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# The mineralization phenotype in *Abcc6*$^{-/-}$ mice is affected by *Ggcx* gene deficiency and genetic background – A model for pseudoxanthoma elasticum

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## Abstract

Pseudoxanthoma elasticum (PXE) is an autosomal recessive disorder characterized by ectopic mineralization of connective tissues, and shows considerable intra- and inter-familial phenotypic variability. PXE is caused by mutations in the *ABCC6* gene, and targeted ablation of *Abcc6* in mouse recapitulates PXE. In this study, we examined the hypothesis that the *GGCX* gene encoding γ-glutamyl carboxylase may interfere with the mineralization process in *Abcc6*$^{-/-}$ mice. Thus, *Abcc6*$^{-/-}$ and *Ggcx*$^{+/-}$ mice were generated on 129S1;C57 and 129S1;129X1;C57 genetic backgrounds, respectively, and backcrossed with C57BL/6J for 5 generations. Thus, these strains differ by the 129X1 contribution to the background of the mice. We then generated *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ and *Abcc6*$^{-/-}$;*Ggcx*$^{+/-}$ mice by crossing *Abcc6*$^{-/-}$ and *Ggcx*$^{+/-}$ mice. The degree of mineralization of connective capsule of vibrissae, a biomarker of the mineralization process in PXE, was evaluated by computerized morphometric analysis and quantified colorimetrically by calcium and phosphate levels in tissues. The mineralization of the vibrissae in *Abcc6*$^{-/-}$ mice takes place at ~5-6 weeks of age and is significantly enhanced at 3 months of age in comparison to wild-type mice (>10-fold, $p<0.001$). However, the onset of mineralization in *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ mice was delayed until between 3-4 months of age, suggesting that the genetic background plays a role in modifying the mineralization process. The mineralization in the *Abcc6*$^{-/-}$;*Ggcx*$^{+/-}$ mice was accelerated in comparison with age-matched *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ mice, with ~3-fold difference at 3, 4 and 9 months of age ($p<0.01$). The mineralization process was also accelerated in these mice by a special custom-designed diet with mineral modifications. These findings suggest a role for both the *GGCX* gene and the genetic background as well as dietary factors in modulating the phenotypic severity of PXE caused by loss-of-function mutations in *ABCC6*.

### Keywords

Pseudoxanthum elasticum; ectopic mineralization; heritable skin diseases; ABC transporters

## Introduction

Pseudoxanthoma elasticum (PXE) is a multi-system disorder characterized by extensive mineralization of the peripheral connective tissues (for review, see refs. [1,2]). While a number of organs demonstrate ectopic mineralization, the clinical findings primarily involve

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**Competing interests** None

three different organ systems, *viz.* the skin, the eyes, and the cardiovascular system. The characteristic cutaneous finding is a small, yellowish papule on the lateral side of the neck, axillae, and the antecubital fossae. The cutaneous lesions then coalesce into plaques of inelastic, leathery skin that depicts loss of recoil and elasticity. With the progression of the disease, essentially all of skin can become involved with the major cosmetic impact on the patient. The ocular manifestations characteristically consist of angioid streaks, associated with neovascularization and bleeding into the eye [3]. The eye involvement then leads to a progressive loss of the central vision and occasionally causes blindness as early as the fourth decade of life. The cardiovascular manifestations include intermittent claudication, bleeding from the gastric arteries and, occasionally, myocardial infarcts in early fourties. However, there is considerable clinical, both intra- and inter-familial, phenotypic variability so that one of the organ systems may be predominant in some families.

The characteristic histopathologic finding in PXE is accumulation of pleiomorphic elastotic material which becomes progressively mineralized [1,2]. Similarly, the angioid streaks reflect mineralization of Bruch's membrane, a thin elastin-rich layer behind the pigmented epithelium of the retina. Mineralization also affects the cardiovascular system primarily by deposition of calcium/phosphate complexes in the elastic lamellae in the arterial blood vessels. Thus, PXE was initially postulated to be a connective tissue disorder with primary abnormalities in the elastic fibers [4]. Subsequent genetic linkage analyses and sequencing of the genes encoding the principal components of the elastic structures, elastin and the elastin-associated microfibrillar proteins, excluded the elastic fibers as the primary sites of genetic lesions [5,6]. Subsequent positional cloning analyses mapped the PXE locus to the short arm of human chromosome 16, to the region 16p13.1 [7,8], and sequencing of the putative candidate genes within the critical interval identified mutations in the *ABCC6* gene, a member of the ATP–binding casette family C, member 6 (for review, see [9]). The *ABCC6* gene encodes a 1,503 amino acid transmembrane protein, ABCC6 (also known as MRP6), which is postulated to serve as an efflux pump, but the precise molecules transported under physiologic conditions are currently unknown.

