

We recently generated two independent *Fgf-23*^{-/-} mouse lines by replacing the entire coding region of the *Fgf-23* gene with lacZ and the neomycin resistance gene (17). In contrast to the normal phenotype of heterozygous mice, homozygous mutants exhibit multiple features resembling premature aging; these mice develop normally until 2 wk of age and are macroscopically indistinguishable from their wild-type littermates. However, visible growth retardation is apparent from 3 wk onwards, associated with uncoordinated sluggish movements, followed by premature lethality between 4 to 13 wk. *Fgf-23*^{-/-} mice can easily be recognized by their small size and marked kyphosis (Fig. 1A). The genital organs of both sexes of *Fgf-23*^{-/-} mice are severely atrophic as observed by both macroscopic (Fig. 1D and E) and microscopic (Fig. 2A and B) examinations; the severe hypogonadism in both sexes results in infertility, a major consequence of aging in both humans and experimental animals (8,26).

Effects of *Fgf-23* ablation on lung and lymphoid system

Fgf-23^{-/-} mice show typical features of emphysema in the lungs (Fig. 2D), which appear as early as 3 wk of age and are consistent with similar emphysematous changes documented in aged populations (27). In addition, severe atrophy of thymus, spleen, and lymph nodes can be noted in *Fgf-23*^{-/-} mice when compared with controls (Fig. 1G and H); total peripheral blood lymphocyte counts are also significantly decreased in homozygous mutant mice (Fig. 1B). To test the functional activity of the mutant T cells, an in vitro proliferation assay was carried out in which cells isolated from lymph nodes, spleen, and thymus were stimulated with mitogens including Con A (Fig. 1C) or PHA-P (data not shown). T cells derived from *Fgf-23*^{-/-} mice showed markedly reduced ability to proliferate (Fig. 1C), a feature usually characteristic of human aging (28).

Abnormal calcification in *Fgf-23* null mice

Widespread thickening of vessel walls with extensive calcifications are observed in *Fgf-23*^{-/-} mice by 6 wk of age (Fig. 2G); small and medium sized arteries in the kidneys are most extensively affected (Fig. 3). Atherosclerosis is a common age-associated vascular change, and there is a growing body of evidence that increased thickening and stiffness of arteries with endothelial dysfunction in apparently healthy elderly persons poses a higher risk for developing cardiovascular diseases (29). Determining the exact role of FGF-23 in premature age-associated vascular changes may help identify new targets for intervention to prevent or delay the course of the atherosclerotic process. *Fgf-23*^{-/-} mice also exhibit widespread soft tissue calcifications in lung, kidney, skeletal muscle, skin, urinary bladder, testes, cardiac muscle, and arterial walls, as detected by von-Kossa staining (Fig. 3A, C, and E and data not shown). Soft tissue calcifications appear as early as 6 wk postnatally and progress

with age. In general, the distribution of ectopic calcification in *Fgf-23*^{-/-} mice is compatible with that of premature aging. Furthermore, there are amyloid accumulations in such tissues as kidney and heart (Fig. 2*F* and *H*). Detailed skeletal analyses of *Fgf-23*^{-/-} mice by radiographic analysis show uneven mineral distribution and reduced radio-density in the bones suggesting osteopenia. Autoradiographic studies of both forelimbs and of hindlimbs showed that the bone mineral density (BMD) was strikingly reduced in *Fgf-23*^{-/-} mice, compared with their control littermates. Dual-energy X-ray absorptiometry (DEXA) analysis showed similar reduction of BMD in 3, 6, and 11 wk of *Fgf-23*^{-/-} mice; an observation again validated by detection of significant decrease in volumetric BMD of the femoral shaft (control 426.6±21.4 vs. knockout 262.7±25) and the femoral metaphysis (control 350.4±36.2 vs. knockout 236.9±15.1) in *Fgf-23*^{-/-} mice by pQCT (17). Histomorphometric findings in these mutant mice also bear a resemblance to those found in senile osteoporosis in humans (30).

