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Original Article

Hypertrophic chondrocyte-specific *Col10a1* controlling elements in Cre recombinase transgenic studies

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Abstract: The type X collagen gene (*COL10A1*) is specifically expressed in chondrocytes undergoing hypertrophy, which is an essential late stage of endochondral ossification during the development of long bones. We have previously localized multiple murine *Col10a1* promoter-enhancer elements and used these elements for transgenic studies with *LacZ* reporter gene or genes of interest. Here, we report two additional transgenic mouse lines in which *Cre* was driven by the 10 kb *Col10a1* promoter/intron and the 300-bp enhancer elements respectively. Cre activity was assessed by breeding the transgenic founders onto the RosA26R genetic background and to examine its β-gal activity (blue staining) via Cre/Lox P recombination. Our results showed that, in addition to the Cre activity in hypertrophic chondrocytes, we also observed blue staining of the bone marrow and the surrounding digits when the 10 kb *Col10a1* promoter/intron element was used, whereas the 300-bp enhancer element could drive Cre expression exclusively within the hypertrophic zone as demonstrated by the blue staining pattern. This is intriguing, as the 10 kb promoter covers the 300-bp enhancer element. We then further reanalyzed the *LacZ* transgenic mice. We did observe non-specific blue staining in 10 kb-*LacZ* mice but not the mice with the 300-bp enhancer. In addition, the Cre reporter construct was on a coat-color vector backbone, which enables direct visual genotyping of the transgenic mice in the FVB/N albino background. Together, our results support that the 300 bp *Col10a1* enhancer provides a more efficient genetic tool to target the hypertrophic zone for studies of skeletal development and disease.

Keywords: Col10a1 enhancer, hypertrophic chondrocytes, Cre transgenic mice

Introduction

Endochondral bone formation is a known well-coordinated process for mammalian skeletal, especially for long bone development [1]. It involves a cartilage intermediate that allows mesenchymal chondrocytes to undergo proliferation, hypertrophy and apoptosis within different zones of the growth plate [2]. As an ending stage of chondrocyte differentiation, chondrocyte hypertrophy and expression of its specific marker, the type X collagen gene (*COL10A1*), are indispensable for endochondral bone formation [3-7]. Schmid metaphyseal chondrodysplasia (SMCD), cleidocranial dysplasia (CCD) and osteoarthritis are known to be caused by mutation and dysfunction of human *COL10A1* [8-15]. Therefore, for the last 2-3 decades, many efforts have been put on the field of *COL10A1* gene regulation, i.e. to identify the specific cis-enhancer elements and transacting factors that direct its specific expression in hypertrophic chondrocytes. Such enhancer elements are potential genetic tools to target the hypertrophic stage and the binding transcription factors are expected to play the vital role during skeletal development and the progression of skeletal diseases. It was previously reported that multiple proximal promoter elements were responsible for low level *Col10a1* expression in different species [16]. Later, we and others have localized the murine *Col10a1*

