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Wnt-mediated reciprocal regulation between cartilage and bone development during endochondral ossification

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ABSTRACT

The role of Wnt signaling is extensively studied in skeletal development and postnatal bone remodeling, mostly based on the genetic approaches of β-catenin manipulation. However, given their independent function, a requirement for β-catenin is not the same as that for Wnt. Here, we investigated the effect of Wnt proteins in both tissues through generating cartilage- or bone-specific *Wls* null mice, respectively. Depletion of *Wls* by *Col2-Cre*, which would block Wnt secretion in the chondrocytes and perichondrium, delayed chondrocyte hypertrophy in the growth plate and impaired perichondrial osteogenesis. Loss of *Wls* in chondrocytes also disturbed the proliferating chondrocyte morphology and division orientation, which was similar to the defect observed in *Wnt5a* null mice. On the other hand, inactivation of *Wls* in osteoblasts by *Col1-Cre* resulted in a shorter hypertrophic zone and an increase of TRAP positive cell number in the chondro-osseous junction of growth plate, coupled with a decrease in bone mass. Taken together, our studies reveal that Wnt proteins not only modulate differentiation and cellular communication within populations of chondrocytes, but also mediate the cross regulation between the chondrocytes and osteoblasts in growth plate.

Introduction

The majority of the skeletons consist of cartilage and bone, which are formed by chondrocytes and osteoblasts through endochondral ossification [1]. Endochondral ossification starts from the chondrogenic condensation of mesenchymal progenitor cells, followed by a sequential differentiation program from resting, proliferating to hypertrophic chondrocytes. These chondrocytes in the anlage, which are organized as a zonal growth plate, have distinct morphology and extracellular matrix. The resting chondrocytes at distal growth plate have round shape, whereas the proliferating chondrocytes are aligned as columnar cells. As they exit the cell cycle, these chondrocytes enlarge and progress into hypertrophic chondrocytes. Meanwhile, there is a shift of major extracellular matrix from Collage II (*Col II*) in the non-hypertrophic chondrocytes to Collagen X (*Col X*) in the hypertrophic chondrocytes. Finally, the hypertrophic chondrocytes undergo mineralization, apoptosis and are replaced by osteoblasts originating from the perichondrium, which are osteochondrogenic progenitor cells surrounding the cartilage anlage.

The cellular development of cartilage and bone is tightly coupled. Previous studies have shown that the prehypertrophic and hypertrophic chondrocytes secrete several important signals, such as Ihh and VEGF, to regulate adjacent perichondrial osteogenic differentiation, primary ossification center (POC) formation and angiogenesis [2–5]. Conversely, the subchondral bone is also important for the maintenance of cartilage homeostasis. For instance, subchondral bone cells from osteoarthritis (OA) patients produce local factors to regulate chondrocyte differentiation in articular cartilage [6].

Wnt signaling has important roles in multiple processes of cartilage and bone development [7]. Wnt proteins consist of 19 members and activate intracellular cascade through β-catenin-dependent or independent pathways, which are usually named as canonical and non-canonical signaling pathway, respectively. Canonical Wnt/β-catenin signaling determines the chondrogenic or osteogenic commitment from mesenchymal progenitor cells at an early stage [8], and promotes the hypertrophic chondrocyte differentiation at late stage [9]. It also promotes osteoblast differentiation and maturation during endochondral and intramembranous ossification [8,10,11]. In addition, β-catenin signaling is precisely controlled in the regulation of articular cartilage homeostasis. Activation or inactivation of β-catenin in articular chondrocytes resulted in OA-like phenotypes [12,13]. Inhibition of Dkk1 prevented joint destruction in arthritic mice [14]. On the other hand, non-canonical Wnt5a and Wnt5b differentially regulate the transition of chondrocyte differentiation within the growth plate [15]. The gradient of Wnt5a/PCP signaling instructs the directional limb outgrowth by establishing polarity in chondrocytes [16,17]. Therefore, Wnt signaling has a crucial role in orchestrating the cartilage and bone development.