Effects of *Fgf-23* ablation on skin and intestine

The hair of *Fgf-23*^{-/-} mice is sparser than that in control littermates. A reduced number of hair follicles with marked decrease in dermal and epidermal thickness and barely detectable subcutaneous fat are consistently observed in mutant mice (Fig. 4*H*). There is less proliferative activity (as determined by BrdU labeling) (Fig. 2*T*) and increased rate of apoptosis (as determined by TUNEL staining) (Fig. 2*R*) in the hair follicles of *Fgf-23*^{-/-} mice, compared with controls (Fig. 4*Q* and *S*), a phenomenon consistent with aging (31,32). Similarly, reduction in the villus height of the intestinal mucosa, resulting in reduced mucosal surface area is a frequently observed feature during advanced age (33). *Fgf-23*^{-/-} mice show focal areas of intestinal distention (Fig. 2*J*), with marked atrophy of intestinal mucosa (Fig. 2*L*); less vascularization and very thin intestinal muscle layers are also documented in the *Fgf-23*^{-/-} mice, as determined by immunohistochemical staining of intestinal sections by α -SMA (Fig. 2*N*). BrdU staining shows decreased proliferation of intestinal cells in *Fgf-23*^{-/-} mice (Fig. 2*P*), an additional feature of age-associated intestinal changes. Figure 2*I*, *K*, *M*, and *O* represents corresponding images of wild-type controls.

Generation of *Fgf-23*^{-/-}/*1 α (OH)ase*^{-/-} compound mutants

The expression of the *1 α (OH)ase* gene was significantly elevated, as determined by quantitative real-time PCR in *Fgf-23*^{-/-} mice (data not shown); such an increased renal expression of *1 α (OH)ase* in *Fgf-23*^{-/-} mice is also reflected by increased serum levels of 1,25 dihydroxyvitamin D₃ (368.1±226.3 pg/ml in knockout mice; 56.4±13.8 pg/ml in wild-type mice) (17). To determine whether increased levels of vitamin D in *Fgf-23*^{-/-} mice could mediate some of the premature aging features documented in *Fgf-23*^{-/-} mice, we generated *Fgf-23*^{-/-}/*1 α (OH)ase*^{-/-} double knockout mice, by cross breeding heterozygous *Fgf-23* mutants with *1 α (OH)ase* mutants. In contrast to *Fgf-23*^{-/-} animals, *Fgf-23*^{-/-}/*1 α (OH)ase*^{-/-} double knockout mice were indistinguishable from wild-type mice, in terms of their appearance, body weight, and physical activities (Fig. 4); serum calcium and phosphate levels were reversed when compared with *Fgf-23*^{-/-} littermates, resulting in the absence of calcifications in all examined tissues, including in the kidney (Fig. 3*B*), heart (Fig. 3*D*), and lung (Fig. 3*F*). The serum calcium level in control mice was 8.5 ± 0.42mg/dl, was increased in *Fgf-23*^{-/-} mice (12.18 mg/dl), but decreased in *Fgf-23*^{-/-}/*1 α (OH)ase*^{-/-} double knockout mice (6.3±0.34mg/dl). Similarly, serum phosphate levels in control mice was 8.93±0.0 5mg/dl and was significantly higher in *Fgf-23*^{-/-} mice (19.63±2.72mg/dl), while markedly reduced in *Fgf-23*^{-/-}/*1 α (OH)ase*^{-/-} double knockout mice (6.36±0.17mg/dl). The size and shape of thymus, intestine, and genital organs in double knockout mice were similar to those of wild-type littermates (data not shown). The pathological changes in skin (Fig. 4*H*) and intestine (Fig. 4*E*) of *Fgf-23*^{-/-} mice were dramatically ameliorated and rescued in *Fgf-23*^{-/-}/*1 α (OH)ase*^{-/-} double mutants (Fig. 4*F* and *I*). The fact that *Fgf-23*^{-/-}/*1 α (OH)ase*^{-/-} double mutants ameliorate systemic premature aging-like features, dramatically improve lifespan (Fig. 4*C*),

and regain fertility, suggests that the induction of “premature aging-like features” in *Fgf-23*^{-/-} mice is partly mediated through abnormal metabolism of vitamin D.