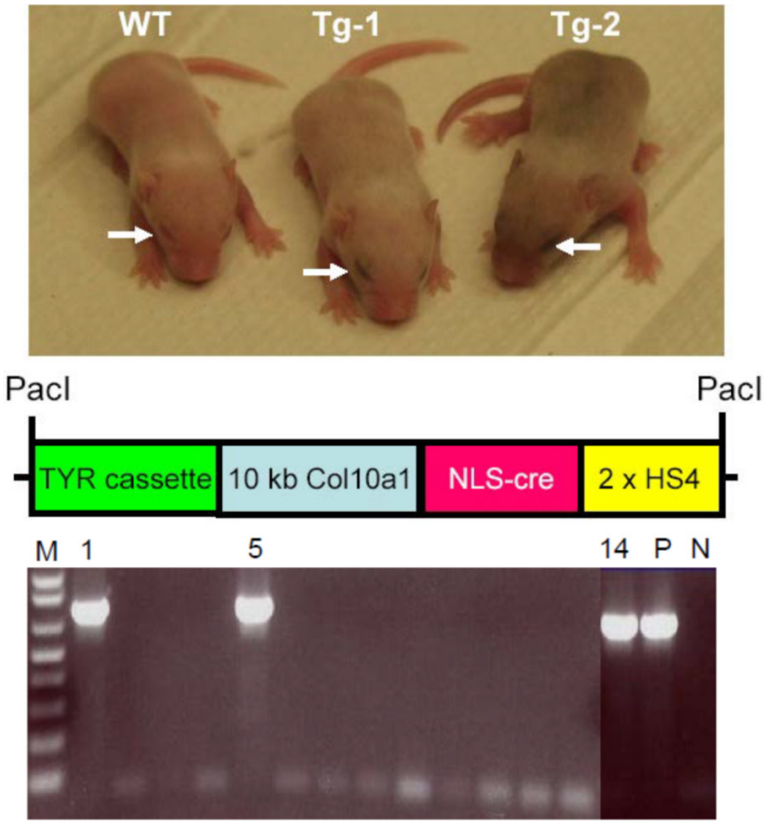

Figure 1. *Col10a1* 10 kb-Cre transgenic mouse line with a coat color background. The coat color cassette used in this transgenic reporter construct was derived from tyrosinase cDNA and chicken β-globin HS4 insulator. It leads to a higher expression rate and allows direct visual genotyping of the coat color (black eyes) in the FVB/N albino background (Top panel, arrows, [22, 30, 31]). Bottom chart indicates structure and release of the transgenic cassette by PacI for microinjection. WT: wild type; Tg: transgenic. M: molecular weight marker. Bottom panel shows PCR genotyping results of 3 *Col10a1*-10 kb-Cre founders. P-positive control, N-negative control.

cis-enhancer to its distal promoter and to approximately a 150 bp region [16, 17]. These cell-specific *Col10a1* promoter elements have been utilized for multiple transgenic studies involving reporter (*LacZ* and *Cre*) and functional studies of genes of interest [18-21]. Here, we show two extra transgenic mouse lines in which *Cre* was driven by our previously defined 10 kb *Col10a1* promoter/intron and the 300-bp enhancer elements respectively [17].

Materials and methods

Generation of Col10a1-10 kb-Cre and Col10a1-4×300 bp-Cre transgenic mice

Our previous transgenic studies have demonstrated that a larger 10 kb *Col10a1* control element will lead to high levels of reporter expression in hypertrophic chondrocytes compared to mice with the 4 kb *Col10a1* proximal promoter [16, 17]. This 10-kb fragment, which contains a distal promoter, a large second intron and a portion of exon III, was used to generate a Cre-containing transgenic reporter construct on a coat-color vector backbone [22]. Specifically, we first cloned the *Col10a1* 10 kb promoter/intron elements into a Cre (with nuclear localization signal) containing vector, pLC2-NLS-cre, then we cloned the 10 kb-NLS-Cre cassette into a coat-colored vector as described [22]. Detailed cloning strategy and release of the transgenic cassette using PacI digestion are available upon request. The tissue specificity of Cre activity in these transgenic founders was tested by breeding onto the RosA26R genetic background, a mouse reporter line that expresses β-gal in cells undergoing Cre recombination via a Cre recombinase/LoxP system [23, 24]. PCR genotyping was used to identify the transgenic founders by following *Cre* primer pairs: Forward: 5'-CCGGTCGATGCAACGAGTGATGAGGTT-3' and Reverse: 5'-CAGGGTGTTATAAGCAATCCCCAGAAATGC-3'. We have also demonstrated that the 300-bp enhancer within *Col10a1* distal promoter could drive a higher-level and more specific reporter expression in hypertrophic chondrocytes in vivo [16, 17]. For this reason, we established another transgenic mouse line by generating a reporter construct, in which Cre recombinase was located on the same coat-color vector under the control of the 300 bp *Col10a1* distal promoter [22]. Detailed cloning strategies are also available upon request. Aforementioned *Cre*-primer pairs were used to identify transgenic founders by PCR genotyping. All animal used and studies were approved by the animal care and oversight committees at