The majority of the studies of Wnt signaling function in skeletal development are based on the genetic alterations in components of

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intracellular Wnt signaling cascade or individual Wnt ligand. For instance, alterations in β-catenin allele have been extensively used to study the function of canonical Wnt signaling in multiple developmental processes. However, β-catenin is also involved in the cell junction complex besides mediating canonical Wnt signaling activity [18]. Meanwhile, the alterations in individual Wnt ligand could not fully reveal its function due to the redundancy among different Wnt family members. In particular, these genetic approaches have disadvantages in investigating the paracrine effect of Wnt proteins on cartilage and bone development.

Wntless (*Wls*), or Gpr177, is a trafficking protein that is essential for the secretion of both canonical and non-canonical Wnt proteins [19–23]. The *Wls* transcript and protein are detected in multiple tissues and organs during mouse embryogenesis [21]. Genome-wide association studies have recently identified it as a susceptibility locus for osteoporosis [24]. The *Wls* conditional knockout mouse is an ideal model to study the function of Wnt signaling pathway in bone development [25,26].

Here we studied the function of *Wls* in cartilage and bone development though generating mice with cartilage or bone-specific *Wls* deficiency. Deletion of *Wls* in chondrocytes and perichondrium with *Col2-Cre* impaired chondrocyte differentiation and organization, as well as endochondral ossification. On the other hand, deletion of *Wls* in osteoblasts with *Col1-Cre* resulted in a shortened hypertrophic zone and an increase of TRAP positive cells in the chondro-osseous junction of growth plate. Our investigations provide novel insights into the Wnt-mediated reciprocal regulations between cartilage and bone formation.

Materials and methods

Mice

Wls$^{c/c}$ [23] mice were crossed with *Col2-Cre* [27], *Col1-Cre* [28] transgenic mice to generate various tissue-specific *Wls* null mice. *Wls*$^{c/c}$ mice were first crossed with *Col2-Cre* or *Col1-Cre* transgenic mice to get *Col2-Cre; Wls*$^{c/+}$ or *Col1-Cre; Wls*$^{c/+}$ mice, then *Col2-Cre; Wls*$^{c/+}$ and *Col1-Cre; Wls*$^{c/+}$ mice were crossed with *Wls*$^{c/c}$ mice to get *Col2-Cre; Wls*$^{c/c}$ and *Col1-Cre; Wls*$^{c/c}$ mice. *Wls*$^{c/c}$ or *Wls*$^{c/+}$ littermates were used as WT controls in all of the experiments. *Wnt5a*$^{-/-}$ embryos were generated by crosses of heterozygous *Wnt5a*$^{+/-}$ mice [29].

Histology and in situ hybridization

Limbs were fixed by 4% paraformaldehyde at 4 °C overnight, embedded in paraffin, and sectioned at 8 μm. Safranin O, von Kossa staining and in situ hybridization with DIG-labeled probes were applied on sections as previously described [27].

Immunohistochemistry (IHC)

Limb frozen sections of 12 μm slices or paraffin sections of 8 μm slices were stained with anti-Wnt5a (R&D, AF645), anti-Wnt5b(R&D, MAB3006), anti-Wnt11 (R&D, AF2647), anti-Wnt10b(Sigma, PRS4619), anti-Gpr177(Santa Cruz, sc133635), anti-Osterix (Abcam, ab22552), anti-BrdU (Sigma, ab6326), anti-phalloidin (Beyotime,C1033) primary antibodies, and Alex fluor 488(Invitrogen, A-21208) secondary antibody. All antibodies were used according to the recommended dilution rate. Labeled sections were counterstained with DAPI fluorescent dye (Southern Biotech), observed using a Leica confocal microscope, and then photographed. Mice received an intraperitoneal injection of bromodeoxyuridine (BrdU) (Sigma) with a dose of 100 μg/g body mass, and were sacrificed 2 h later, then embedded in paraffin for sectioning. For BrdU incorporation and Tunel staining, sections were treated according to the manufacturer's instructions.