Expression of *klotho* in *Fgf-23* null mice

Mice homozygous for hypomorphic alleles of the *klotho* gene exhibit a number of premature aging features, similar to above-described features of *Fgf-23*^{-/-} mice; these include kyphosis, ectopic calcifications, atherosclerosis, hypogonadism, emphysema, osteopenia, generalized organ atrophy, and short lifespan (34). We, therefore, studied the level of *klotho* expression in *Fgf-23*^{-/-} mice. By quantitative real-time PCR, we found less expression of *klotho* in the kidney of both *Fgf-23*^{-/-} mice and *Fgf-23*^{-/-}/*1α(OH)ase*^{-/-} compound mutants (Fig. 5A).

DISCUSSION

We present here a novel role of Fgf-23 in aging and longevity. Our results showed that genetically ablated *Fgf-23* mice develop extensive premature aging-like features, which include, but are not limited to reduced lifespan, infertility, osteopenia, atherosclerosis, and atrophy of the skin and other internal organs, bearing the resemblance to that observed during human aging. All these extensive age-associated phenotypes were caused by the disruption of a single gene, *Fgf-23*, a secreted protein that appears to exert its effects through binding with cell surface receptors; *Fgf-23*^{-/-} mice, along with *klotho* mutant mice, thus are very different from described previously premature-aging models that are mostly due to dysregulation of nuclear proteins (1,3,7,8). Despite the fact that *Fgf-23*^{-/-} mice show systemic premature aging-like phenotypes, the expression of the *FGF-23* gene is very restricted and mainly documented in bone (osteoblasts and osteocytes). Generalized involvement of tissues and organs outside the skeletal system in *Fgf-23*^{-/-} mice suggests that these premature aging-like features are likely to be noncell-autonomous phenomena, exerted through the pleiotropic hormonal function of Fgf-23.

Fgf-23^{-/-} mice show increased vitamin D activities and abnormal mineral ion homeostasis leading to widespread soft tissue calcifications (17,35). Interestingly, abolition of vitamin D activities from *Fgf-23*^{-/-} mice, by genetical ablation of the *1α(OH)ase* gene, rescued a number of systemic aging phenotypes, suggesting that “premature aging-like phenotypes” of *Fgf-23*^{-/-} mice are partly exerted through abnormal regulation of vitamin D.

Fgf-23^{-/-} mice show extremely high biochemical and morphological similarities with mice homozygous for hypomorphic alleles of the *klotho* gene (34). We found a decreased expression of *klotho* in kidney of *Fgf-23*^{-/-} mice. Based on the strikingly similar biochemical and morphological phenotypes of *Fgf-23*^{-/-} and *klotho* mutant mice (34) and reduced expression of *klotho* in *Fgf-23*^{-/-} mice, we presume that part of the premature aging process may be regulated through a common humoral signaling pathway in both these strains of mice. Similar to *Fgf-23*^{-/-} mice, *klotho* mutant mice display abnormal mineral ion homeostasis and increased serum levels of 1,25 dihydroxyvitamin D₃; lowering serum levels of vitamin D by dietary restriction results in alleviation of some of the aging phenotypes in *klotho* mutants, suggesting that they are downstream events resulting from elevated 1,25 dihydroxyvitamin D₃ (36); these observations are very similar to the rescue of premature aging-like phenotypes of *Fgf-23*^{-/-} mice by genetic ablation of the *1α(OH)ase* gene. From current results, and taking into account earlier studies, we propose a new model of interactions among Fgf-23, vitamin D, and *klotho* (Fig. 5B). Both Fgf-23 and *klotho* are negative regulators of vitamin D metabolism by suppressing the expression of the *1α(OH)ase* gene. It has been shown that 1,25 dihydroxyvitamin D₃ can induce *klotho*, as demonstrated by up-regulation of renal expression of *klotho* following 1,25 dihydroxyvitamin D₃ injection into wild-type mice (36). However, despite significantly increased levels of 1,25 dihydroxyvitamin D₃, we found decreased renal expression of *klotho* in *Fgf-23*^{-/-} mice, suggesting that interactions of vitamin D and *klotho*