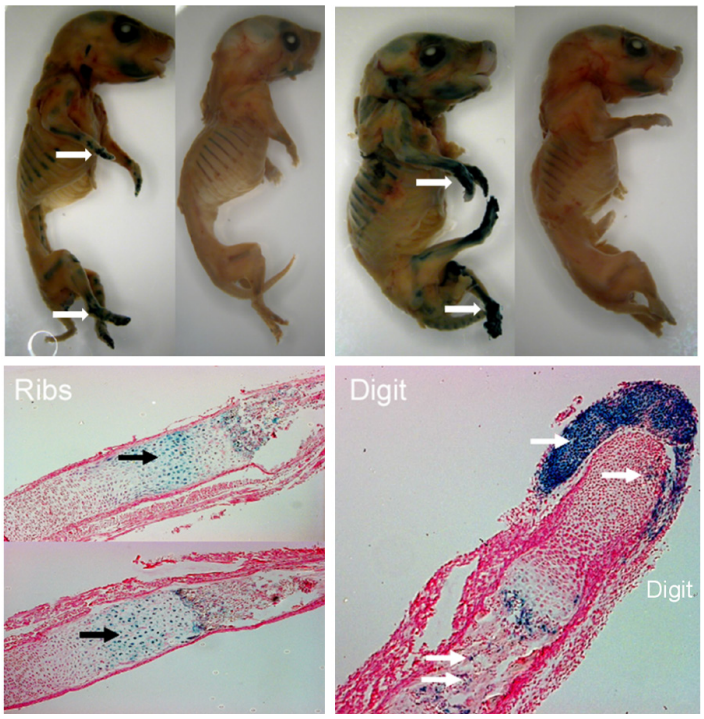

Figure 2. *Col10a1* 10 kb-Cre expression on a RosA26R genetic background. *Col10a1* 10 kb-Cre transgenic founder mice were bred with the RosA26R reporter mice. Cre reporter activity was examined by X-gal staining of the mouse embryos showing β-galactosidase activity in cells undergo Cre recombination. Whereas whole staining and histological analysis of two representative transgenic mouse ribs and digit sections showed Cre expression throughout hypertrophic zone (left panel, black arrows and data not shown), non-specific Cre activity was also seen in bone marrow, soft tissue surrounding the digits and in the resting chondrocytes of the digits of both lines (white arrows).

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Analysis of transgenic reporter mice

The transgenic cassette containing the *Col10a1* distal promoter elements and the *Cre* gene were released by *Pac I*, and then purified by QIXII purification kit (Qiagen). Purified DNA was microinjected into fertilized mouse eggs and then implanted into FVB pseudopregnant foster mothers using the Axiovert 200 transgenic apparatus (Carl Zeiss, Germany) or conducted at the Transgenic Production Service core facility at the University of Illinois (UIC). PCR genotyping was executed by using *Cre*-specific primers pairs [23]. The transgenic founder mice were crossed with RosA26R reporter mice and the offspring at E15.5 (embryonic day 15.5) were subjected to whole mount X-gal staining as mentioned above [18, 19]. Mice at the P1 stage (postnatal day 1) were X-gal stained, paraffin embedded, sectioned, counterstained with nuclear fast red (Poly Scientific R&D Corp) so as to show the expression level of *LacZ* reporter [18, 19]. Blue staining of the sagittal sections of the limb growth plate from both transgenic and wild-type littermates were detected using Nikon microscope (Nikon Eclipse 80i, Nikon Instruments Inc., Melville, NY) and the Qcapture Suite software (version, 2.95.0, Quantitative Imaging Corp., USA). At least 30 sections were analyzed for each growth plate. The animal studies were approved by the animal care and oversight committees at Baylor College of Medicine and Jiangsu University.

Statistical analysis

All data were shown as mean ± SD (standard deviation) and were set up in triplicates. All statistical calculations were performed using GraphPad Prism software version 5.0. Student's t test was used to evaluate the significance of differences and a *P* value less than 0.05 was considered statistically significant (*P < 0.05; **P < 0.01).

Results

Establishment of Col10a1-10 kb-Cre transgenic mice

Our transgenic studies have demonstrated that the 10 kb *Col10a1* control element can direct high-level reporter expression in hypertrophic chondrocytes compared to mice with the 4 kb *Col10a1* proximal promoter [16, 17]. We have generated reporter construct on a coat-color vector backbone in which Cre recombinase was placed under the regulation of this 10 kb *Col10a1* element (**Figure 1**. middle panel) [22]. By visual (coat color and eye color, top panel) and PCR genotyping (bottom panel), we obtained three transgenic mice (lanes 1, 5, and 14), Tg: transgenic, WT: wild type. P: positive control, N; negative control.

