Molecules and Cells

BK Channel Deficiency in Osteoblasts Reduces Bone Formation via the Wnt/β-Catenin Pathway

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Global knockout of the BK channel has been proven to affect bone formation; however, whether it directly affects osteoblast differentiation and the mechanism are elusive. In the current study, we further investigated the role of BK channels in bone development and explored whether BK channels impacted the differentiation and proliferation of osteoblasts via the canonical Wnt signaling pathway. Our findings demonstrated that knockout of Kcnma1 disrupted the osteogenesis of osteoblasts and inhibited the stabilization of β-catenin. Western blot analysis showed that the protein levels of Axin1 and USP7 increased when Kcnma1 was deficient. Together, this study confirmed that BK ablation decreased bone mass via the Wnt/β-catenin signaling pathway. Our findings also showed that USP7 might have the ability to stabilize the activity of Axin1, which would increase the degradation of β-catenin in osteoblasts.

Keywords: β-catenin, BK channel, bone development, Kcnma1, osteoblasts

INTRODUCTION

Bone maintains basic functionality, including supporting the body, making blood cells, storing minerals and maintaining structural integrity (Matsushita et al., 2020). Bone homeostasis includes bone formation and bone resorption, which depend on the interaction between osteoblasts and osteoclasts (Chen et al., 2018). Therefore, the imbalance in bone remodeling in response to decelerated bone construction and accelerated bone resorption results in low bone mass and skeletal fragility. Osteoblasts underpin the major functions of the skeleton, as they can produce bone matrix, secrete collagen type I, regulate mineralization of bone matrix and play an important role in regulating osteoclast differentiation. Bone marrow-derived mesenchymal stem cells (BMSCs) differentiate into osteoblasts (Zhang et al., 2020), which are one of the main functioning cells in bone formation. In the process of osteoblast differentiation and matrix mineralization, Osterix is expressed at a high level in skeletal progenitor stem cells and bone-forming osteoblasts (Nakashima et al., 2002) and has been widely used as a tool in bone research (Hu et al., 2021; Wang et al., 2021).

Large conductance calcium-activated potassium (BK) channels (also named the maxi-K or Slo1 channels) are among the K^+ channels that have unusually large single-channel conductance (Gonzalez-Perez and Lingle, 2019) and are distinctive in being regulated by two physiological stimuli, including elevations in cytosolic Ca^{2+} and membrane depolarization (Castillo et al., 2016). BK channels encoded by the Kcnma1 (also called Slo1 or KCa1.1) gene are more widely expressed in a larger range of cells than many other plasma membrane ion channels. Kcnma1, which undergoes extensive alternative pre-mRNA splicing, has been found to play an important

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role in many diseases (Bailey et al., 2019). The functions of BK channels in primary osteoblasts (Hirukawa et al., 2008), osteoblast cell lines (Hei et al., 2016) and progenitors such as mesenchymal stem cells (MSCs) (Zhang et al., 2014) in bone remodeling have attracted considerable attention but are not entirely defined.

The Wnt signaling pathway plays an essential role in bone formation (Oh et al., 2020; Zhou et al., 2020). The subcellular localization of β-catenin is dynamically regulated in the canonical Wnt/β-catenin signaling pathway, and the localization of β-catenin is commonly thought to be regulated by ligand–receptor interactions on the surfaces of cells (Lesage et al., 2004). Lack of β-catenin in the osteoblast lineage results in reduced cell proliferation and differentiation and increased bone resorption by osteoclasts (Choi et al., 2018). As a key scaffolding protein, Axin1 has a profound effect on Wnt/β-catenin signaling. The ubiquitin-proteasome system regulates the stability of Axin1 (Zhang et al., 2011) and thus coordinates bone dynamics (Matsumoto et al., 2017). As a ubiquitin-specific protease (USP) family deubiquitinase, USP7 is participated in a diverse array of cellular processes including osteogenic differentiation (Bhattacharya et al., 2018). However, it is not clear whether ablation of the BK channel α-subunit will cause any damage in bone through the canonical Wnt pathway. In this study, we constructed conditional knockout (CKO) mice to explore the role of Kcnma1 in osteoblasts and attempted to determine the relationship between BK channels and the Wnt/β-catenin signaling pathway in bone tissues.

MATERIALS AND METHODS

Animals

Using CRISPR/Cas9 technology, we successfully designed and constructed sgRNA targeting on exon 4 of Kcnma1 *in vitro*. A mixture of sgRNA and Cas9 mRNA was microinjected into the fertilized eggs of C57BL/6 mice (Bioray Laboratories, China). After the mice were born, DNA was extracted and the founder mice were identified by polymerase chain reaction (PCR). The newborn F1 mice were also genotyped by PCR. The Osterix-Cre transgenic mice were purchased from Bioray Laboratories. To delete Kcnma1 specifically in osteoblasts, the CKO C57BL/6 mouse line was generated by crossing Kcnma1-floxed mice ($Kcnma1^{f/f}$) with Osterix-Cre transgenic mice. We selected Osterix-Cre(+); $Kcnma1^{f/f}$ as experimental mice and Osterix-Cre(–); $Kcnma1^{f/f}$ as control mice. All the study protocols were approved by the Animal Ethical Committee of School of Pharmacy, Fudan University (No. 2017-03-YL-ZXM-01). Mice were weighed every week one month after birth.

The genotypes were determined by PCR amplification of genomic DNA isolated from the tails of mice 4 weeks after birth. DNA was extracted using an Animal Genomic DNA Quick Extraction Kit for PCR Analysis (Beyotime Biotechnology, China). PCR amplification was carried out on a CFX96 Real-Time System thermal cycler (Bio-Rad Laboratories, USA) with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation (94°C, 20 s), annealing (57°C, 30 s), and extension (72°C, 20 s), and a final round at 72°C for 5 min. Two percent agarose (Yesen, China) gels stained with 0.1% YeaRed (Yesen) were used to visualize the PCR products. The PCR products were also delivered to San Gon Biotech (China) to analyze the ordering of the sequences. The sequences of the primers and product sizes used for genotyping are listed in Table 1.

Microcomputed tomography (micro-CT)

Tibial samples from 4-month-old female mice were dissected free of soft tissue, fixed overnight in 70% ethanol and analyzed by micro-CT with a Quantum GX2 scanner (PerkinElmer, USA). For tibia reconstruction, the trabecular bone volume of interest (VOI) was drawn to include 200 slices of metaphyseal spongiosa starting from 0.9 mm below the growth plate. The trabecular bone mineral density (Tb.BMD), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular bone volume/tissue volume (BV/TV) as well as the cortical bone mineral density (Ct.BMD) and cortical bone thickness (Ct.Th) were calculated using the software supplied with the instrument.

