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Conditional expression of constitutively active estrogen receptor α in chondrocytes impairs longitudinal bone growth in mice

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ABSTRACT

Estrogen plays important roles in the regulation of chondrocyte proliferation and differentiation, which are essential steps for longitudinal bone growth; however, the mechanisms of estrogen action on chondrocytes have not been fully elucidated. In the present study, we generated conditional transgenic mice, designated as caERα^{ColII}, expressing constitutively active mutant estrogen receptor (ER) α in chondrocytes, using the chondrocyte-specific type II collagen promoter-driven Cre transgenic mice. caERα^{ColII} mice showed retardation in longitudinal growth, with short bone lengths. BrdU labeling showed reduced proliferation of hypertrophic chondrocytes in the proliferating layer of the growth plate of tibia in caERα^{ColII} mice. *In situ* hybridization analysis of type X collagen revealed that the maturation of hypertrophic chondrocytes was impaired in caERα^{ColII} mice. These results suggest that ERα is a critical regulator of chondrocyte proliferation and maturation during skeletal development, mediating longitudinal bone growth *in vivo*.

1. Introduction

Estrogen has been assumed to function as a sex steroid hormone with various physiological activities, including regulation of longitudinal bone growth. Longitudinal bone growth occurs at the growth plate cartilage through a process called as endochondral ossification, in which cartilage is formed and replaced by the bone tissue. The growth plate has a specific zonal morphology with the resting zone of immature chondrocyte at the epiphyseal plate, moving distally into the proliferating zone with more mature flat-typed chondrocytes, the hypertrophic zone with large chondrocytes, and a band of ossifying cartilage [1]. Around the end of puberty, the epiphyseal chondrocytes stop duplicating and the epiphyseal plate becomes calcified, forming a visible line known as the epiphyseal line. Estrogen is assumed to stimulate this process of epiphyseal closure [2]. The discovery of a male patient with an inactivating mutation in the estrogen receptor α (*ERα*) has confirmed this hypothesis [3]. The patient showed longitudinal growth into adulthood because of the absence of epiphyseal plate fusion, resulting in a tall stature as well as severe osteoporosis. In addition, similar phenotypes have been found in patients deficient in aromatase, which is responsible for the biosynthesis of estrogens [4,5]. These findings imply that estrogen regulates chondrocyte differentiation/proliferation – the main determinants of longitudinal growth and bone quality.

Ovariectomy in rodents stimulates longitudinal growth, and the growth is inhibited by estrogen [6,7]. Interestingly, several studies with hypophysectomized rats have shown that estrogen inhibits longitudinal growth [8,9] by directly influencing the activity of epiphyseal plate chondrocytes. *ERα* and *ERβ* are expressed in epiphyseal plate chondrocytes during development, and estrogen directly affects the structure of epiphyseal plates [10,11]. Despite accumulating evidence of the estrogen action on longitudinal bone growth, the results of several *in vitro* experiments have been inconclusive with reports of stimulatory [12], inhibitory [13], and no effect [14] of estrogen on chondrocyte proliferation.

The physiological functions of estrogen are mediated by *ERα* and *ERβ* [15]. ERs are transcription factors that regulate the expression of estrogen-targeted genes in response to hormone binding. Several animal models including *ERα*, *ERβ*, and aromatase knockout mice have been used to investigate longitudinal growth regulation by estrogen [16–18]. These animals have unaffected or inhibited longitudinal growth, in contrast to the patient with the *ERα* mutation [17,19]. However, more recently, a mouse model with chondrocyte-specific inactivation of *ERα* was developed and high-dose estrogen treatment in this model failed to reduce epiphyseal plate height in adult mice [20]. Nevertheless, whether or not ERα

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functions in chondrocytes during embryonic development and before sexual maturation remains unknown. Gain-of-function studies of chondrocyte-specific ERα have not been presented.