PXE-like cutaneous manifestations have also been described in a number of other clinical situations, including β-thalassemia and sickle cell anemia, as well as a sequela of long-term treatment with D-penicillamine [9-11]. More recently, it has also been demonstrated that patients with mutations in the *GGCX* gene develop not only vitamin K-dependent coagulation factor deficiency but also PXE–like cutaneous findings [12,13]. Most interestingly, we have recently reported a family in which patients with PXE harbored heterozygous mutations in *ABCC6* and *GGCX* genes, suggesting digenic inheritance of PXE in these individuals [14]. The latter studies suggested that *GGCX* may serve as a modifier gene that complements the loss-of-function mutations in the *ABCC6* gene, thus enhancing the connective tissue mineralization and leading to PXE-like cutaneous findings.

Well over 300 distinct mutations have been disclosed in the *ABCC6* gene in different families with PXE. Careful examination of the mutation database in the context of clinical manifestations in different families with PXE has not provided clear-cut genotype/phenotype correlations that would explain the considerable phenotypical variability [9]. We have recently developed an *Abcc6*$^{-/-}$ mouse which recapitulates the genetic, histopathologic and ultrastructural features of PXE [15]. Specifically, these mice demonstrate mineralization in the skin, eyes, and the arterial blood vessels similar to that in patients with PXE. In this study, we have utilized this mouse model to explore the contribution of genetic background, and particularly the expression of the *GGCX* gene, to the mineralization process.

## Materials and methods

### Mice and the special diets

The PXE mouse model was developed by targeted ablation of the *Abcc6* gene, as described previously [15]. Heterozygous alleles were backcrossed for five generations into the C57BL/6J background (N5) and interbred to generate *Abcc6*$^{-/-}$ knock-out (KO) and *Abcc6*$^{+/+}$ wild-type (WT) mice. The *Ggcx* heterozygous mice were obtained from University of Michigan (courtesy of Dr. David Ginsburg) [16]. Heterozygous alleles for *Ggcx* were backcrossed into C57BL/6J mice for five generations (N5). Compound *Abcc6*; *Ggcx* transgenic mice were generated by intercrossing *Abcc6*$^{-/-}$ mice with *Ggcx*$^{+/-}$ mice. The mice, fostered in the Animal Facility of the Thomas Jefferson University, had free access to water and were maintained in a temperature- and humidity-controlled environment under 12-hour light/dark cycles.

The mice were fed either a standard rodent diet (Laboratory Rodent Diet 5010; PMI Nutrition, Brentwood, MO, USA) or an experimental diet with specific mineral modifications (Harlan Teklad, Rodent diet TD.00442, Madison, WI, USA). In comparison with the standard rodent diet, the percentage of absorbable phosphorus (non-phytate) was increased by 2-fold (from 0.43% to 0.85%), vitamin D3 was increased by 22-fold (from 4.4 IU/g to 100 IU/g). In addition, there was a decrease in calcium (from 1.0% to 0.4%) and magnesium (from 0.22% to 0.04%).

The animal studies were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Histopathology and quantification of tissue mineralization by computerized morphometric analysis

For histopathological analysis of mineralization of vibrissae, muzzle skin biopsies were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin, Alizarin Red, or von Kossa using standard methods. Computerized morphometric analysis of hematoxylin and eosin-stained sections of muzzle skin was performed as described elsewhere [17]. The sections were examined with a Nikon model Te2000 microscope furnished with an Auto Quant Imaging system (Watervliet, New York, NY). The number of vibrissae with and without evidence of mineralization was determined in all sections, and the extent of mineralization was described as the percentage of area of mineralization per total area of vibrissae.

### Quantification of calcium and phosphate deposition

To quantify the calcium/phosphate depositions in mouse vibrissae, the muzzle skin which contains the vibrissae, was harvested and decalcified with 0.15 N HCl for 48 hours at room temperature. The calcium content was determined colorimetrically by the *o*-cresolphthalein complexone method (Calcium (CPC) Liquicolor; Stanbio Laboratory, Boerne, TX). The phosphate content was determined with Malachite Green Phosphate Assay kit (BioAssay Systems, Hayward, CA). The values for calcium and phosphate were normalized to tissue weight. Calcium and phosphorus in the serum samples were quantitatively assayed as above.