Histological analysis

The tibiae were immediately fixed with a 2% buffered paraformaldehyde solution after the animals were sacrificed. These tissues were decalcified, dehydrated and embedded in paraffin. Then, the tissues were cut into section slices on a rotary microtome. The sections were prepared for H&E staining and tartrate-resistant acid phosphatase (TRAP) activity. All staining procedures were performed according to standard methods.

Cell culture and differentiation

ROS 17/2.8 cells were cultured with DMEM/Nutrient Mixture F-12 Ham (DMEM/F12) containing 5% fetal bovine serum (FBS). MC3T3-E1, a mouse osteoblastic cell line, was main-

Table 1. Primer sequences for genotyping

	Sequence	Product size (bp)
Kcnma1-L-loxp	Forward: 5′-CCCAACGCAAAGAAAAG-3′	Wild type: ~195
	Reverse: 5′-CACAATGCCAGGAAACG-3′	Flox: ~229
Kcnma1-R-loxp	Forward: 5′-CTCTACTTTGGCTTGCG-3′	Wild type: ~275
	Reverse: 5′-GCTTCAAACCCTTCCTT-3′	Flox: ~309
Osterix-Cre	Forward: 5′-ATTTGCCTGCATTACCGGTCG-3′	~309
	Reverse: 5′-CAGCATTGCTGTCACTTGGTC-3′	
Kcnma1-Seq	Forward: 5′-TCTCCATACTCCCTCCCTT-3′	~1,500
	Reverse: 5′-CCTAATCCTACAAGCCTTCACT-3′	

tained in α-MEM medium supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. At 90% confluence, ROS 17/2.8 and MC3T3-E1 cells were seeded into 6- and 96-well plates for subsequent experiments.

BMSCs were flushed out from the femurs and tibiae of mice with Dulbecco's modified Eagle's medium (DMEM). The primary osteoblasts were cultured with osteogenic medium containing 10% FBS (Cyagen, USA), 100 U/ml penicillin G, and 100 mg/ml streptomycin (Gibco, USA) at 37°C with 5% CO_2. Osteogenic medium was prepared in advance by supplementing the maintenance medium with 10 nM dexamethasone (Sigma, USA), 50 mg/ml ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma). Osteoblast differentiation is characterized by alkaline phosphatase and mineralization (Marie, 2008). For ALP staining, the cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde solution, and stained for ALP for 30 min in the dark using the BCIP/NBT alkaline phosphatase color development kit (Beyotime Biotechnology) by a standard procedure. For alizarin red staining, the primary osteoblasts were stained with 40 mM alizarin red solution (Yeasen, China) at 37°C for 30 min and washed with distilled water.

Cell viability assays

ROS 17/2.8 cells were seeded into 96-well cluster dishes at 5,000 cells/well. After the cells grew overnight in 100 μl of culture medium, different concentrations of paxilline (MCE, USA) were added to the plates. Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) solution was used to measure cell viability. The absorbance was measured at 450 nm according to the manufacturer's instructions. This dataset contained the experimental results repeated four times, and significant differences were assessed using Student's *t*-test.

BK overexpression

We utilized transcription activator-like effector nuclease (TALEN) technology to establish a BK knockout cell line on MC3T3-E1 cells (Hei et al., 2016). The BKα-myc plasmid was transfected into MC3T3-E1 cells in 6-well plates using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's instructions. The cells were collected for protein analysis two days later.

Quantitative real-time PCR

Total RNA from the bone powder and cells was extracted using TRIzol reagent according to the standard protocol. RNA was reverse transcribed into cDNA using PrimeScript™ RT Master Mix (TaKaRa, China). Gene expression was determined with a SYBR-Green (TaKaRa) PCR assay. The sequences are listed in Table 2.

Table 2. Primers used for RT-PCR

Gene	Primer
Runx2	Forward: 5′-TTCAACGATCTGAGATTTGTGG-3′
	Reverse: 5′-GGATGAGGAATGCGCCCTA-3′
Alp	Forward: 5′-CCAACTCTTTTGTGCCAGAGA-3′
	Reverse: 5′-GGCTACATTGGTGTTGAGCTTTT-3′
Kcnma1	Forward: 5′-CGCAAGTGATGCCAAAGAAGT-3′
	Reverse: 5′-CTTCAAAATAGATCAGCCGCC-3′
Gapdh	Forward: 5′-AGGTCGGTGTGAACGGATTTG-3′
	Reverse: 5′-TGTAGACCATGTAGTTGAGGTCA-3′

Western blot analysis

Total proteins from long bones and cells were analyzed by western blotting. The bone powder and cells were lysed with RIPA buffer supplemented with 1% protease inhibitor cocktail (P0013B; Beyotime Biotechnology). To collect the supernatants, insoluble materials were removed by centrifuging at 13,300 rpm for 15 min. The protein amount was quantified with the bicinchoninic acid (BCA) protein assay (P0012; Beyotime Biotechnology) using different concentrations of bovine serum albumin (BSA) as a standard. Protein lysates were separated by electrophoresis in 10% polyacrylamide gels and were then transferred to polyvinylidene difluoride (PVDF) membranes. Subsequently, the membranes were blocked with 5% skimmed milk solution at room temperature and incubated at 4°C overnight with primary antibodies against BK (ab3586; Abcam, UK), β-catenin (51067-2-AP; Proteintech, USA), Runt-related transcription factor 2 (Runx2) (ab76956; Abcam), Col1a1 (A1352; Abclonal, USA), GSK3β (12456T; Cell Signaling Technology, USA), p-GSK3β (67558-1-Ig; Proteintech), USP7 (66514-1-Ig; Proteintech), and Axin1 (AF3287; R&D Systems, USA). The gray densities of the protein bands were all normalized by applying GAPDH density as an internal control.

Immunohistochemical (IHC) staining

After dewaxing and hydration, antigen retrieval was performed. The tissue section slices were treated with 3% H_2O_2 for 20 min and incubated with β-catenin primary antibody (1:100) overnight at 4°C. Finally, 100 μl of HRP-conjugated secondary antibody was incubated for 30 min at room temperature. Representative images were captured by a microscope.

Statistical analyses

Data with more than two groups were analyzed with one-way ANOVA. Differences between two groups were analyzed using Student's *t*-test. All statistical analyses were performed with GraphPad Prism (ver. 5.0; GraphPad Software, USA) from at least three independent samples. A P value < 0.05 was considered statistically significant.