Cre expression of Col10a1-10 kb-Cre transgenic mice

Col10a1 10 kb-Cre transgenic founder mice were bred with the RosA26R reporter mice. Cre

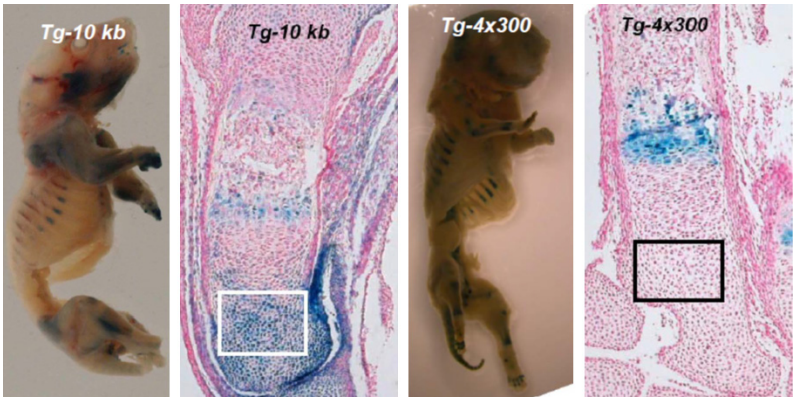

Figure 3. Differential *LacZ* reporter expression in *Tg-10 kb* and *Tg-4×300* mice. We performed a detailed histological analysis of our previous *Col10a1*-10 kb-LacZ and *Col10a1*-4×300 bp-LacZ transgenic mice. Left panel is a sagittal section of an X-gal stained *Tg-10 kb* P1 mouse digit. Non-specific blue staining showing LacZ reporter activity can also be seen in the resting chondrocytes, perichondrium and surrounding soft tissues in addition to the hypertrophic zone (white box and vicinity area). Sections from *Tg-4×300* only show reporter activity throughout hypertrophic zone (right panel). No staining is observed in the resting chondrocytes or surrounding tissues (right panel, black box and surrounding area).

reporter activity was examined by X-gal staining of the mouse embryos (E15.5) showing β-galactosidase activity in cells undergo Cre recombination [23]. Whereas whole staining and histological analysis of representative transgenic mouse ribs and digit sections showed Cre expression throughout hypertrophic zone. Surprisingly, non-specific Cre activity was also seen in bone marrow, soft tissue surrounding the digits and in the resting chondrocytes of the digits of both lines (**Figure 2**).

Comparison of Col10a1-10 kb-LacZ and 300 bp-LacZ transgenic mice

We then performed detailed histological analysis of our *Tg-10 kb* and *Tg-4×300* transgenic reporter mice. The result showed that *Tg-10 kb* mice also show non-specific β-galactosidase activity (blue staining) in or around the digits, but this non-specific staining was not seen in the *Tg-4×300* mice (**Figure 3**). Therefore, we have performed a similar transgenic study, i.e. we generated another construct using 4 copies of the tissue specific 300 bp element, upstream of the *Col10a1* basal promoter driving Cre as a reporter as described [17].

Establishment of Col10a1 300-bp-Cre transgenic mice

A similar Cre-expressing transgenic reporter construct was driven by applying the tissue-specific 4×300 bp element upstream of the *Col10a1* basal promoter (Xbp), instead of the *Col10a1* 10 kb promoter/intronic fragment (bottom). Since the basal promoter ends in intron I, a splicing acceptor sequence was added in the construct (**Figure 4**, top panel). Transgenic founder mice were identified based on eye color (top panel left, white arrow and data not shown) and PCR genotyping. We obtained four transgenic mice (lanes 4, 5, 9, and 10), Tg: transgenic, WT: wild type. P: positive control, N: negative control.

Tissue-specific Cre expression of Col10a1 300-bp-Cre transgenic mice

Col10a1 300 bp-Cre transgenic founder mice were bred with the RosA26R (ROSA) reporter mice. Cre reporter activity was examined by X-gal staining of the mouse embryos (E15.5) showing β-galactosidase activity in cells undergo Cre recombination. Whole staining and histological analysis of representative transgenic mouse ribs, other long bone sections showed Cre activity around hypertrophic zone of proximal and distal humerus (**Figure 5**).