### Statistical analysis

The results in different groups of mice receiving various diets were first analyzed for normal distribution using Hamilton's test. Comparisons of continuous measures across all groups were completed using two-sided Kruskal–Wallis non-parametric tests [18]. The Kruskal–Wallis test is comparable to one-way analysis of variance, but without the parametric

assumptions. For each of the paired group comparisons, an exact two-sided Wilcoxon's test was computed. All statistical computations were completed using SPSS version 15.0 software.

## Results

### Connective tissue mineralization in *Abcc6*$^{-/-}$ mice

In this study, we have utilized *Abcc6*$^{-/-}$ knock-out mice (KO) as a model system for ectopic mineralization in PXE. These mice were developed by targeted ablation of the mouse *Abcc6* gene resulting in deletion of exons 15-18 [15]. The background of the initial KO mice was 129S1/SvImJ;C57BL/6J, and they were backcrossed for 5 generations into C57BL/6J background (N5). As reported previously [15], these mice demonstrate extensive ectopic mineralization of connective tissues in a number of organs (not shown). In particular, an early, reproducible biomarker of mineralization was found to be the presence of calcium/phosphate deposits in the connective tissue capsules surrounding the vibrissae, and this is reliably noted to begin at 5-6 weeks after birth and progresses so that at 3 months of age, mineralization in *Abcc6*$^{-/-}$ mice is obvious (Fig. 1b) [19]. No mineralization in the vibrissae of the corresponding wild-type mice is noted at 3 months of age (Fig. 1a) or during the subsequent follow-up to two years of age [15]. Thus, the *Abcc6*$^{-/-}$ mice demonstrate ectopic connective tissue mineralization recapitulating the histopathologic features of PXE.

### The role of the *Ggcx* gene in the mineralization process

Recent studies have revealed that the *GGCX* gene, when deficient, can result in vitamin K-dependent coagulation factor deficiency as well as PXE-like cutaneous findings [12,13]. Furthermore, we have demonstrated that individuals heterozygous for an *ABCC6* mutation when combined with a heterozygous *GGCX* mutation also can result in PXE-like cutaneous findings [14]. To examine the role of the *Ggcx* gene as a potential modifier gene for mineralization, the *Abcc6*$^{-/-}$ mice were crossed with *Ggcx*$^{+/-}$ mice which were initially developed at 129S1/SvImJ; 129X1/SvJ; C57BL/6J background [16]. These mice were kept on standard control diet. The mineralization in the vibrissae was quantitated by computerized morphometric analysis of hematoxylin and eosin (H & E) stained sections (Fig. 1, top row), and the presence of calcium/phosphate deposits was demonstrated by special stains (Alizarin Red and von Kossa stains; Fig. 1, middle and bottom rows, respectively). Examination of *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ mice revealed no mineralization at 3 months of age (Fig. 1c) while the original *Abcc6*$^{-/-}$ mice displayed distinct mineralization at the same age (Fig. 1b). Furthermore *Abcc6*$^{-/-}$ mice with haploinsufficiency of the *Ggcx* gene, *i.e. Abcc6*$^{-/-}$; *Ggcx*$^{+/-}$, demonstrated mineralization of vibrissae at 3 months of age (Fig. 1d) while the *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ littermates did not (Fig. 1c). Thus, mineralization in the *Abcc6*$^{-/-}$ mice, when on Ggcx$^{+/+}$ background, was delayed as compared to the original *Abcc6*$^{-/-}$ mice. However, deficiency in the *Ggcx* gene (*Ggcx*$^{+/-}$) accelerated the mineralization at three months, indicating a role for the *Ggcx* gene in this process. Nevertheless, the *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ mice demonstrated mineralization at four months of age (Fig. 1e), and this progressively increased between four and nine months of age (Fig. 1g). At the same time, mineralization in the *Abcc6*$^{-/-}$;*Ggcx*$^{+/-}$ also progressively increased between three and nine months of age (Fig. 1d,f,h) and exceeded that noted in *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ littermates (Table 1).