RESULTS

Generation of Kcnma1 osteoblast CKO mice

To investigate the effect of Kcnma1, which encodes the pore-forming α-subunits of BK in osteogenesis, we generated transgenic mice with CKO of Kcnma1 in osteoblasts. Kcnma1 CKO mice were generated by deleting exon 4 using the CRISPR/Cas9 strategy (Fig. 1A). Osterix-Cre mice were crossed with Kcnma1$^{f/f}$ mice, and their offspring were bred to obtain osteoblast-specific CKO mice (Fig. 1B). We performed PCR analysis to identify the genotype of each mouse (Figs. 1C and 1D), and the genomic DNA sequences were also confirmed by sequencing (Fig. 1E). mRNA levels were significantly de-

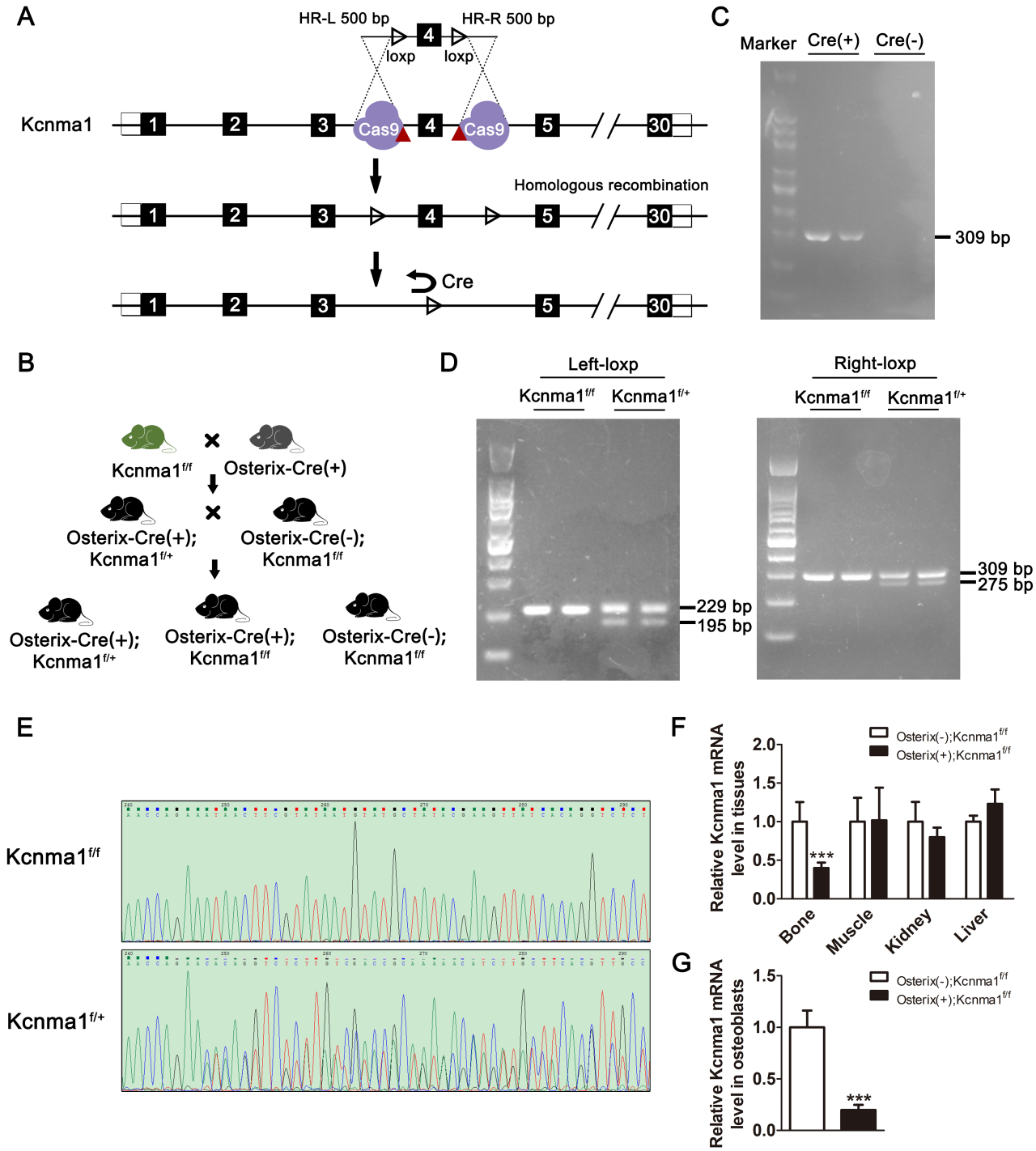

Fig. 1. Generation of Kcnma1 CKO mice. (A) Schematic outlining the generation of Kcnma1 CKO mice using the CRISPR/Cas9 system. (B) The mating system of gene-modified mice. (C and D) The targeted fragment of Osterix-Cre and Kcnma1 was amplified by PCR using genomic DNA templates. (E) DNA sequencing map for Kcnma1$^{f/f}$ and Kcnma1$^{f/+}$ mice. (F) qRT-PCR analysis of Kcnma1 mRNA levels in bone, muscle, kidney and liver from Osterix-Cre(-); Kcnma1$^{f/f}$ and Osterix-Cre(+); Kcnma1$^{f/f}$ mice. (G) Kcnma1 gene expression was significantly down-regulated in primary osteoblasts from Osterix-Cre(+); Kcnma1$^{f/f}$ mice. The Kcnma1 mRNA levels in all tissues and primary osteoblasts from Osterix-Cre(+); Kcnma1$^{f/f}$ mice were normalized to those in Osterix-Cre(-); Kcnma1$^{f/f}$ mice. Data are presented as mean ± SD (n = 3). $***P < 0.001$.

creased in bones isolated from Kcnma1$^{f/f}$ mice infected with Osterix-Cre, while there were no significant differences in other tissues (Fig. 1F). We performed quantification of Kcnma1 expression to examine the efficiency of Kcnma1 knockout in primary osteoblasts (Fig. 1G). These data demonstrated that Kcnma1 is efficiently deleted in osteoblasts in Osterix-Cre(+); Kcnma1$^{f/f}$ mice. The Osterix-Cre(+); Kcnma1$^{f/f}$ mice were viable; however, they showed a smaller and weaker appearance than the Osterix-Cre(–); Kcnma1$^{f/f}$ mice (Supplementary Fig. S1).

Kcnma1 ablation induced defective bone formation

We recorded the body weights of the mice (Fig. 2A) from the age of one month and found that Osterix-Cre(+); Kcnma1$^{f/f}$ mice were smaller than control littermates from birth; thus, we estimated that Kcnma1 ablation might induce skeletal dysplasia. The length of the tibiae in Osterix-Cre(+); Kcnma1$^{f/f}$ mice was significantly shorter than that in normal mice (Figs. 2B and 2C). Therefore, we carried out micro-CT (Fig. 2D) and H&E staining (Fig. 2F) to analyze the skeletal development of mice. A reduction in trabecular bone and reduced thickness of cortical bone in the tibiae were observed in Osterix-Cre(+); Kcnma1$^{f/f}$ female mice at the age of four months. Bone parameters (Fig. 2E), including the Tb.BMD and Tb.Th as well as the Ct.BMD, Ct.Th and BV/TV, were decreased in the tibiae of Osterix-Cre(+); Kcnma1$^{f/f}$ female mice; however, Tb.Sp was slightly increased. Osterix-Cre(–); Kcnma1$^{f/f}$ mice were used as the control.