Discussion

The type X collagen gene (*COL10A1*) is especially expressed by hypertrophic chondrocytes and guide the sedimentary of other matrix molecules to this area, and thus provides an appropriate environment for hematopoiesis, mineralization, and skeletal modeling which are main point for endochondral ossification. Dysfunction of the type X gene have been closely related to chondrocyte maturation which has been noticed in various skeletal dysplasia, bone regeneration, bone reconstruction, and pathogenesis of osteoarthritis [4, 11, 15, 25-27]. Therefore, identifying the specific cis-enhancer elements and finding out the transacting factors have been focus of the field of the type X collagen gene regulation [28, 29]. The tissue-specific *Col10a1* enhancer elements can be

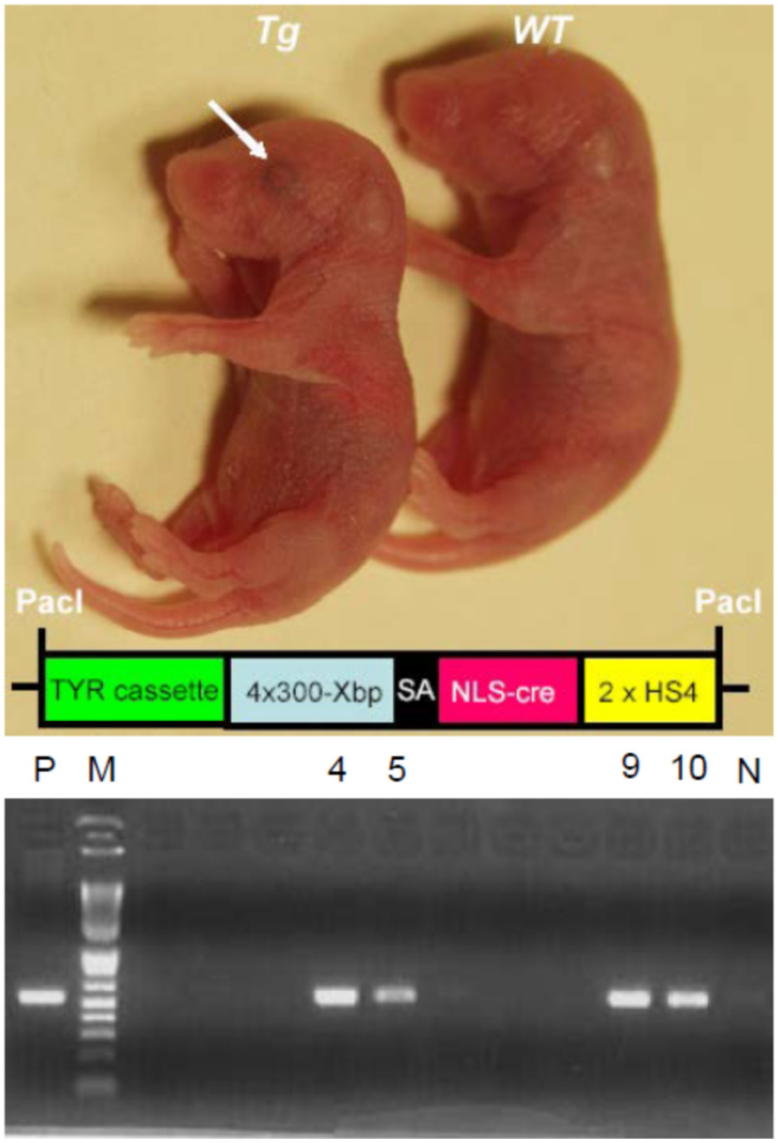

Figure 4. *Col10a1* 4×300-Xbp-SA-Cre transgenic mouse line with a coat color background. A similar Cre-expressing transgenic reporter construct was generated by using the tissue-specific 4×300 bp element upstream of the *Col10a1* basal promoter (110 bp) instead of the *Col10a1* 10 kb promoter/intronic fragment (bottom). One transgenic founder mouse was identified based on eye color and PCR genotyping (left, white arrow and data not shown). Since the basal promoter ends in intron I, a splicing acceptor adaptor (SA) was then inserted upstream of the Cre gene for the cassette. WT: wild type; Tg: Transgenic. Bottom chart shows structure and release of the transgenic cassette by PacI for microinjection. WT: wild type; Tg: transgenic. Bottom panel shows PCR genotyping results of 4 *Col10a1*-300 bp-Cre founders. P-positive control; N-negative control; M: molecular weight marker.

used as genetic tools to target genes of interest within the hypertrophic zone.