To verify the accumulation of calcium and phosphate in the mineralized areas of the vibrissae, the amounts of calcium and phosphate were separately determined by chemical assays and normalized to the tissue weight of the biopsies of the muzzle skin. The amounts of calcium and phosphate (expressed as Ca x P products in vibrissae per g tissue) confirmed

the results of histopathologic analysis of *Abcc6*$^{-/-}$; *Ggcx*$^{+/+}$ and *Abcc6*$^{-/-}$; *Ggcx*$^{+/-}$ mice at three, four, and nine months of age (Fig. 2).

### Modulation of mineralization by genetic background (129X1/SvJ)

As indicated above, the genetic background of the *Ggcx*$^{+/-}$ mice seemed to delay the mineralization process in *Abcc6*$^{-/-}$ mice apparently due to differences in the genetic make-up of the embryonic stem (ES) cells used in development of the original KO mice. Specifically, the development of *Ggcx*$^{+/-}$ mice utilized R1 ES cells while the *Abcc6*$^{-/-}$ mice were developed using W9.5 ES cells. These two ES cells differ by the presence of 129X1/SvJ in R1 cells. To examine the influence of the genetic background in these mice, apart from the *Ggcx* deficiency, we crossed the *Abcc6*$^{-/-}$ mice with mice of 129X1/SvJ strain (Fig. 3a). The heterozygous mice were then interbred to generate *Abcc6*$^{-/-}$ mice with a contribution from the 129X1/SvJ genetic background, *i.e. Abcc6*$^{-/-}$; 129X1/SvJ. These mice were compared with the original *Abcc6*$^{-/-}$ mice on C57BL/6J background. As shown in Fig. 3b, mineralization of the connective tissue in vibrissae was delayed in *Abcc6*$^{-/-}$ with 129X1/SvJ background contribution, and took place between the third and fourth month of age, as compared to at 5-6 weeks in the original *Abcc6*$^{-/-}$ mice (Fig. 1a,b).

### Experimental diet with mineral modifications alters the mineralization process

Early retrospective analyses of patient cohorts with PXE have suggested that ingestion of high quantities of dairy products, enriched in calcium and phosphate, during childhood and adolescence may accelerate the phenotypic manifestations of PXE [20,21]. More recently, we have demonstrated that diet with enrichment of magnesium counteracts the mineralization in *Abcc6*$^{-/-}$ mice [22]. Thus, we proceeded to explore the effects of a special diet with mineral modifications in our PXE mouse model system, consisting of *Abcc6*$^{-/-}$; *Ggcx*$^{+/+}$ mice, with the hypothesis that this special custom-designed diet may enhance the mineralization process. The mice were placed on the special experimental diet (see Materials and methods) at four weeks of age at the time of weaning and continued on this diet for another two months. At three months of age, the degree of mineralization in the vibrissae was first examined by computerized morphometric analysis. As shown in Fig. 4a, the mice on experimental diet demonstrated extensive mineralization of the connective tissue capsule of the vibrissae which was significantly more extensive than that noted in the corresponding mice kept on normal control diet (Fig. 1c and Table 1).

The acceleration of mineralization in *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ mice on the experimental diet, as compared to those on control diet, was also confirmed with chemical assay of calcium and phosphate contents of the muzzle skin containing the vibrissae (Fig. 2). Furthermore, comparison of *Abcc6*$^{-/-}$; *Ggcx*$^{+/+}$ mice with *Abcc6*$^{-/-}$; *Ggcx*$^{+/-}$ mice, both kept on experimental diet, revealed that the latter mice showed a 3-fold enhancement of mineralization, as determined by calcium and phosphate assays (Fig. 2, right hand columns) ($p<0.05$). Necropsy of the *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ mice kept on experimental diet revealed extensive mineralization in the kidneys, lungs, spleen and the eyes attesting to the multi-organ involvement in this mouse model of PXE, and similar results were noted in *Abcc6*$^{-/-}$;Ggcx$^{+/-}$ mice kept on the experimental diet. Finally, assay of the calcium and phosphorus concentrations in the serum of mice placed on experimental diet revealed values comparable to those measured in mice kept on control diet, and the serum Ca/P ratio was unaltered (Table 2).