are mediated through Fgf-23. More importantly, in *Fgf-23^{-/-}/1 α (OH)ase^{-/-}* compound mutants, where synthesis of the active form of vitamin D was completely abolished, expression level of *klotho* was similar to those found in *Fgf-23^{-/-}* mice, again emphasizing that vitamin D-mediated expression of *klotho* is regulated through Fgf-23. Furthermore, when we injected control mice with recombinant FGF-23, we did not find any significant changes in the renal expression of *klotho* compared with the vehicle treated mice, although these FGF-23-treated mice did show low level of serum phosphate, compared with vehicle treated control mice. However, when *Fgf-23^{-/-}* mice were treated with FGF-23, we found induction of renal expression of *klotho*; despite high levels of active vitamin D, renal expression of *klotho* is low in *Fgf-23^{-/-}* mice; however, as soon as these mice were exposed to exogenous FGF-23, we could observe induction of *klotho* expression.

Another aspect that needs further study is to determine the role of serum phosphate on the expression of *klotho*. In our study, the expression of *klotho* in both *Fgf-23^{-/-}* mice and *Fgf-23^{-/-}/1 α (OH)ase^{-/-}* compound mutants was very similar, despite the fact that *Fgf-23^{-/-}* mice are hyperphosphatemic and *Fgf-23^{-/-}/1 α (OH)ase^{-/-}* compound mutants are being hypophosphatemic; it is therefore unlikely that less expression of *klotho* is influenced by serum phosphate, at least in our two studied models. The influence of serum calcium, phosphate, and PTH on the expression of *klotho* in normal and abnormal mineral ion homeostasis needs further investigation. However, the premature aging features observed in *Fgf-23^{-/-}* mice are unlikely to be due to reduced expression of *klotho*; therefore, to clarify this issue, we are planning further studies to determine whether pharmacological or genetic restoration of *klotho* has any effect on the aging phenotypes in *Fgf-23^{-/-}*. Our proposed model (Fig. 5B) should provide the basis for further molecular studies to establish the exact mechanisms by which Fgf-23 interacts with *klotho* to regulate mineral ion homeostasis and to produce premature aging-like phenotypes.

In conclusion, our results provide novel genetic links among Fgf-23, vitamin D, and multiple premature aging-like phenotypes; the result that genetic ablation of Fgf-23 leads to premature aging with reduced lifespan supports the novel concept that a humoral factor(s) can regulate the aging process. Finally, *Fgf-23^{-/-}* mice may provide an important tool to study the effects of genetic or pharmacological interventions to delay premature aging process. A better understanding of intrinsic factors influencing aging processes will lead to better insights into the mechanisms by which extrinsic factors can have differential effects on aging individuals.

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Figure 1.

Premature aging features of *Fgf-23^{-/-}* mice. **A)** Mutant mouse (*top*) shows small body size, reduced muscle mass, and kyphosis when compared with normal control littermate (*bottom*) at 6 wk of age. **B)** Number of lymphocytes in peripheral blood of *Fgf-23^{-/-}* animals ($n=9$, blue bar) was significantly decreased ($P<0.0001$), when compared with controls ($n=13$, red bar). **C)** Mitogenic response of T cells isolated from thymus, lymph nodes, or spleen to concanavalin A. Shown are mean values (quadruplicates) of controls ($n=3$, red circles) and *Fgf-23^{-/-}* animals ($n=2$, blue squares). Red and blue cross-lines represent mean of 12 measurements in controls (red lines) and 8 measurements in mutants (blue lines). **D)** Atrophy of mutant uterus and ovary (*right*) when compared with normal (*left*) at 6 wk of age leads to hypogonadism and infertility in females. **E)** Atrophied testes of *Fgf-23^{-/-}* animals (*right*) also causes infertility in *Fgf-23^{-/-}* males. **F)** Quantitative assessment of the weight of spleen and thymus ($n=3$, $P<0.05$). Data are presented as fold over control (=1) after each organ weight was normalized to total body weight of matching animal. Macroscopic picture of an atrophied thymus (**G**) and spleen (**H**) of *Fgf-23^{-/-}* animals (*right*) when compared with normal control littermates (*left*).

Figure 2.