To examine the role of human ERα in a mouse model, we used a constitutively active human ERα (caERα) mutant with a substitution of tyrosine by serine at codon 537 (ERα Y537S) [21]. Previous studies had shown that caERα exhibits high transcription activity even in the absence of ligand stimulation [21,22]. We thus assumed that caERα would be useful for analyzing ERα gains-of-function *in vivo*. In the present study, we generated conditional transgenic mice, expressing caERα in chondrocytes, by using Col II-Cre transgenic mice in which Cre recombinase expression is controlled by the chondrocyte-specific collagen type II (ColII) promoter [23]. These mice exhibit shorter bone length, and impaired chondrocyte proliferation and differentiation. This mouse model points to a crucial role of ERα in chondrocytes and would improve our understanding of ERα functions *in vivo*.

2. Materials and methods

2.1. Construction of the transgene

Constitutively active ERα (caERα) containing the Y537S substitution has been described previously [22]. CAG–GFP^{floxed}–caERα transgene plasmid was constructed using pCALwL vector containing CAG promoter [22], which is a composite promoter consisting of a cytomegalovirus IE enhancer, a chicken *β-actin* promoter, 2 functional loxP sites, and a rabbit *β-globin* polyadenylation signal. The cDNAs for GFP fused to histone H2B and caERα were subcloned into sites between the 2 loxP sites and upstream of the polyadenylation signal, respectively.

2.2. Generation of transgenic mice

The CAG–GFP^{floxed}–caERα plasmid was linearized by restriction enzyme digestion. Transgenic mice were produced by microinjecting the linearized plasmid into the pronuclei of the fertilized eggs from C57BL/6 mice, as described previously [22,24]. Transgenic mice were identified by polymerase chain reaction (PCR) assays of genomic DNA extracted from the tail, and transgene expression was monitored by GFP fluorescence under a stereomicroscope. Primers derived from the 5′- and 3′- flanking sequences of GFP (5′-ACGTGCTGGTTGTTGTGCTGTCTCTCATCA-3′ and 5′-TGATTTGATC CCCGGGTACCGAGCGAC-3′) were used to amplify a 1.9 kb product. ColII-Cre mice were kindly provided by Dr. Behringer [23], and ROSA26LacZ reporter mice were obtained from The Jackson Laboratory (Bar Harbor, ME). For Cre transgenic mice, primers derived from Cre (5′-CCTGGAAAATGCTTCTGTCCGTTTGCC-3′ and 5′-GAGTTGATAGCTGGCTGGTGGCAGATG-3′) were used to amplify a 653-bp product. For ROSA26LacZ transgenic mice, primers 5′-GCGAAGAGTTTGTCCTCAACC-3′, 5′-AAAGTCGCTCTGAGTTGTTAT-3′ and 5′-GGAGCGGGAGAAATGGATATG-3′ were used to amplify a 340-bp product for the transgene and a 650-bp product for the wild-type allele (The Jackson Laboratory). CAG–GFP^{floxed}–caERα transgenic mice were mated with ColII-Cre transgenic mice to produce caERα^{ColII} conditional transgenic mice. ColII-Cre/ROSA26LacZ reporter mice were generated by cross breeding ROSA26LacZ and ColII-Cre mice and were used to track ColII promoter activity throughout the ontogeny of the mouse. All animal experiments were approved by the Institutional Animal Care and Use Committee.

2.3. Quantitative reverse transcriptase-PCR

Total RNA was extracted from the cartilage of 15-week-old female wild-type (WT) and caERα^{ColII} mice using ISOGEN reagent (Nippon Gene, Tokyo, Japan). To examine transgene expression, quantitative reverse transcriptase-PCR (qRT-PCR) was performed as described previously [25]. To assess the caERα transgene expression, primers 5′-ATGGACTACAAGGACGATGATGAC-3′ and 5′-GCAGTAGGGCCATCCCAGAT-3′ corresponding to Flag and human ERα sequences, respectively, were used. A comparison of the qRT-PCR product amounts was carried out using the comparative cycle threshold (Ct) method, with *Gapdh* as a control. The experiments were independently repeated at least 3 times, with each performed in triplicate. The results are shown as mean ± SD. Statistical analysis was carried out using Student's *t* test.

