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Stanniocalcin 2 is associated with ectopic calcification in α-klotho mutant mice and inhibits hyperphosphatemia-induced calcification in aortic vascular smooth muscle cells

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ABSTRACT

Ectopic calcification of soft tissues can have severe clinical consequences especially when localized to vital organs such as heart, arteries and kidneys. Mammalian stanniocalcin (STC) 1 and 2 are glycoprotein hormones identified as calcium/phosphate-regulating hormones. The mRNA of STCs is upregulated in the kidney of α-klotho mutant (kl/kl) mice, which have hypercalcemia, hyperphosphatemia and hypervitaminosis D and exhibit a short life span, osteopenia and ectopic calcification. In the present study, we investigated the distribution and localization of STCs in kl/kl mice. Quantitative RT-PCR revealed that renal mRNA expression of STC2 was increased in both kl/kl mice and fibroblast growth factor 23 (Fgf23)-null mice compared with wild type mice. Interestingly, STC2 protein was focally localized with the calcified lesions of renal arterioles, renal tubular cells, heart and aorta in kl/kl mice. In vitro analysis of rat aortic vascular smooth muscle (A-10) cells showed that inorganic phosphate (Pi) stimulation significantly increased STC2 mRNA levels as well as that of osteocalcin, osteopontin and the type III sodium-dependent phosphate co-transporter (PiT-1), and induced STC2 secretion. Interestingly, the knockdown with a small interfering RNA or the over-expression of STC2 showed acceleration and inhibition of Pi-induced calcification in A-10 cells, respectively. These results suggest that the up-regulation of STC2 gene expression resulting from abnormal α-klotho-Fgf23 signaling may contribute to limitation of ectopic calcification and thus STC2 represents a novel target gene for cardio-renal syndrome.

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Introduction

Patients with chronic kidney disease and osteoporosis and as well as the elderly people are at greater risk of heterotopic ossification. The pathogenesis of vascular mineralization involves both passive and active processes [1,2]. Passive mineralization is a deposition of supersaturated mineral on the region of cellular damage and degeneration. The active mechanism of calcification has many similarities with ossification via expression of osteogenic differentiation markers, such as Runx2, osteocalcin (OCN) and osteopontin (OPN). Recently, in vivo and in vitro studies have demonstrated that hyperphosphatemia is a crucial factor for vascular calcification [3,4]. Elevated inorganic phosphate (Pi) leads to transformation of vascular smooth muscle cells (VSMC) into osteoblast-like cells by mediating upregulation of osteogenic markers and initiation of extracellular matrix calcification [4–6]. Meanwhile, it has also been reported that Pi-induced calcification is caused by apoptosis of VSMC [7], although vesicles derived from apoptotic VSMC may perform similar functions to bone-specific matrix vesicles [8].

The serum level of Pi is regulated by dietary phosphorus, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], parathyroid hormone (PTH), fibroblast growth factor-23 (Fgf23), and other factors [9,10]. Recently, it has been reported that α-klotho acts as an essential cofactor of Fgf23 [11,12]. α-Klotho mutant (kl/kl) mice exhibited hypercalcemia, hyperphosphatemia and hypervitaminosis D [13]. After weaning, kl/kl mice display various phenotypes resembling human aging syndromes, including short life span, poor growth, cognitive deficit, osteopenia and ectopic calcification of various soft tissues, especially the arteries and kidneys [13,14]. Indeed, Fgf23-null mice exhibit the similar phenotype to kl/kl mice [14,15]. Various age-related disorders shown in kl/kl mice and Fgf23-null mice are ameliorated by feeding a low-Pi diet [16,17]. Fgf-23 and type IIa sodium-dependent phosphate cotransporter

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(NaPi-2a) double-knockout mice, which do not have hyperphosphatemia, are viable and rescued from some diseases [18]. Together, these data suggest that high serum levels of Pi play a key role in these disorders.