Macroscopic and microscopic features of various organs at 6 wk of age ($n=8$). Hematoxylin- and eosin stained sections of wild-type (**A**) and *Fgf-23*^{-/-} (**B**) testes and of control (**C**) and *Fgf-23*^{-/-} (**D**) lungs (**A–D**: $\times 10$). Mutant lung exhibits typical emphysematous features as observed during aging. **E**) Toluidine blue staining of an *Fgf-23*^{-/-} kidney at 6 wk of age. Arrows depict kidney stones found only in the mutants. **F**) Congo-red staining indicates amyloid deposition found in small and medium sized arteries of the mutant kidney and heart (**H**). **G**) von Kossa staining demonstrates severe calcification of the aortic wall in *Fgf-23*^{-/-} animals. Macroscopic image of small intestine of wild-type (**I**) and *Fgf-23*^{-/-} animal (**J**) ($n=6$), showing ballooning of mutant intestine. Hematoxylin/eosin staining of cross sections of wild-type (**K**) and *Fgf-23*^{-/-} littermate (**L**) (**K** and **L**: $\times 5$). Reduced height of intestinal villi, and atrophy of intestinal mucosa are shown. Immunohistochemical evaluation by α -smooth muscle actin antibody staining presents dramatic reduction in vascularization and smooth muscle coat in intestine of mutant animals (**N**), when compared with normal littermates (**M**). BrdU staining demonstrates striking diminution of proliferative cells in mutants (**P**) vs. wild-type (**O**) mice (**M** to **P**: $\times 10$). **R**) Increased apoptosis in the mutant skin is visible by positive TUNEL staining

mostly located in hair follicles when compared with normal (*Q*). BrdU labeling of the skin resulted in dramatic decrease in proliferative cells in *Fgf-23*^{-/-} (*T*) vs. normal (*S*) littermate (*Q* to *T*: ×10).

Figure 3.

Ectopic calcifications. Von Kossa staining of paraffin sections of kidney (**A**), heart (**C**; muscle and valves), and lung of *Fgf-23*^{-/-} (**E**) animals demonstrating severe calcifications of these organs. Images presented in **B**, **D**, and **F** show complete elimination of abnormal calcification pattern in littermates that are deficient for both *Fgf-23* and *1α(OH)ase* gene ($n=8$; **A** to **F**: $\times 10$).

Figure 4.

A) Macroscopic image of a wild-type (WT), *Fgf-23*^{-/-}, and *Fgf-23*^{-/-}/*1α(OH)ase*^{-/-} double mutant at ~6 wk of age. **B)** Body weight curve and survival curve **(C)** of control, *Fgf-23*^{-/-} and *Fgf-23*^{-/-}/*1α(OH)ase*^{-/-} mice (*n*>15) showing gain of weight and prolonged lifespan in compound mutants. Hematoxylin/eosin staining of intestinal sections of wild-type **(D)**, *Fgf-23*^{-/-} **(E)**, and *Fgf-23*^{-/-}/*1α(OH)ase*^{-/-} double knockout animals. Single *Fgf-23*^{-/-} mice exhibit **(E)** severe atrophy of intestine, which is significantly improved by deletion of the *1α(OH)ase* gene. Similar improvement was also noted in the skin section of *Fgf-23*^{-/-}/*1α(OH)ase*^{-/-} double knockout animals, compared with *Fgf-23*^{-/-} mice; wild-type **(G)**, *Fgf-23*^{-/-} **(H)**, and *Fgf-23*^{-/-}/*1α(OH)ase*^{-/-} **(I)** double knockout animals **(D to I: ×10)**.

Figure 5.

A) Quantitative real-time PCR was performed for *klotho* expression in kidney ($n>3$). Relative expression of *klotho* mRNA in kidneys of *Fgf-23*^{-/-} animals ($P<0.01$) and in *Fgf-23*^{-/-}/*1α(OH)ase*^{-/-} double knockout animals ($P<0.05$) is significantly decreased when compared with controls. Data shown is the *klotho* mRNA expression as fold over control (=1). **B)** Schematic representation of a model showing the possible interactions among *Fgf-23*, vitamin D, and *klotho*. *Fgf-23* activity is required for vitamin-D mediated expression of *klotho*.