Collectively, these data suggest that Kcnma1 may play an important role in bone development, especially in osteoblasts.

Kcnma1 contributed to osteoblast differentiation

To further determine whether deletion of Kcnma1 would influence the differentiation of osteoblasts, western blotting and reverse transcription PCR (RT-PCR) were applied to measure the expression of osteogenic markers, including Runx2, Col1a1, and Alp. Runx2 and collagen genes are involved in bone anomalies in various metabolic bone diseases, including the thickness of compact bones and the length of long bones (Funato et al., 2020). The results showed that the relative protein expression levels of Runx2 and Col1a1 were significantly reduced in Osterix-Cre(+); Kcnma1$^{f/f}$ mice (Figs. 3A and 3B). Moreover, the mRNA levels of Alp and Runx2 were also decreased in long bones from Osterix-Cre(+); Kcnma1$^{f/f}$ mice (Fig. 3C). Osteoprotegerin (OPG) protects the skeleton from excessive bone resorption by binding to receptor activator of nuclear factor-kappa B ligand (RANKL) and preventing it from binding to its receptor, RANK. As an important determinant of bone metabolism, the Rankl/Opg ratio reflects the bone-resorptive function to a certain extent. To explore the osteoblast-mediated indirect effect on osteoclasts, we detected the ratio of the gene expression of Rankl to Opg, which was slightly increased in Osterix(+); Kcnma1$^{f/f}$ mice, while it showed no significant difference (Fig. 3D). In addition, there was no significant difference in osteoclast activity as measured by trap staining between the Osterix-Cre(–); Kcnma1$^{f/f}$ and Osterix-Cre(+); Kcnma1$^{f/f}$ mice (Fig. 3E); thus, we focused on the functional changes in osteoblasts. To test osteoblast function, we extracted primary osteoblasts from femurs and

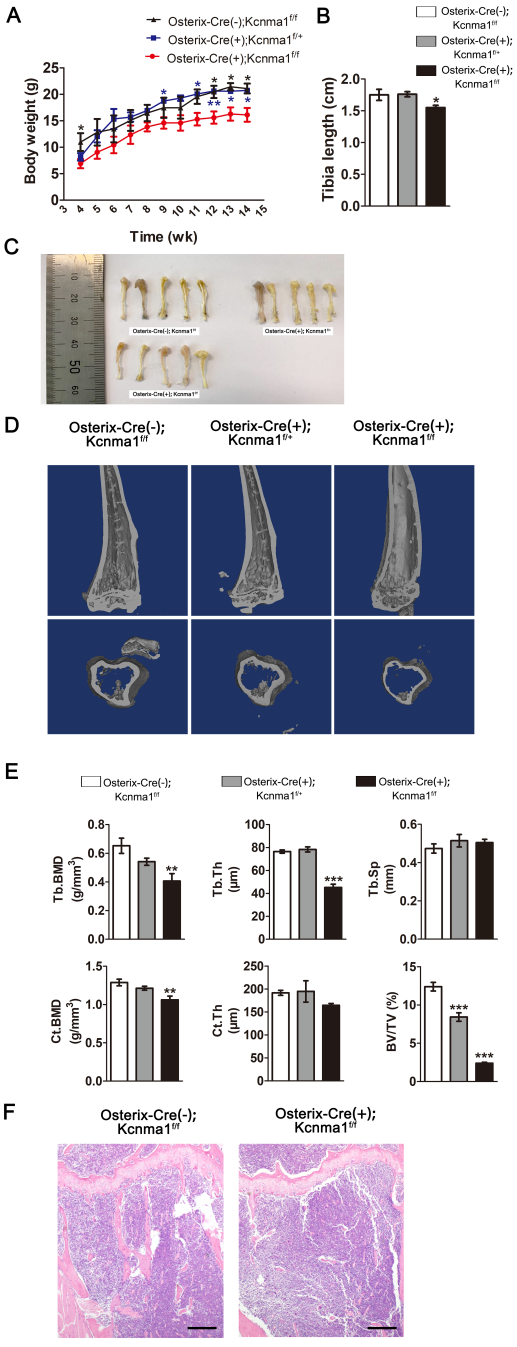

Fig. 2. Deletion of Kcnma1 impairs bone formation in mice. (A) Body weight was recorded every week beginning one month after the mice were born. Osterix-Cre(+); Kcnma1$^{f/f}$ mice were used as the control. (B) The length of the tibiae was measured in 4-month-old mice, n = 5. (C) Decreased length of tibiae of Osterix-Cre(+); Kcnma1$^{f/f}$ mice compared to control littermates. (D) Representative micro-CT images of trabecular and cortical bone in the tibiae of female mice at 4 months of age are shown. (E) Micro-CT measurements for Tb.BMD, Tb.Th, Tb.Sp, Ct.BMD, Ct.Th, and BV/TV from the groups of mice indicated, n ≥ 4. (F) H&E staining of the tibiae of Osterix-Cre(-); Kcnma1$^{f/f}$ and Osterix-Cre(+); Kcnma1$^{f/f}$ mice. Scale bars = 200 μm. Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