The stanniocalcin (STC) family of glycoproteins consists of STC1 and STC2 which have 35% conserved sequence identity [19]. STC was first identified from the corpuscles of Stannius in bony fish, as a regulator of mineral homeostasis, having effects on calcium influx [20,21]. Like fish STC, it is thought that mammalian STC1 and STC2 exhibit similar roles in regulating calcium (Ca) and Pi homeostasis [19]. Indeed, it has been shown that STC1 stimulates phosphate reabsorption in the small intestine and proximal tubules of the kidney [22,23], and renal STC1 mRNA expression is increased by $1,25(OH)_2D_3$ in rat [24]. We have previously characterized the human STC2 gene and shown that human STC2 decreases Pi uptake activity and human NaPi-2a gene promoter activity in opossum renal proximal tubular cell line (OK cells). We also observed that the STC2 gene is widely expressed in mice; however, its expression in hypophosphatemic (Hyp) mice, which is a model of human X-linked hypophosphatemic vitamin D-resistant rickets (XLH), was down-regulated in many organs [25]. Our more recent work identified the opossum STC2 gene and observed that its expression and secretion in OK cells were positively and negatively controlled by $1,25(OH)_2D_3$ and PTH [26]. Interestingly, Yahata et al. indicated the up-regulation of STC1 and STC2 gene expressions in the kidney of kl/kl mice [27]. However, the role of STCs in kl/kl mice has been unclear.

In the present study, to clarify the role of increased STC2 in kl/kl mice, we analyzed the gene expression and localization of STC2. We found that STC2 localized to ectopic calcification sites in kidney, heart and aorta of kl/kl mice. Furthermore, we investigated the role of STC2 in ectopic calcification by using rat aortic smooth muscle cells.

Materials and methods

Experimental animals

Heterozygous α-klotho mutant mice were purchased from CLEA Japan (Osaka, Japan) and mated to produce wild-type (WT) mice and homozygous kl/kl mice. Mice were maintained with 12 h:12 h light–dark cycles with free access to regular mouse chow and water under pathogen-free conditions. Mice genotypes were confirmed using genomic DNA extracted from tail clippings and amplified by PCR using specific primers: 5′-TGGAGATTGGAAGTGGACG-3′, 5′-CAAGGACCAGTTCATCATCG-3′ and 5′-TTAAGGACTCCTGCATCTGC-3′ [28]. Mice were weaned at 3 week (wk) of age and were given free access to water and regular mouse chow. Male and female heterozygous-*Fgf23*$^{+/-}$ mice were bred to attain WT and *Fgf23*$^{-/-}$ at 6 wk. Routine PCR was used to identify the genotypes of various mice as described previously [29]. The breeding and handling of α-klotho mutant mice and *Fgf23*$^{-/-}$ mice in experiments were approved by the Animal Experimentation Committee of the University of Tokushima and the institutional animal care and use committee at the Harvard Medical School, respectively.

Table 1
Sequence of oligonucleotide primers for quantitative RT-PCR analysis.

Name	GenBank accession no.	Primer sequence
Mouse STC1	NM_009285	5′-AAGCGCAACCCGGAAGCCATCACTG-3′
		5′-GGCTGTCTCTGATTGTACTGACCGTG-3′
Mouse STC2	NM_011491	5′-AGCCCAGGAGAACGTCGGTGTGATTG-3′
		5′-CAGCAGCAGGTTCACAAGGTCCACATAG-3′
Mouse β-actin	NM_007393	5′-CTGACCCTGAAGTACCCCATTGAACA-3′
		5′-CTGGGGTGTTGAAGGTCTCAAACATG-3′
Rat STC1	NM_031123	5′-CCAAGGTCTTCCTTGCCATT-3′
		5′-TGCTGCAAACATTGAGCTTG-3′
Rat STC2	NM_022230	5′-TACCAGTTGCAGAGGGAATG-3′
		5′-TTACAAGGTCCACGTAGGGT-3′
Rat PiT1	NM_031148	5′-CCCATCAGCACAACACATTG-3′
		5′-TAGGGACGGTGACAAACCAG-3′
Rat OCN	NM_013414	5′-CTGCATTCTGCCTCTCTGAC-3′
		5′-CCGGAGTCTATTCACCACCT-3′
Rat OPN	NM_012881	5′-GATAGCTTGGCTTACGGACTG-3′
		5′-CTTGATAGCCTCATCGGACTC-3′
Rat β-actin	NM_031144	5′-CTAAGGCCAACCGTGAAAAGA-3′
		5′-TGGTACGACCAGAGGCATACA-3′