## Discussion

PXE is a pleiotropic, multi-system disorder with considerable intra- and inter-familial heterogeneity [1,2,20]. While the cardinal clinical manifestations revolve around three organ

systems, *i.e.*, the skin, the eyes, and the cardiovascular system, there is considerable variability in terms of the age of onset of the disease and extent of the clinical findings. While PXE is an autosomal recessive disease with complete penetrance [23], the manifestations are not present at birth, and the average age of diagnosis is around 13 years of age [20]. In some families, the skin manifestations are predominant with relatively little involvement of the eyes or the cardiovascular system, while in other families, the ocular findings or cardiovascular problems predominate [20]. The reasons for this phenotypic variability are currently unknown. In this study, we have explored the reasons for the phenotypic variability utilizing a mouse model that recapitulates features of human PXE, *i.e.*, the *Abcc6*$^{-/-}$ mouse [15]. This mouse model reliably demonstrates progressive, late-onset mineralization of skin, retina, and arterial blood vessels after 3-6 months of age, but the first signs of mineralization are evident in the connective tissue capsule of vibrissae of the mice around 5-6 weeks of age. This mineralization process, which is progressive, can be followed by quantitative computerized morphometric analysis of histopathologic sections of the muzzle skin containing the vibrissae, and can also be quantitated by chemical assay of calcium and phosphate in the corresponding skin biopsies.

Our studies have previously demonstrated that the mineralization in the *Abcc6*$^{-/-}$ mice is progressive up to two years of age, while the corresponding wild-type littermates do not show any mineralization in the tissues affected in PXE [15,19]. In this study, we first explored genetic factors that might alter the mineralization phenotype in *Abcc6*$^{-/-}$ mice. First, we examined the role of the *Ggcx* gene in modifying the mineralization in *Abcc6*$^{-/-}$ mice. The interest in the *Ggcx* gene is derived from recent demonstrations that loss-of-function mutations in the *GGCX* gene in patients can result in development of vitamin K-dependent coagulation factor deficiency and PXE-like skin manifestations [12,13]. Even more strikingly, the presence of combined heterozygous mutations in the *ABCC6* and *GGCX* genes have been shown to result in PXE-like cutaneous findings, suggesting digenic inheritance of PXE in selected patients [14]. The *GGCX* gene encodes γ-glutamyl carboxylase, an enzyme necessary for activation of vitamin K-dependent coagulation factors, and thus, homozygous or compound heterozygous mutations in this gene lead to bleeding tendency [24]. This γ-carboxylation activation of the coagulation factors is dependent on the presence of reduced vitamin K in hepatocytes. The activated coagulation factors are then secreted into circulation but in the absence of γ-carboxylation reaction, the coagulation factors remain inactive and bleeding diathesis ensues. γ-Glutamyl carboxylase is also expressed in peripheral cells, such as fibroblasts where it has been shown to activate matrix gla-protein (MGP) also by γ-carboxylation reaction [24-26]. The active form of MGP serves as a powerful anti-mineralization factor, as attested by the fact that MGP KO mice display profound mineralization of connective tissues, particularly in the cardiovascular system [27]. Thus, mutations in the *GGCX* gene can explain the bleeding disorder and PXE-like cutaneous findings in the same patients due to deficient γ-glutamyl carboxylation of Gla-proteins.

The physiologic substrate transported by ABCC6, which is expressed primarily in the baso-lateral surface of the hepatocytes [28,29], is currently unknown. However, it has been postulated that one of the substrates might be a vitamin K derivative [30]. In the absence of ABCC6 transporter activity such derivative may not get transported from hepatocytes and becomes deficient in the peripheral connective tissue cells, such as in dermal fibroblasts. Consequently, the absence or reduced quantity of vitamin K co-factor in the peripheral tissue could result in reduced MGP activation and as a result, connective tissue mineralization takes place [2,26]. Consequently, identification of the critical transport substrate for ABCC6 would allow the development of a treatment strategy to directly introduce the missing factor to the circulation bypassing the liver, thus resulting in potential amelioration, and perhaps cure, for PXE, a currently intractable disease.