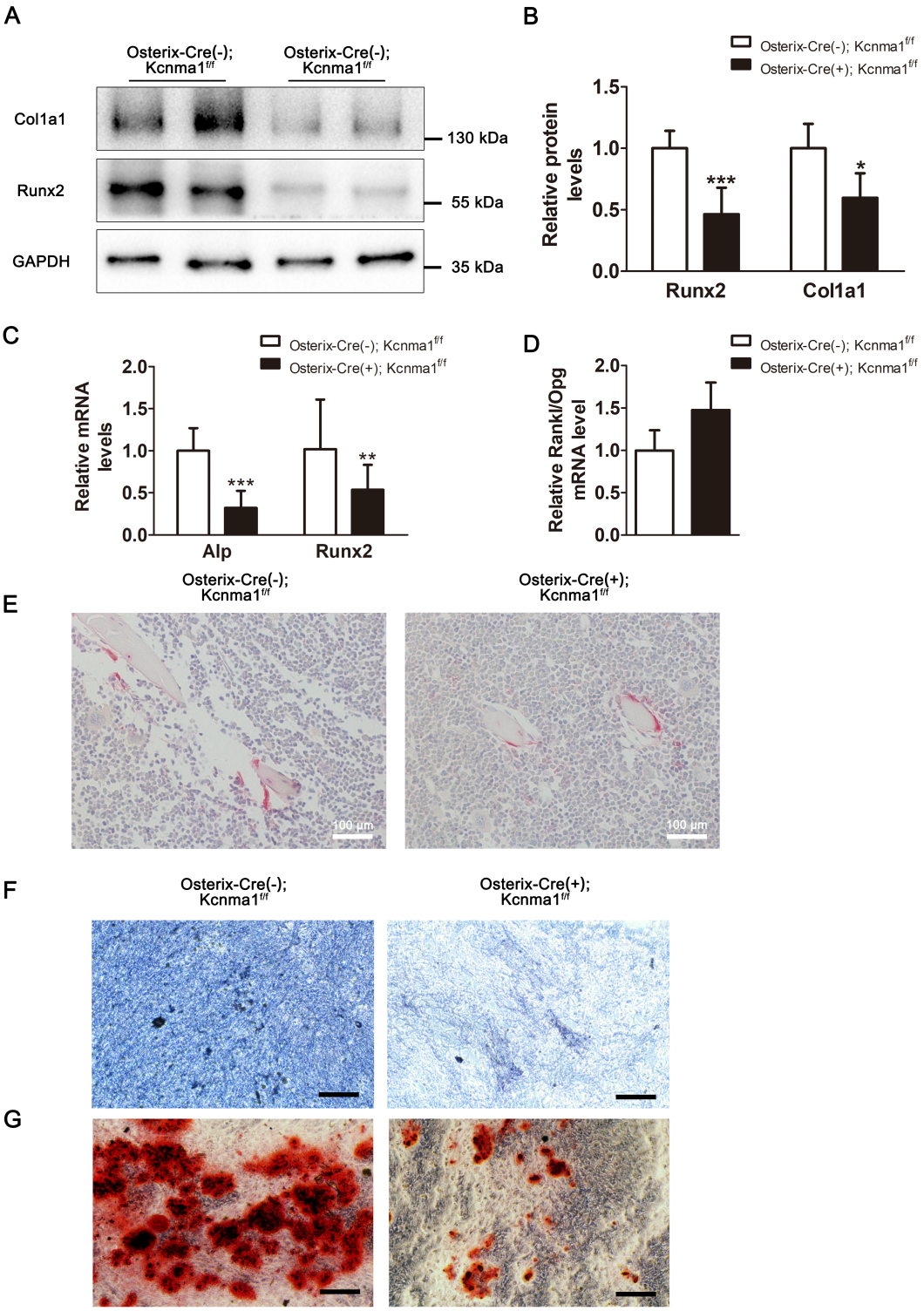

Fig. 3. Lack of Kcnma1 decreases the capacity of osteoblast differentiation. (A and B) Expression and quantitative analysis of osteogenic markers, including Runx2 and Col1a1, by western blot. Quantification of protein levels was normalized to GAPDH. (C) qRT-PCR analysis of Alp and Runx2 in long bones from Osterix-Cre(-); Kcnma1$^{f/f}$ and Osterix-Cre(+); Kcnma1$^{f/f}$ mice. (D) The ratio of Rankl/Opg were determined in the bone tissues of Osterix-Cre(-); Kcnma1$^{f/f}$ and Osterix-Cre(+); Kcnma1$^{f/f}$ mice by RT-PCR. (E) Representative images of TRAP staining in the trabecular region of the tibiae. Scale bars = 100 μm. (F) ALP staining of primary osteoblasts isolated from the indicated mice after 14 days of culture. Scale bars = 100 μm. (G) Representative images of Alizarin Red staining of osteoblasts cultured in DMEM supplemented with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. Scale bars = 100 μm. Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