Real-time RT-PCR and western blot analysis

Total RNA was extracted with RNA iso Plus reagent (Takara Bio Inc., Shiga, Japan) and then dissolved in RNase-free water. First-strand cDNA was synthesized from 2.5 μg of total RNA using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Two microliters of the cDNA was then used for quantitative PCR, with 20 to 30 cycles of amplification, and the PCR products were then separated by electrophoresis using 2% agarose gels. The primer sequences used for PCR amplification are described in Table 1. Real-time quantitative RT-PCR analysis was performed using the LightCycler™ (Roche Diagnostics, Tokyo, Japan) as previously described [26].

For western blot analysis, the conditioned medium from A-10 cells (see below) was centrifuged at 1500×*g* for 10 min at 4 °C. The

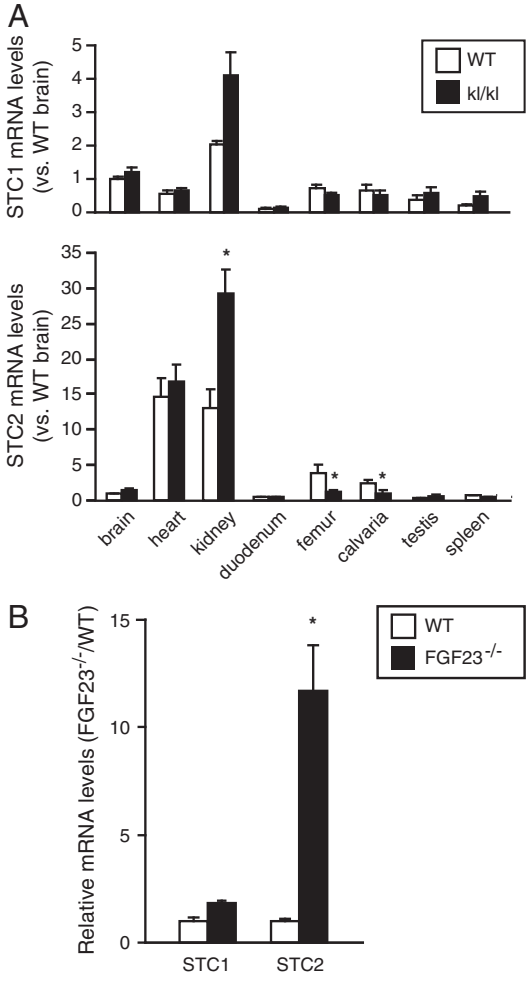

Fig. 1. The mRNA expressions of STC1 and STC2 in various tissues of kl/kl mice and kidney of *Fgf23*$^{-/-}$ mice.The mRNA levels of STC1 and STC2 were determined by real-time quantitative RT-PCR analysis using (A) brain, heart, kidney, duodenum, muscle, femur, calvaria, testis and spleen from 6 to 7-wk-old WT and kl/kl mice, and (B) kidney of 6-wk-old *Fgf23*$^{-/-}$ mice. Results were normalized to the mRNA level of β-actin. The data in A are represented as mean fold increases ± SD (n = 3–5) above the levels of WT mice brain, and the data in B are represented as mean fold increases ± SD (n = 3–5) above the levels of WT mice $^*p<0.05$ compared with WT mice.

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