2.4. Histological procedures

β-Galactosidase (*β-gal*) activity was assessed as described, with minor modifications [24]. Frozen sections (10-μm thick) of neonatal ColII-Cre/ROSA26LacZ mice (P0) were fixed with a 0.25% glutaraldehyde solution for 10 min. The sections were then washed 3 times and incubated with a staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 0.1% phosphate buffer at pH 7.5, 5 mM potassium ferrocyanide, 5-mM potassium ferricyanide, 0.02% NP-40, 0.01% sodium deoxycholate, and 2 mM $MgCl_2$) for 4–5 h in order to β-gal activity. *In situ* hybridization was performed using standard techniques [24]. Briefly, frozen sections (10-μm thick) of tibia from caERα^{ColII} embryos (16.5 d.p.c.) were hybridized with a DIG-labeled anti-sense cRNA riboprobe for mouse collagen type X, according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

2.5. Radiographic analysis of the femur

Radiographic analysis of femora from 8-week-old female WT and caERα^{ColII} mice was performed using mFX-1000 (Fujifilm, Tokyo, Japan). Femur lengths were measured, and the mean ± SD was calculated ($n = 6$).

2.6. BrdU analysis

Pregnant mice bearing 16.5 d.p.c. embryos were i.p. injected with BrdU labeling reagent (10 μL/g body weight; Zymed Laboratories); 2 h later, the mice were killed. Embryonic tibias were dissected and sectioned. The incorporated BrdU was detected using a BrdU staining kit (Zymed Laboratories) to distinguish actively proliferating cells. The tissue sections were measured using a micrometer, and the average number of BrdU-positive cells/mm^2 cartilage ± SD was calculated.

3. Results

3.1. Generation of conditional transgenic mice expressing constitutively active ERα (caERα) in chondrocytes

To assess the function of ERα in chondrocytes *in vivo*, we first generated a floxed GFP- constitutively active ERα (caERα) transgenic mouse line bearing the CAG–GFP^{floxed}–caERα transgene [22]. caERα is a constitutively active mutant of human ERα with a Y537S substitution that enhances transcription, regardless of estrogen [17,22]. The CAG promoter has the potential for ubiquitous expression in various tissues. Mice bearing this construct will express GFP instead of caERα because of the poly(A) signal sequence located immediately following the GFP sequence. In the presence of Cre recombinase, the GFP sequence will be deleted and caERα expression will emerge under the control of the CAG promoter (Fig. 1A). We obtained 2 lines of CAG–GFP^{floxed}–caERα transgenic mice, lines #2 and #6. Transgene integration in both