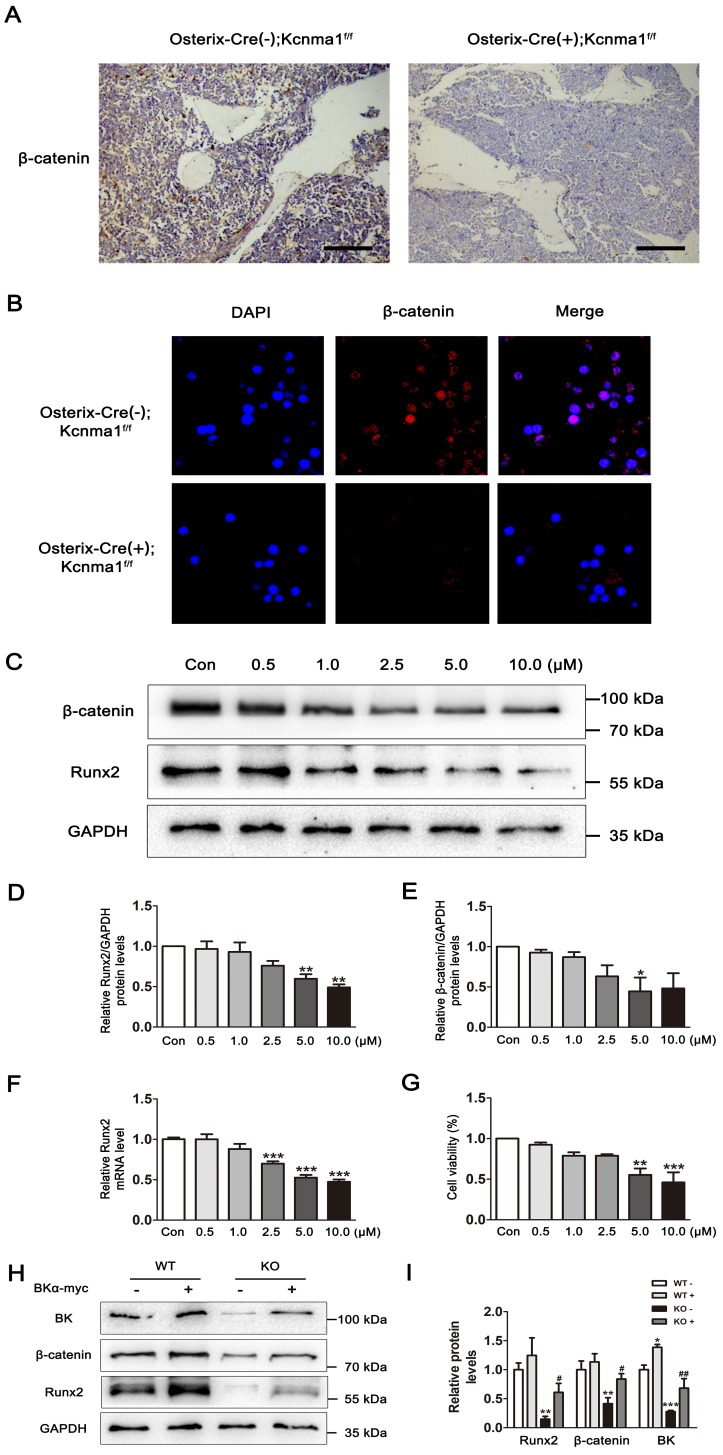

Fig. 4. Kcnma1 ablation leads to a decrease in β-catenin. (A) Representative images of β-catenin staining of tibiae from the indicated groups of mice. Scale bars = 100 μm. (B) Immunofluorescent staining of β-catenin in osteoblasts isolated from the indicated mice. (C) Protein expression of Runx2 and β-catenin in ROS 17/2.8 cells determined by western blot. ROS 17/2.8 cells were treated with different concentrations of paxilline for 48 h. (D and E) Quantitative analysis of Runx2 and β-catenin *in vitro*. (F) The Runx2 mRNA expression levels in ROS 17/2.8 cells treated with different concentrations of paxilline. (G) Cell viability of ROS 17/2.8 cells was assessed by CCK-8 assay. Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (H) Western blot analysis of Runx2, β-catenin and BK after the BKα-myc plasmid was transfected into MC3T3-E1 cells. (I) Quantitative analysis of Runx2, β-catenin and BK in BKα-myc plasmid transfected cells. Quantification of protein levels was normalized to GAPDH. Data are presented as mean ± SD, versus wild type (WT) -: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, versus KO -: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

tibiae, and cells were cultured in osteogenic medium. Consistently, the results implied that deletion of Kcnma1 induced reduced ALP activity, and the numbers of mineralized nodules of the primary osteoblasts were decreased by Alizarin Red staining after 21 days of differentiation (Figs. 3F and 3G), indicating that Kcnma1 deficiency inhibited osteogenic differentiation.

Taken together, these data show the importance of BK channels in osteoblast differentiation and bone formation.

Deficiency of Kcnma1 suppressed β-catenin expression

Given the observation that Kcnma1 deficiency impairs osteoblast function, we further explored the possible mechanisms leading to the results. We examined the protein expression of β-catenin by western blotting to verify our results. Kcnma1 deficiency reduced the protein levels of β-catenin in bones and primary osteoblasts. Similar effects were also observed in tibiae from Osterix-Cre(–); Kcnma1$^{f/f}$ and Osterix-Cre(+); Kcnma1$^{f/f}$ mice by immunohistochemistry (Fig. 4A).

Simultaneous immunofluorescence detection demonstrated that the expression of β-catenin in primary osteoblasts extracted from Osterix-Cre(+); Kcnma1$^{f/f}$ mice was significantly decreased compared with that in Osterix-Cre(–); Kcnma1$^{f/f}$ mice (Fig. 4B).

In addition, we added different concentrations of paxilline, a potent BK channel inhibitor, to ROS 17/2.8 osteoblast-like cells to investigate the effect of BK channels on osteoblast function. Intriguingly, the relative protein expression levels of Runx2 and β-catenin were downregulated (Figs. 4C-4E) in the presence of paxilline, and as shown in Fig. 4F, the decreased mRNA expression of Runx2 indicated that the intracellular downstream molecule was affected in Wnt/β-catenin signaling. We then detected the cell growth-inhibitory action of the BK channel inhibitor and found that the inhibitory rate of cell growth was dose-dependent (Fig. 4G). We next conducted the experiment to determine whether overexpressing Kcnma1 could rescue the dysfunctional phenotypes from deletion of Kcnma1 in osteoblasts. The results indicated that overexpression of BK led to increased protein levels of β-catenin and Runx2 in Kcnma1 knockout MC3T3-E1 cells (Figs. 4H and 4I). The intranuclear translocation of β-catenin, which is the central molecule in canonical Wnt signaling, promotes the transcriptional activity of osteogenesis-associated factors and the osteoblast differentiation of BMSCs (Xiang