In the present study, besides the specific modulation of the mineralization process in the *Abcc6*$^{-/-}$ mice by haploinsufficiency of the *Ggcx* gene, the genetic background associated with the *Ggcx* heterozygous mice, 129X1/SvJ, was shown to delay the onset of the mineralization in PXE mice. In fact, while the *Abcc6*$^{-/-}$ mice reliably demonstrate mineralization of the vibrissae at 5-6 weeks, the same mice with genetic contribution from 129X1/SvJ develop mineralization not until between 3 and 4 months of age. While the precise gene content of this mouse strain is largely unknown it is conceivable that such modifier genes may also exist in humans and they modulate both the age of onset and the extent of mineralization in patients with PXE, providing partial explanation for the phenotypic variability. In this context, it is of interest that a few recent studies have also suggested genetic modulation in PXE. For example, single nucleotide polymorphisms in the promoter of the secreted phosphoprotein 1 (SPP1, also known as osteopontin) were shown to be associated with PXE, contributing to the susceptibility to this disease [31]. In another study, polymorphisms in the xylosyltransferase genes were shown to be involved in the severity of the disease [32]. Specifically, variations in the *XYLT* were shown to associate with higher internal organ involvement, while a missense variation (p. A115S) in the *XT-I* gene resulted in high serum xylosyltransferase activity, potentially reflecting increased remodeling of the extracellular matrix. Finally, genetic variations in the antioxidant genes are risk factors for early disease onset of PXE [33]. This observation is in concurrence with our previous demonstrations that oxidative stress may play a role in the development of PXE [34].

In addition to the genetic modulation, we explored the causes of phenotypic variability in PXE by placing the *Abcc6*$^{-/-}$;*Ggcx*$^{+/-}$ and *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ mice on a special, custom-designed diet with mineral modifications. Our recent studies have demonstrated that high content of magnesium in the mouse diet abolishes the development of mineralization at least up to three months of age [22]. Consequently, we tested the possibility that an experimental diet with mineral modifications, including low magnesium content, might enhance the mineralization process. Indeed, feeding of the mice with this experimental diet resulted in acceleration of the mineralization process in *Abcc6*$^{-/-}$; *Ggcx*$^{+/-}$ and *Abcc6*$^{-/-}$;*Ggcx*$^{+/+}$ mice as compared to the corresponding mice kept on standard control diet. These findings support the notion that changes in the diet can alter the age of onset and extent of mineralization in PXE. Specifically, our recent studies have shown that supplementation of the diet with magnesium can prevent and stop the progression of mineralization in the Abcc6$^{-/-}$ mouse model of PXE [22]. Collectively, our results of the study demonstrate that mineralization in a PXE mouse model can be modulated by genetic factors and diet, thus implying that PXE is a metabolic disease at the genome-environment interface [35].

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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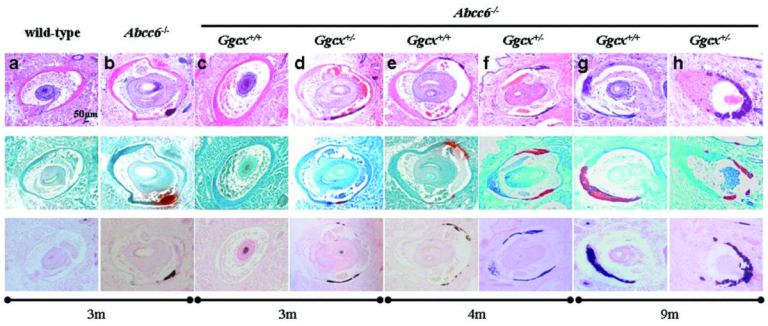


**Fig. 1.**

Histopathologic demonstration of mineralization in the vibrissae of *Abcc6*$^{-/-}$ mice crossed with *Ggcx*$^{+/-}$ heterozygous mice. The resulting *Abcc6*$^{-/-}$ mice either on *Ggcx*$^{+/+}$ (columns c, e and g) or *Ggcx*$^{+/-}$ (d, f, h) background as well as the original *Abcc6*$^{-/-}$ mice (b) and their corresponding wild-type littermates (a) were biopsied at 3, 4 or 9-months of age, and the muzzle skin samples were processed for histopathology with staining with H & E (top row), or with Alizarin Red or von Kossa stains (middle and lower rows, respectively) for calcium/phosphate.