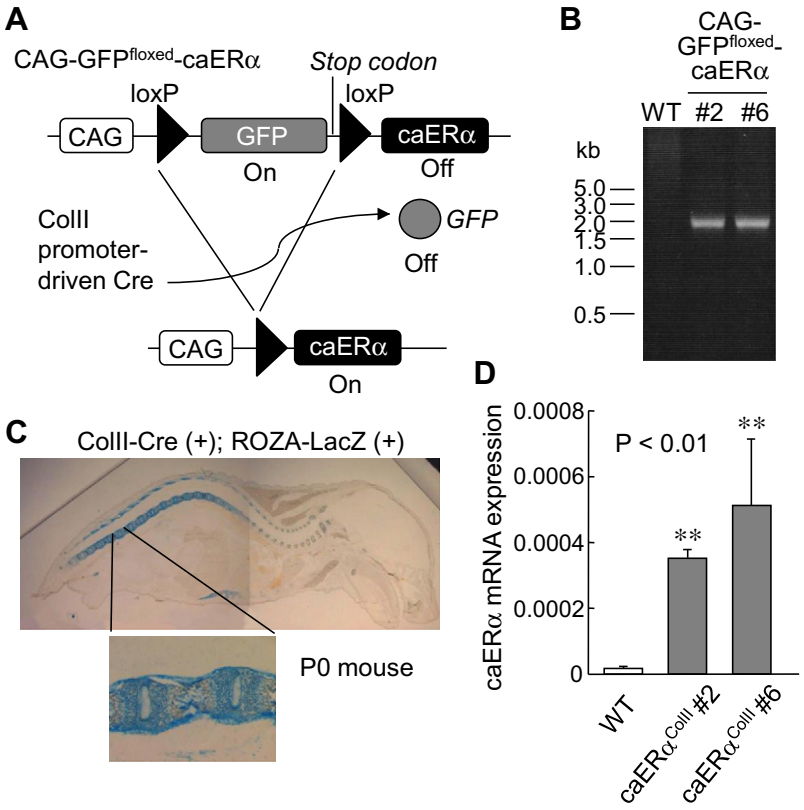

Fig. 1. Generation of conditional transgenic mice expressing constitutively active ERα (caERα) in chondrocytes. (A) Schematic representation of a CAG–GFP^{floxed}–caERα transgene: the construct expresses express GFP instead of caERα since the loxP-flanked GFP gene has a stop codon at the end of the gene. In the presence of Cre recombinase, the GFP sequence is excised and caERα, rather than GFP, is expressed under the CAG promoter control. (B) Genotyping of caERα transgenic mouse lines #2 and #6. Transgenic mice were identified by PCR assays of genomic DNA extracted from mouse tails using primers derived from the 5′- and 3′-flanking sequences of GFP. A positive signal was shown by a 1.9-kb product. (C) A ColII-Cre transgenic mouse was mated with a ROSA26LacZ tester mouse and spines from ColI-Cre+; ROSA26LacZ + newborn mice (P1) were stained with X-gal. (D) Total RNA was extracted from the cartilage of 15-week-old female wild-type (WT) and caERα^{ColII} mice (lines #2 and #6), and subjected to quantitative reverse transcriptase-PCR (qRT-PCR) with primers for amplifying the caERα transgene. The results are shown as mean ± SD and statistical analysis was performed using Student's *t* test. $^{**}P < 0.01$.

transgenic lines was verified by genomic PCR with GFP-specific primers (Fig. 1B).

To generate mice with conditional caERα expression in chondrocytes, we employed ColII-Cre transgenic mice in which Cre recombinase expression is under the control of the chondrocyte-specific collagen type II (ColII) promoter [23]. In order to inspect Cre expression in chondrocytes, we generated mice heterozygous for ColII-Cre and ROSA26LacZ transgenes by breeding homozygous ROSA26LacZ mice with homozygous ColII-Cre mice. Spines were prepared from heterozygous neonates at P0 and monitored by X-gal staining (Fig. 1C). LacZ-positive signals were detected within chondrocytes in these tissues. Chondrocyte-specific expression of Cre recombinase in ColII-Cre transgenic mice was demonstrated by Ovchinnikov et al. [23]. We then crossed CAG–GFP^{floxed}–caERα transgenic mice and ColII-Cre mice to generate conditional transgenic mice caERα^{ColII}, in which caERα protein is expressed specifically in chondrocytes. Transgene expression was confirmed in cartilage tissues prepared from caERα^{ColII} mice (Fig. 1D).

3.2. Short bone length of mice expressing caERα in chondrocytes

We noted that caERα^{ColII} mice were shorter in stature than their WT littermates Fig. 2. A shows the representative appearance of 8-week-old female WT and caERα^{ColII} mice. The body lengths of caERα^{ColII} mice from transgenic lines caERα^{ColII} #2 and #6 were shorter than that of WT mice. Comparison of heads also revealed decreased vertex to nose length in caERα^{ColII} mice. We then performed radiographic analysis of the femora to determine whether long-bone growth was affected in caERα^{ColII} mice. As shown in Fig. 2B, femora were shorter in caERα^{ColII} mice than in WT mice. Statistical analysis revealed significantly reduced femur length in both transgenic mouse lines. These results indicate that long bone longitudinal growth is impaired in caERα^{ColII} mice.