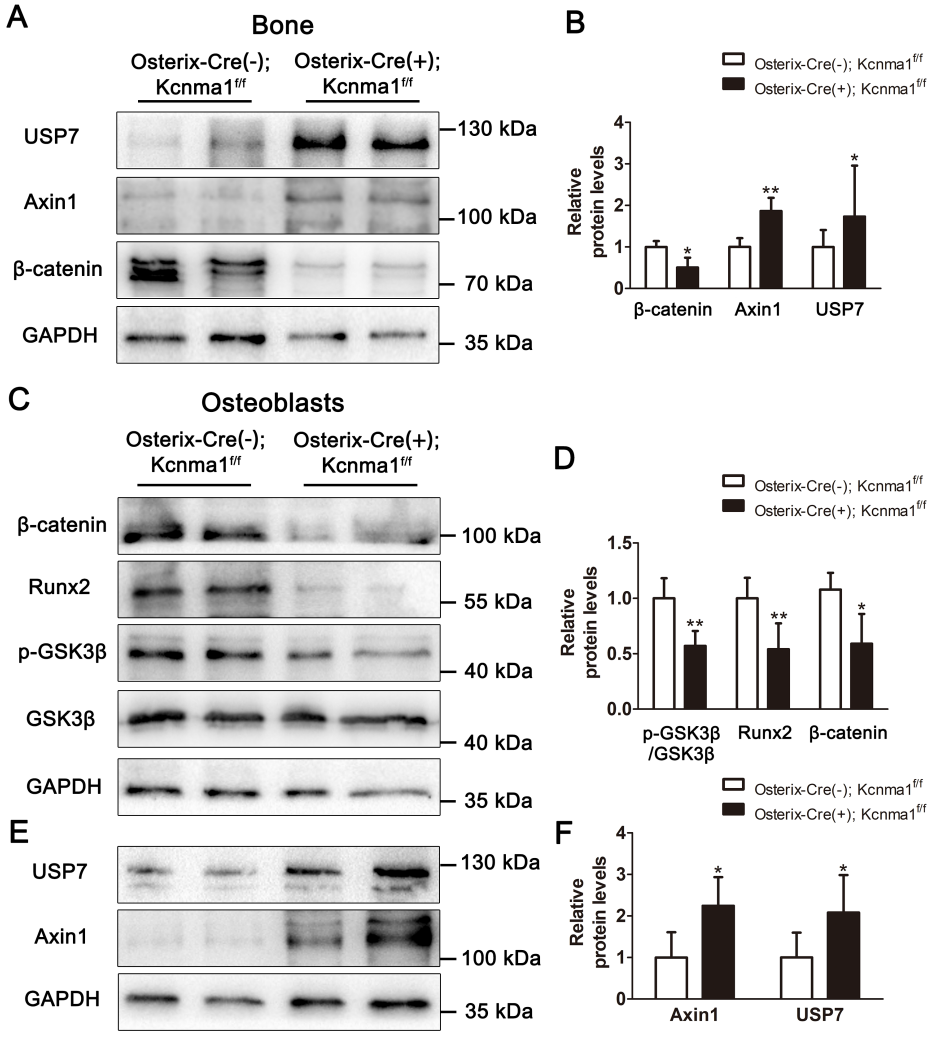

Fig. 5. Kcnma1 regulates β-catenin expression via canonical Wnt signaling. (A and B) Protein expression and quantitative analysis of β-catenin, Axin1 and USP7 in bone tissues from Osterix-Cre(-); Kcnma1$^{f/f}$ and Osterix-Cre(+); Kcnma1$^{f/f}$ mice. (C and D) western blot analysis of β-catenin, Runx2 and phosphorylated levels of GSK3β in osteoblasts isolated from the indicated mice. (E and F) Protein expression levels and quantitative analysis by western blot of Axin1 and USP7 in osteoblasts. Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$.

et al., 2020). Therefore, we speculated that the quantitative change in β-catenin was related to canonical Wnt signaling.

BK is involved in the canonical Wnt signaling pathway in osteoblasts and bone formation

To clarify whether BK channels can regulate β-catenin by modulating the canonical Wnt signaling pathway, the expression levels of multiprotein complexes, including GSK3β and Axin, were determined by western blot analysis. As shown in Figs. 5C and 5D, the phosphorylation levels of GSK3β were reduced in primary osteoblasts obtained from Osterix-Cre(+); Kcnma1$^{f/f}$ mice.

We next detected the expression of Axin, another functional component that plays an important role in the regulation of the stability of β-catenin in the canonical Wnt signaling pathway in previous studies (Alula et al., 2021). The protein expression of Axin1 increased in both long bones (Fig. 5A and 5B) and osteoblasts (Figs. 5E and 5F) obtained from Osterix-Cre(+); Kcnma1$^{f/f}$ mice compared with Osterix-Cre(–); Kcnma1$^{f/f}$ mice. Additionally, the expression of USP7, which seems to have a direct interaction with the Wnt/β-catenin pathway (Friese et al., 2019), was increased in the bones and osteoblasts of Osterix-Cre(+); Kcnma1$^{f/f}$ mice.

These results demonstrate that Kcnma1 deficiency inhibits osteogenic differentiation of primary osteoblasts via the Wnt/β-catenin pathway and that the deubiquitinating enzyme USP7 seems to participate in osteogenesis.

DISCUSSION

In the present study, we evaluated the effects of BK ablation in osteoblasts and explored the possible mechanisms of the interaction between BK channels and β-catenin. Our data showed that Kcnma1 deficiency impaired skeletal integrity and inhibited normal osteoblast differentiation and proliferation. The decrease in β-catenin in bones and osteoblasts obtained from Osterix-Cre(+); Kcnma1$^{f/f}$ mice might be attributed to the connection between Axin1 and USP7. The increase in USP7 in Osterix-Cre(+); Kcnma1$^{f/f}$ mice might stabilize the function of Axin1, which promoted the degradation of β-catenin in osteoblasts (Fig. 6).

Global deletion of BK channel (Kcnma1$^{-/-}$) mice show some abnormal behavior such as ataxia (Niday and Bean, 2021), abnormal gait (Typlt et al., 2013), hearing loss (Föller et al., 2012), circadian imbalances (Whitt et al., 2018), and urinary bladder incontinence (Li et al., 2009). Most studies focus on the effect of BK channel on the nervous system, while more and more research on the relationship between other diseases and BK channel has appeared in recent years. Both of Kcnma1$^{-/-}$ mice and Osterix(+); Kcnma1$^{f/f}$ mice show decreased body weight, which is probably associated with the balance between differentiation of adipocytes and osteoblasts. As a candidate bone mechanoreceptor, BK channels are reported to affect the cell number and function of MG63 cells and primary human osteoblasts (Henney et al., 2009). The mechanosensitivity of the BK channels seems to imply complicated interplay with signals because they will be open in response to membrane stretching or hypotonic shock (Rezzonico et al., 2003). Previous studies have demonstrated that knockout of BK channels may cause osteopenia because autonomous release of cathepsin K in osteoclasts, one of the major enzymes in the degradation of bone, induces an increase in matrix osteoclast resorption (Sausbier et al., 2011). In our previous study, we found that global knockout of the BK channel is

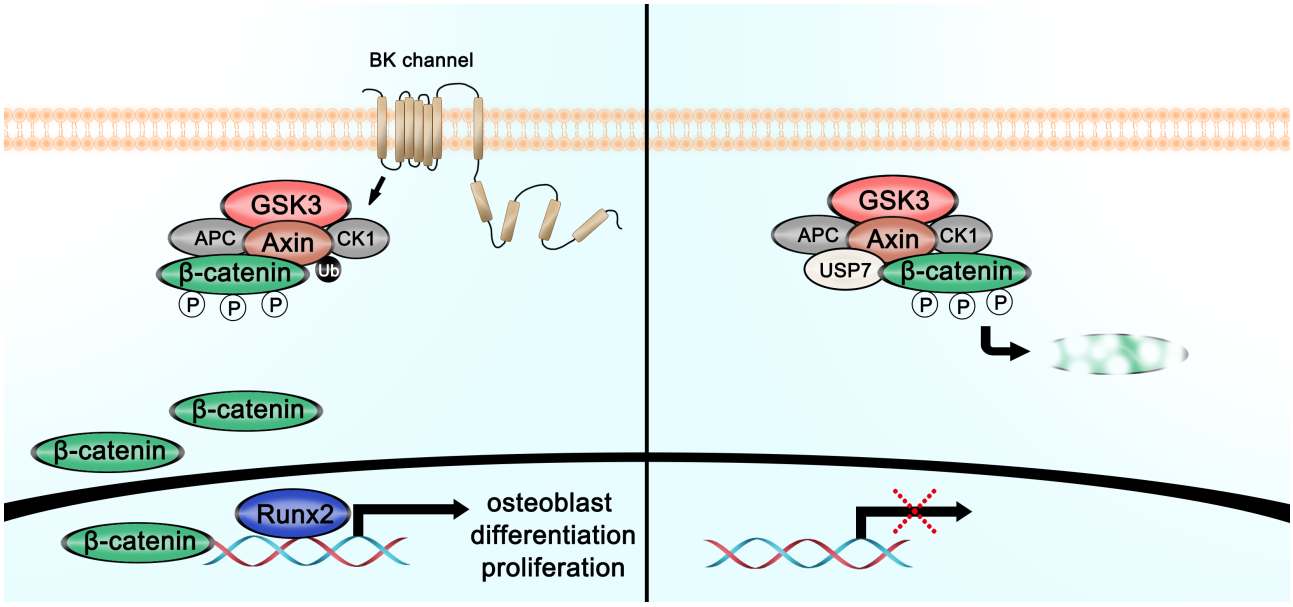

Fig. 6. Schematic diagram of how Kcnma1 regulates bone formation through Wnt/β-catenin signaling. In the process of bone development, the BK channel plays an essential role in the activation of osteoblast proliferation and osteogenic differentiation. When BK channels are unable to function normally, they stunt bone growth by inhibiting cytosol-nucleus transportation of β-catenin transportation in the canonical Wnt signaling pathway. USP7 maintains the stability of Axin1 and facilitates the degradation of β-catenin in osteoblasts, which decreases the expression of the transcription factor Runx2 and results in bone disorders.