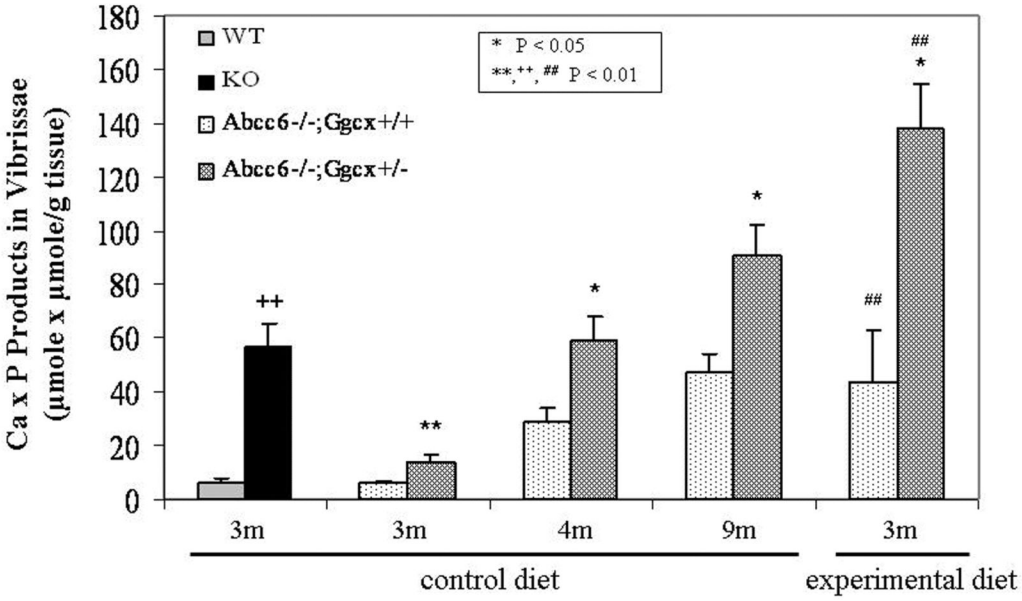


**Fig. 2.**

Quantitation of the mineralization by chemical assay of calcium and phosphate in the vibrissae of mice. A group of mice was kept on standard control diet, while others were placed at 4 weeks of age on experimental diet. The genetic background of WT and KO mice was C57BL/6J, while the other mice have a genetic contribution from 129X1/SvJ. The values for Ca x P were normalized to the weight of the tissue. The statistical significance was calculated by Wilcoxon non-parametric ranking test (n = 4-15 mice). Statistical significance: *, **, = in comparison to age-matched *Abcc6*$^{-/-}$; *Ggcx*$^{+/+}$ mice on the same diet. $^{+,++}$ = in comparison to age-matched WT mice. $^{\#,\#\#}$ = in comparison to age-matched *Abcc6*$^{-/-}$; *Ggcx*$^{+/+}$ or *Abcc6*$^{-/-}$; *Ggcx*$^{+/-}$ mice on control diet.

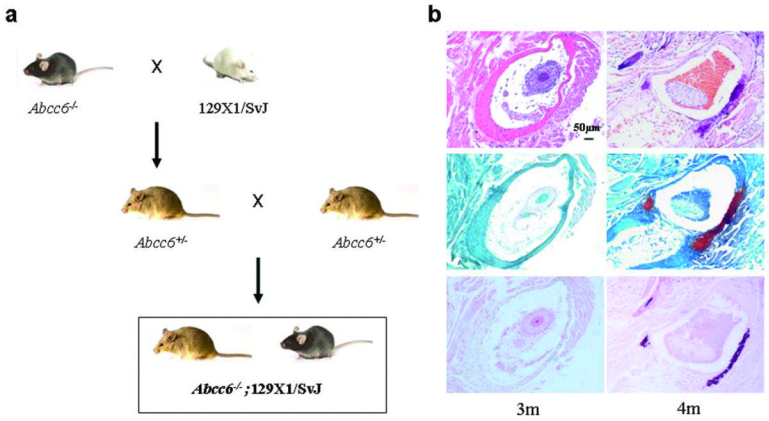


**Fig. 3.**

Schematic presentation of the crossing of *Abcc6*$^{-/-}$ mice on C57BL/6J background with mice of strain 129X1/SvJ. Note the agouti coat color in heterozygotes while Abcc6$^{-/-}$; 129X1/SvJ mice showed variable color (Box) (a). Histopathologic examination of the mineralization in the vibrissae of *Abcc6*$^{-/-}$ mice with 129X1/SvJ genetic contribution (b). Note that the mineralization, as visualized by H & E, Alizarin Red or von Kossa stains (top, middle and bottom rows, respectively) reveals that mineralization takes place between 3 and 4 months of age.