3.3. Reduced proliferation and differentiation of chondrocytes in caERα^{ColII} mice

Since femur lengths in caERα^{ColII} mice were significantly shortened, BrdU labeling experiments were performed in 16.5 d.p.c. mouse embryos to assess chondrocyte proliferation (Fig. 3A and B). The rate of BrdU-positive cells in hypertrophic chondrocytes was significantly reduced, by approximately 50%, in caERα^{ColII} transgenic mice, when compared with WT mice. These results suggest that caERα suppresses chondrocyte proliferation. We also examined the expression of type X collagen, a marker for hypertrophic and prehypertrophic chondrocytes, by *in situ* hybridization (Fig. 4). At 16.5 d.p.c., type X collagen-expressing hypertrophic chondrocyte zones were reduced in caERα^{ColII} mice in comparison to their WT counterparts.

4. Discussion

In the present study, we used a Cre/loxP system to generate conditional transgenic mice to investigate the *in vivo* function of

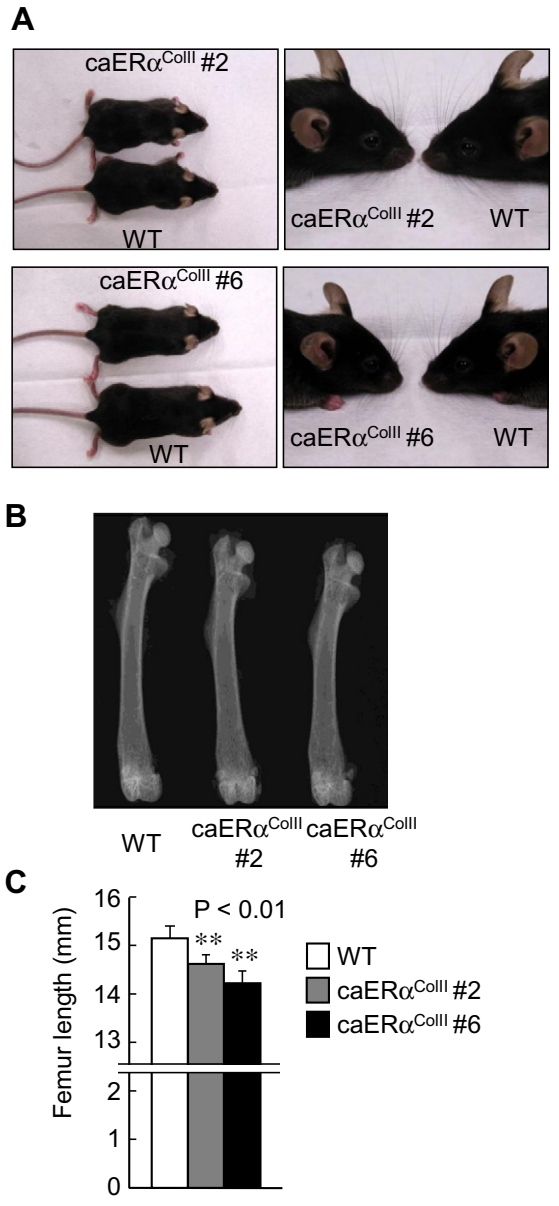

Fig. 2. Short bone lengths in caERα^{ColII} mice. (A) Dorsal views of 8-week-old females from caERα^{ColII} #2 and #6 lines and their WT mice. (B) Representative radiographs of femurs from caERα^{ColII} #2 and #6 mice. (C) Shorter femur lengths in caERα^{ColII} mice. Femur lengths were measured and the average ± SD was calculated (n = 6). Statistical analysis was performed using Student's t test. $^{**}P < 0.01$.

