Klotho expression in osteocytes regulates bone metabolism and controls bone formation

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Osteocytes within the mineralized bone matrix control bone remodeling by regulating osteoblast and osteoclast activity. Osteocytes express the aging suppressor Klotho, but the functional role of this protein in skeletal homeostasis is unknown. Here we identify Klotho expression in osteocytes as a potent regulator of bone formation and bone mass. Targeted deletion of *Klotho* from osteocytes led to a striking increase in bone formation and bone volume coupled with enhanced osteoblast activity, in sharp contrast to what is observed in *Klotho* hypomorphic (*kl/kl*) mice. Conversely, overexpression of *Klotho* in cultured osteoblastic cells inhibited mineralization and osteogenic activity during osteocyte differentiation. Further, the induction of chronic kidney disease with high-turnover renal osteodystrophy led to downregulation of *Klotho* in bone cells. This appeared to offset the skeletal impact of osteocyte-targeted *Klotho* deletion. Thus, our findings establish a key role of osteocyte-expressed *Klotho* in regulating bone metabolism and indicate a new mechanism by which osteocytes control bone formation.

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Klotho is a type I membrane protein that was originally identified as a senescence-related protein because mice carrying hypomorph *Klotho* alleles (*kl/kl*) develop a syndrome that resembled human aging.[1] The principal role of *Klotho* is to form a specific receptor complex with fibroblast growth factor (FGF) receptor 1 (FGFR1) through which it mediates the biological function of FGF23.[2,3] In the kidney, FGF23-Klotho signaling inhibits renal phosphate reabsorption by internalizing the sodium-dependent phosphate cotransporters Napi2a and Napi2c and suppresses 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] synthesis by altering the vitamin D–metabolizing enzymes CYP27b1 and CYP24a1.[4–6] The loss of Klotho hampers the binding of FGF23 to FGFR1 and leads to severe hyperphosphatemia and hypervitaminosis D. These conditions are considered to be the explanation for most features of premature aging observed in *kl/kl* mice.[7,8] Klotho is also expressed in the parathyroid gland, where FGF23-Klotho signaling inhibits the synthesis and secretion of parathyroid hormone (PTH).[9]

The FGF23-Klotho system plays an important role in the pathogenesis of chronic kidney disease (CKD)-mineral and bone disorder.[10] Circulating FGF23 levels increase during the progression of CKD, presumably as a compensatory response to maintain a normal phosphate balance.[11,12] While increased FGF23 leads to decreased 1,25$(OH)_2$D production and secondary hyperparathyroidism in early-to-moderate CKD, it fails to exert its physiological effects in end-stage renal disease owing to the absence of a functioning kidney and the systemic downregulation of Klotho.[13–16] In this setting, extremely elevated FGF23 may exert toxic effects on the cardiovascular and immune systems in a Klotho-independent manner.[17,18]

Importantly, recent investigations have discovered the presence of Klotho in osteocytes.[19] These are the most abundant cells in bone and play key regulatory roles in bone remodeling and mineral ion homeostasis.[20] Senile osteoporosis, characterized by low bone volume and low bone turnover, is one of the definitive features of *kl/kl* mice.[1] However, the functional role of Klotho in osteocytes is poorly characterized, and it is unknown whether the skeletal phenotype of *kl/kl* mice results from a functional defect of Klotho in osteocytes or systemic disturbances in mineral metabolism associated with

disrupted FGF23-Klotho signaling. Moreover, it is unknown whether Klotho expressed in osteocytes plays a role in the pathogenesis of CKD-mineral and bone disorder, including renal osteodystrophy, and if so, how.

We generated a mouse model using an osteocyte-specific deletion of the Klotho gene to explore the functional role of Klotho in osteocytes. We were able to identify a novel role of Klotho in controlling bone formation in osteocytes and determine how the function of Klotho in osteocytes is altered in the presence of CKD.

RESULTS

Targeted deletion of *Klotho* in osteocytes

Mice with osteocyte-specific deletion of *Klotho* were generated using Cre-LoxP recombination. We first crossed floxed *Klotho* (*Klotho*$^{fl/fl}$) mice with tdTomato reporter (*tdTomato*$^{fl/fl}$) mice and then crossed these mice with *Dmp1*-Cre mice (Figure 1a and b). The *Dmp1* promoter has been previously shown to exclusively induce Cre activity in osteocytes.[21,22] The presence of the tdTomato reporter supported the analysis of recombination specificity and efficiency. For our