able to affect bone formation; however, whether it directly affects osteoblasts or functions through indirect regulation is not well defined. In this study, we successfully constructed osteoblast-specific Osterix promoter-driven Kcnma1-CKO mice, which provide a useful model to explore the functions of Kcnma1 in bone formation. Our observations indicated that deficiency of BK channels induced skeletal development retardation and osteogenic dysdifferentiation in Kcnma1-CKO transgenic mice.

Osteoblasts were originally derived from BMSCs (Zhang et al., 2017). In the final stage of bone formation, there are three different fates of osteoblasts: differentiation into osteocytes, transfer to the bone surface to become temporarily inactive osteoblasts, and programmed cell death. During the process of bone formation, some osteoblasts are left in the mineralized bone matrix to form early osteocytes, which are also called osteoid-osteocytes. Early osteocytes are able to emit a large number of synapses that extend to the surface of bones and blood vessels, making extensive connections with osteoblasts, osteoclasts, and endothelial cells (Qing and Bonewald, 2009); thus, bone metabolism is a dynamic process related to complex signal transduction. The Wnt/β-catenin signaling pathway is one of the important signals responsible for osteoblast differentiation and proliferation, and some specific Wnt ligands participate in bone metabolism without affecting cartilage formation (Appelman-Dijkstra and Papapoulos, 2018; Song et al., 2020). Activation of the destruction complex increases the degradation of β-catenin and inhibits its interaction with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF), resulting in a decline in osteoblast transcription factor Runx2 in the nucleus (Saidak et al., 2015). Wnt/β-catenin signaling prevents a cell fate shift of BMSCs to adipocytes and inhibits excessive proliferation and activation of osteoclasts by regulating mature osteoblasts (Matsushita et al., 2020).

The association between the α-subunit of the BK channel and β-catenin may be direct; this interaction is important in mediating the surface expression of the BK channel through its S10 region (Bian et al., 2011). A previous study proved that the canonical Wnt/β-catenin pathway can regulate the surface expression of BK channels. The destruction complex, including Axin, glycogen synthase kinase-3 (GSK-3), adenomatous polyposis coli (APC) protein, and casein kinase 1 (CK1), regulates the stabilization of β-catenin. Axin1, the rate-limiting factor of the destruction complex, is considered the central scaffold of the complex (Li et al., 2012). External stimuli may inhibit GSK-3β phosphorylation, which regulates the degradation and nuclear import of β-catenin. GSK-3β is probably responsible for the phosphorylation of both BK and β-catenin. The relationship between BK channels and β-catenin seems to maintain stable surface expression of BK (Kwan et al., 2013). Moreover, it has been reported that deubiquitinating enzymes (DUBs) are critical for regulating the Wnt/β-catenin pathway (Park et al., 2020). A previous study demonstrated that iberiotoxin (IbTX), an inhibitor of BK channels, could downregulate β-catenin via transmembrane depolarization without obviously affecting the expression patterns of BK channels (Schickling et al., 2015). Here, our study showed that deletion of BK channels in osteoblasts induced decreases in β-catenin expression in both bone tissues and primary osteoblasts. The inhibition of β-catenin in osteoblasts leads to an increase in osteoclast formation (Shu et al., 2020); however, trap staining in the trabecular regions showed no significant difference in our study. Runx2 is the main transcription factor in the process by which MSCs differentiate into osteoblasts. The activation of Runx2 triggers a cascade reaction of downstream osteoblast markers. The phosphorylation levels of GSK3β were reduced in primary osteoblasts obtained from Osterix-Cre(+); Kcnma1$^{f/f}$ mice; however, there was no obvious variation in bone tissues (data not shown). We assumed that the reduction in p-GSK3β could not be detected in whole bone tissues because nonosteoblast-lineage cells still maintained regular phosphorylation levels of GSK3β.

Deubiquitinases (DUBs) can reverse the enzymatic process by which proteins are modified with ubiquitin chains. USP7, a ubiquitin hydrolyzing enzyme belonging to the USP family, plays a vital role in cell signaling. The interactions between USP7 and its substrates and different signal transduction pathways affect its expression and function, and USP7 plays different roles in cells depending on the various environments (Kim and Sixma, 2017). BK ablation may influence the intracellular environment and complex intramolecular mechanisms, which have effects on the expression and activity of USP7. To date, we have only known that ubiquitination levels of the BK α-subunit (Wang et al., 2013) and the BK β1-subunit (Qian et al., 2020) affect the degradation of BK channels as well as BK channel activity. It has been reported that USP7 may be a negative regulator of Wnt/β-catenin signaling and directly interacts with Axin through its N-terminal TRAF domain, which impacts the stabilization of Axin (Ji et al., 2019) and the degradation of β-catenin. However, some previous studies declared that Wnt/β-catenin signaling is downregulated with the use of a USP7 inhibitor through the suppression of deubiquitination of β-catenin (An et al., 2017); thus, there is no final conclusion on the argument of the role of USP7 in Wnt signaling and bone formation. Our results demonstrated that USP7 and β-catenin exhibited opposite trends; however, it remains unclear how USP7 mediates the deubiquitination of Axin in the absence of Kcnma1 in osteoblasts. In addition, there is a need to further explore the relationship between BK channels and deubiquitinases.

In summary, our studies demonstrated that the absence of BK channels in osteoblasts impaired normal bone development. To the best of our knowledge, we did not find any related work on how BK channels regulate osteogenesis through modulating Wnt/β-catenin signaling in the process of bone development. This paper provides evidence that Kcnma1 plays a critical role in controlling skeletal development and may provide evidence for further bone-related research.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

L.J. and Q.Y. were responsible for the design of the experiment and the acquisition, analysis and interpretation of data and drafted the manuscript. J.G. provided professional advice. J.Y. and J.H. critically revised the content and helped complement the experiment. X.Z. and H.X. approved the final version. All aspects of the manuscript writing and revision were carried out by the authors.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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