human ERα in chondrocytes by expressing a caERα mutant. CAG–GFP^{floxed}–caERα transgenic mice [22] expressing GFP but not caERα were mated with ColII-Cre mice to allow for caERα expression in chondrocytes. caERα^{ColII} mice exhibited shorter femur lengths compared with WT mice. BrdU labeling experiments revealed that chondrocyte proliferation was significantly decreased in caERα^{ColII} mice. Furthermore, the areas of expression of type X collagen, a marker for hypertrophic and prehypertrophic chondrocytes, were reduced in the tibias of caERα^{ColII} mice. These results suggest that chondrocyte-specific constitutive activation of ERα in mice leads to early termination of chondrocyte proliferation and differentiation, retarding longitudinal bone growth.

When considering ERα functions in bone growth, it is interesting to compare the result of our mouse model with the clinical phenotypes of patients with ERα or aromatase gene mutations. A male patient with disruptive ERα gene mutation exhibited tall stature with unfused epiphyses and osteoporosis [3]. In addition, patients with estrogen deficiency due to mutations in the aromatase gene also display continuing linear growth into adulthood, owing to a lack of epiphyseal fusion in long bones [26,27]. Estrogen supplementation caused growth plate closure in patients with aromatase deficiency but not in patients with a mutation in the *ERα* gene [3,5,28].

It is also notable that our mouse model contrasts with a recent mouse model of cartilage-specific *ERα* inactivation, which exhibited continuous bone growth with increased adult femur length, in spite of normal skeletal growth during sexual maturation [20]. Moreover, high-dose estrogen treatment of adult cartilage-specific *ERα* knockout mice does not reduce growth plate heights. These reports suggest that *ERα* is required, within growth plate cartilage, to reduce chondrocyte proliferation and longitudinal bone growth in adult mice. Conversely, in the present study, caERα^{ColII} mice exhibited short femur lengths. We consider that our mouse model may reflect a high-dose estrogen status. Since the proliferation and area of hypertrophic chondrocytes were reduced in caERα^{ColII} mice, we speculate that caERα expressed in chondrocytes will inhibit maturation of hypertrophic chondrocytes and thus restrict endochondral ossification to a smaller area, resulting in shorter lengths for the long bone. In humans, premature secretion of estrogen causes female precocious puberty, in which patients exhibit short stature due to the early fusion of the epiphyseal plate [29]. In our mouse model, the short bone phenotype was observed by 8 weeks of age, suggesting that overactivation of ERα signaling in chondrocytes induces short stature before puberty.

It is also presumable that *ERα* will exert its suppressive effects on the differentiation/maturation of chondrocytes through interaction with other transcription factors and signaling molecules. Interestingly, Stallcup et al. reported that estradiol-bound ERα interacts with Runt-related transcription factor 2 (*Runx2*) and represses its transcription activity in osteoblastic cells and breast cancer cells [30]. Runx2 has been known as a master regulator of chondrocyte and osteoblast differentiation [31,32]. Runx2 is expressed in prehypertrophic and hypertrophic chondrocytes, stimulating differentiation through the upregulation of Runx2 target genes, including type X collagen [33]. The regulation of chondrogenesis by Runx2 was further confirmed by reports that transgenic mice overexpressing a dominant negative form of Runx2 in chondrocytes showed dwarfism and skeletal malformations, with suppression of chondrocyte maturation and endochondral ossification [34]. In addition, hypertrophic chondrocytes are known to produce vascular endothelial growth factor (Vegf), which is considered to have an important role not only in angiogenesis, but also in chondrocyte differentiation and endochondral ossification [35]. Estrogen has been reported to stimulate Vegf expression in cultured chondrocyte cells and ovariectomized rats [36]. Estrogen receptors may mediate their functions within growth plates through Vegf regulation. Several other molecules including growth factors, inflammatory cytokines, and matrix metalloproteinases are also assumed to mediate estrogen's effects on cartilage [37]. G protein-coupled receptor GPR30, identified as an intracellular transmembrane estrogen receptor, has been shown to mediate estrogenic responses in epiphyseal plates [38]. Although longitudinal bone growth is thought to be integrally regulated by such estrogen signaling molecules, our gene-engineered animal model provides a useful tool for understanding the direct effects and cell-specific functions of ERα *in vivo*.