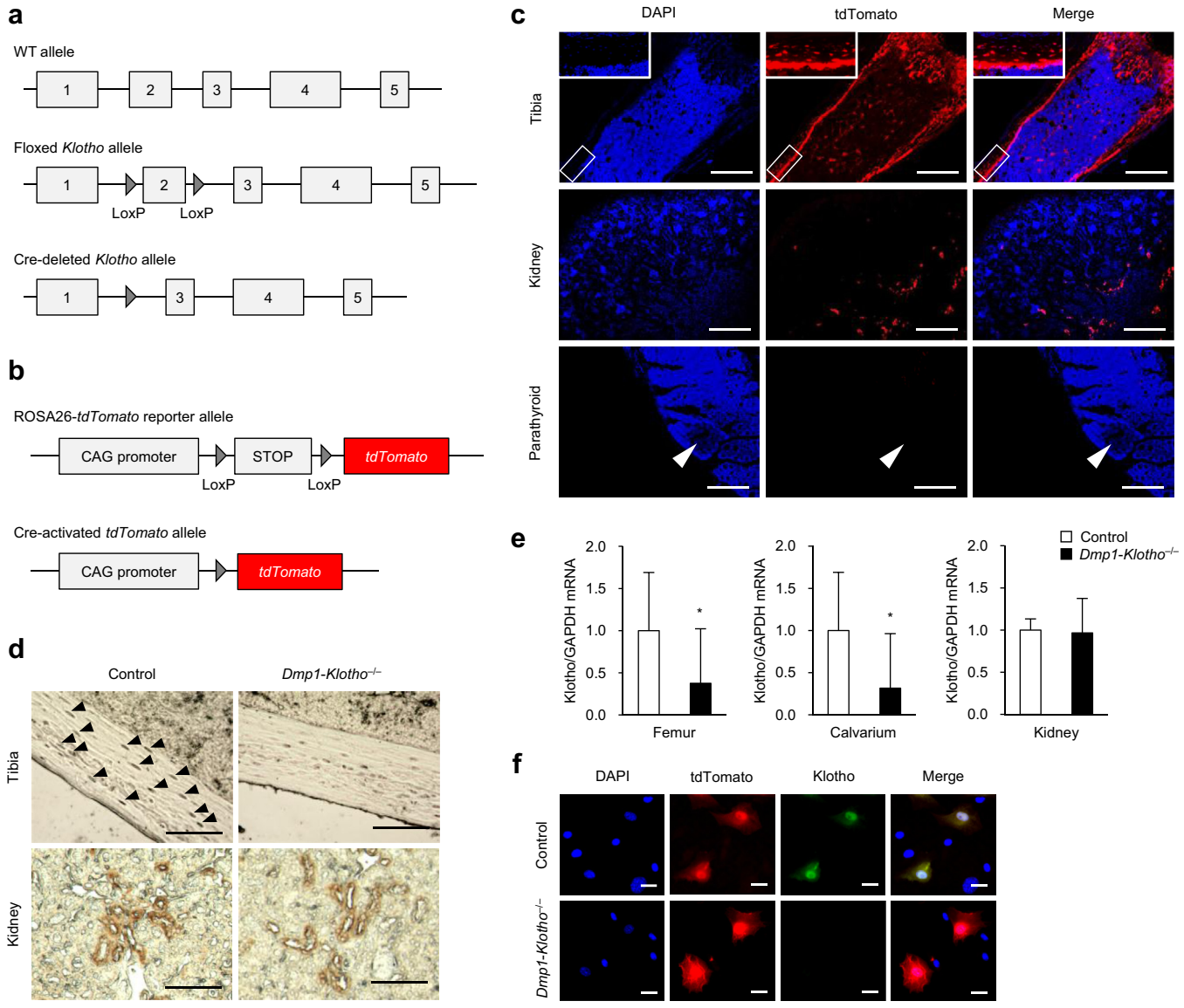

Figure 1 | Generation of mice with osteocyte-specific *Klotho* deletion and *tdTomato* expression. (**a**) Schematic representation of wild-type (WT) allele (top) and floxed *Klotho* allele before (middle) and after Cre-mediated deletion (bottom). (**b**) Schematic representation of floxed STOP *tdTomato* allele before (top) and after Cre-mediated recombination (bottom). (**c**) Localization of tdTomato fluorescence in frozen sections from 5-week-old control mice (*Dmp1-tdTomato*). tdTomato is expressed at high levels in osteocytes and is also present in the kidney medulla but is not expressed in the renal proximal and distal tubular cells and parathyroid cells (arrowheads). Bar = 300 μm. (**d**) Immunohistochemical staining of Klotho in tibial cortical bone and kidney from control mice and *Dmp1-Klotho*$^{-/-}$ mice: arrowheads indicate positive Klotho staining in osteocytes. Bar = 100 μm. (**e**) Quantitative real-time polymerase chain reaction analysis of *Klotho* transcript in the femur, calvarium, and kidney from control mice and *Dmp1-Klotho*$^{-/-}$ mice. Data are represented as mean ± SD; *$P < 0.05$ by *t* test, n = 5–7 in each group. (**f**) Immunofluorescence staining of Klotho in osteocytes isolated from the long bones of control mice and *Dmp1-Klotho*$^{-/-}$ mice. Bar = 20 μm (also refer Figure S1). DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.