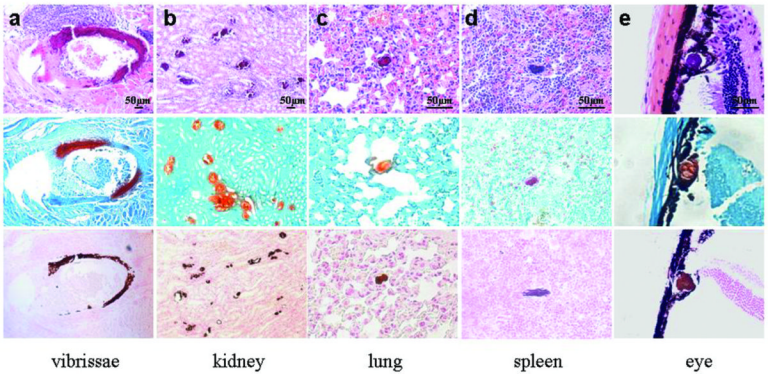


**Fig. 4.**

Illustration of extensive mineralization in various organs of *Abcc6*$^{-/-}$; *Ggcx*$^{+/+}$ mice, on C57BL/6J genetic background with contribution from 129X1/SvJ, at three months of age when placed on experimental diet. H & E, Alizarin Red and von Kossa stains (upper, middle, and bottom rows, respectively).

**Table 1**

Quantitation of connective tissue mineralization of vibrissae[1]

| Mouse | Diet | Age (months) | No. of mice | Mineralized vibrissae (per mouse) | Total vibrissae examined (per mouse) | Percent mineralized[2] | Area of mineralization per total area of vibrissae (%)[2] | Fold[3] | *P*-value[4] | *P*-value[5] |
|---|---|---|---|---|---|---|---|---|---|---|
| wild-type | control | 3 | 10 | 0 | | 0 | 0 | 0.00 | | |
| *Abcc6* $^{-/-}$ | control | 3 | 15 | 5.8 | 10.7 | 60.7 ± 5.2 | 0.73 ± 0.14 | 1.00 | | |
| *Abcc6* $^{-/-}$ *;Ggcx* $^{+/+}$ | control | 3 | 10 | 0.1 | 12.3 | 1.4 ± 1.4 | 0 | 0.00 | | |
| *Abcc6* $^{-/-}$ *;Ggcx* $^{+/-}$ | control | 3 | 8 | 2.1 | 9.3 | 27.7 ± 6.8 | 0.34 ± 0.11 | 0.47 | 0.006 | |
| *Abcc6* $^{-/-}$ *;Ggcx* $^{+/+}$ | control | 4 | 6 | 4.3 | 9.0 | 52.2 ± 9.4 | 0.88 ± 0.23 | 1.21 | | |
| *Abcc6* $^{-/-}$ *;Ggcx* $^{+/-}$ | control | 4 | 6 | 5.7 | 7.8 | 73.9 ± 11.3 | 2.45 ± 0.66 | 3.36 | 0.001 | |
| *Abcc6* $^{-/-}$ *;Ggcx* $^{+/+}$ | control | 9 | 6 | 5.3 | 8.8 | 63.8 ± 9.6 | 1.04 ± 0.61 | 1.43 | | |
| *Abcc6* $^{-/-}$ *;Ggcx* $^{+/-}$ | control | 9 | 4 | 5.8 | 8.5 | 71.1 ± 10.0 | 3.49 ± 0.49 | 4.78 | 0.011 | |
| *Abcc6* $^{-/-}$ *;Ggcx* $^{+/+}$ | experimental | 3 | 5 | 7.2 | 20.0 | 39.1 ± 5.3 | 1.03 ±0.46 | 1.41 | | 0.001 |
| *Abcc6* $^{-/-}$ *;Ggcx* $^{+/-}$ | experimental | 3 | 4 | 8.0 | 14.3 | 61.4 ± 6.7 | 3.10 ±0.78 | 4.25 | 0.065 | 0.006 |

[1] Skin sections containing vibrissae were stained with H&E and examined by computerized morphometric analysis.

[2] Values presented as mean ± S.E.

[3] Calculated using the 3-month-old *Abcc6*$^{-/-}$ mice on the control diet as 1.00.

[4] Compared with age-matched *Abcc6*$^{-/-}$*;Ggcx*$^{+/+}$ mice on the same diet.

[5] Compared with age-matched *Abcc6*$^{-/-}$*;Ggcx*$^{+/+}$ or *Abcc6*$^{-/-}$*;Ggcx*$^{+/-}$ mice on control diet.