Using the conditional expression system described here, we generated conditional transgenic mice expressing caERα in osteoblasts using collagen type I promoter-driven Cre transgenic mice [22]. These mice revealed the stimulatory effect of ERα on bone mineral density by osteoblasts *in vivo* and revealed the gene regulation associated with suppression of osteoclast differentiation. Given that other cell-specific or tissue-specific Cre-expressing

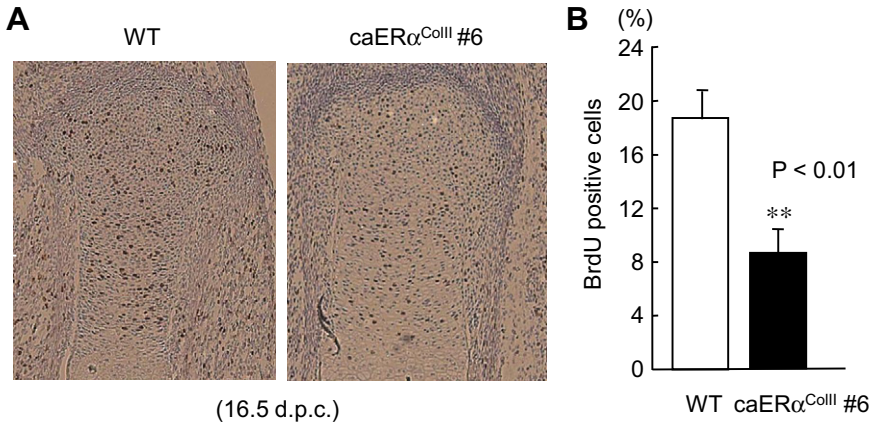

Fig. 3. Decreased BrdU incorporation rate in hypertrophic chondrocytes in caERα^{ColII} mice. BrdU incorporation into proliferating proximal tibia chondrocytes was monitored at 16.5 d.p.c. The numbers of BrdU-positive cells/mm^2 cartilage were measured and the results are shown as mean ± SD. Statistical analysis was performed using Student's *t* test. $^{**}P < 0.01$.

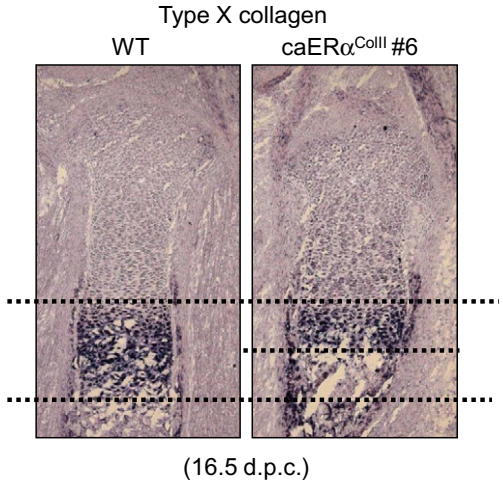

Fig. 4. Decreased collagen type X expression in hypertrophic chondrocytes in caERα^{ColII} mice. *In situ* hybridization of sections of 16.5 d.p.c. WT and caERα^{ColII} mice tibias was performed using DIG-labeled anti-sense cRNA riboprobe for mouse type X collagen. Note the decrease in the type X collagen-expressing hypertrophic zone in caERα^{ColII} mice.

mice are utilized instead of ColI-Cre or ColII-Cre mice, our conditional transgenic mice will be valuable for clarifying the *in vivo* function of ERα in specif cells or tissues as well as in osteoblasts and chondrocytes.

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