In this work, we used our previously defined 10-kb *Col10a1* control element to generate a Cre-containing transgenic reporter construct on a coat-colored vector background [22, 30, 31]. The tissue specificity of Cre activity in these transgenic founders was tested by breeding onto the RosA26R genetic background, a reporter line that expresses β-gal in cells that undergo Cre recombination via a Cre recombinase/Lox P recombination system [23] (**Figure 1**). We preformed X-gal staining of the mouse embryos showing β-galactosidase activity, and histological analysis of two representative transgenic mouse ribs and other long bone sections, shown that the 10 kb *Col10a1* control element can direct high-level reporter expression in hypertrophic chondrocytes compared to mice with the 4 kb *Col10a1* proximal promoter [16]. Surprisingly, in addition to the Cre activity in hypertrophic chondrocytes, reporter activity was also detected in bone marrow and in tissues surrounding digits (**Figure 2** and [16]). Previously, a Cre-expressing mouse line driven by 1 kb *Col10a1* proximal promoter which didn't include the defined enhancer was reported [32, 33]. Not surprisingly, the reporter expression was restricted to lower hypertrophic chondrocytes which is consistent with its promoter activity as described [16]. Some previously studies has been reported that a bacterial artificial chromosome (BAC) construct that contains the entire *Col10a1* gene and large flanking sequences has been shown to control efficient and explicit *LacZ* expression in hypertrophic chondrocytes in transgenic mice [34]. These results suggest the consistency of the endogenous promoter element in mediating transgene expression. Therefore, we

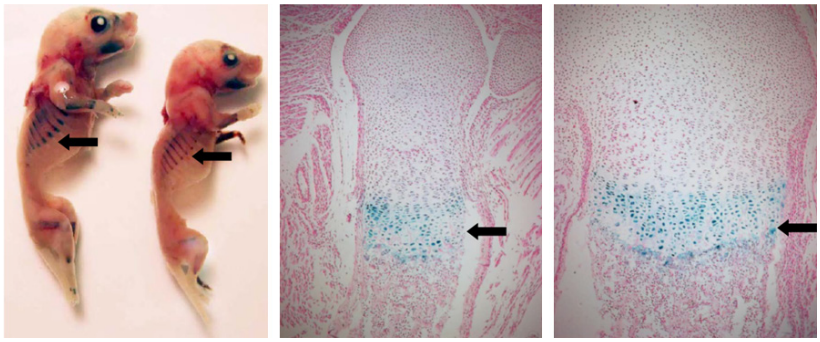

Figure 5. *Col10a1* 300 bp-Cre expression on a RosA26R genetic background. *Col10a1* 300 bp-Cre transgenic founder mice were bred with the RosA26R reporter mice. Cre reporter activity was examined by X-gal staining of the mouse embryos showing β-galactosidase activity in cells undergo Cre recombination. Whole staining and histological analysis of representative transgenic mouse ribs and other long bone sections showed Cre expression throughout hypertrophic zone of proximal and distal humerus (left panel, black arrows and data not shown).

established Cre-expressing transgenic mouse lines using the tissue-specific 4×300 bp element upstream of the *Col10a1* basal promoter [17, 35]. Whole staining and histological analysis of representative transgenic mouse ribs and long bone sections showed Cre expression around hypertrophic area of proximal and distal humerus (**Figure 5**). Such reporter mice will allow for the conditional targeting genes of interest selectively to hypertrophic chondrocytes.

The coat color cassette used in this transgenic reporter construct was derived from tyrosinase cDNA and chicken β globin HS4 insulator. It allows visual genotyping of the coat color directly in the FVB/N albino background (black eyes) and enables more than 90% of the founders to obtain the capability of transgene expression [22, 31].

In summary, we have successfully established two Cre-expressing transgenic mouse lines using our previously defined 10 kb and 300 bp Col10a1 control elements respectively. In the Cre-expressing transgenic mouse lines using 10 kb *Col10a1* promoter/intronic element, in addition to its activity in hypertrophic chondrocytes, Cre-mediate recombination was also seen in skin, perichondrium and resting chondrocytes surrounding the digits. Meanwhile, Cre expression was exclusively restricted to the hypertrophic zone in the transgenic mice using the tissue-specific 4×300 bp element upstream of the *Col10a1* basal promoter [17, 35]. This engineered mouse model provides us a new and unique genetic tool. As it will help to find out the molecular regulation of endochondral ossification and studies of skeletal diseases relating to chondrocyte hypertrophy, including the pathophysiology and progression of osteoarthritis [36-38].

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Disclosure of conflict of interest

None.

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