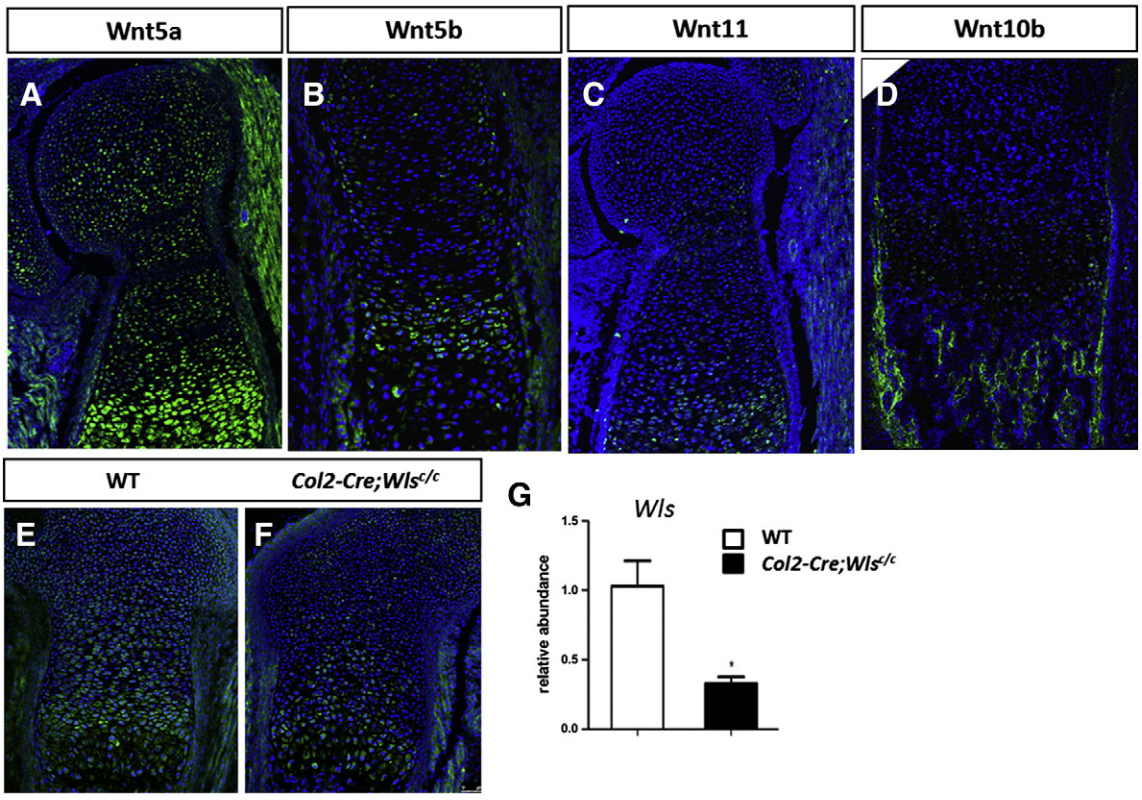

Fig. 1. Expression of *Wls* and Wnts in differentiating chondrocytes. A–D. Expression of Wnt5a (A), Wnt5b (B), Wnt11 (C) and Wnt10b (D) is highly detected in prehypertrophic or hypertrophic chondrocytes of E16.5 humerus. E–F. IHC of *Wls* in E15.5 humerus. Expression of *Wls* protein is reduced in the chondrocytes of *Col2-Cre; Wls*$^{c/c}$ (F) compared to WT (E). A–C, distal humerus; D–F, proximal humerus. G. qRT-PCR of Wls in the E15.5 rib cartilage. β-Actin was used as internal control. *Wls* mRNA expression is reduced in the rib cartilage of *Col2-Cre; Wls*$^{c/c}$ compared to WT. RNA from one WT and mutant embryo was analyzed.

Skeletal analysis

P0 newborn mice were skinned, eviscerated, and fixed in 95% ethanol. Skeletal preparations were performed as previously described [27].

RNA extraction and quantitative real-time PCR

Rib cartilage was homogenized and then extracted using the Trizol reagent (Invitrogen) according to standard procedures. SuperScriptII Reverse Transcriptase (Invitrogen) was used to reverse-transcribe 800 ng of RNA. Real-time PCR was performed on ABI Prism 7500 Sequence Detection System (Applied Biosystems) using a SYBR Green Kit (Roche). The samples were normalized to actin expression.

Analysis of cell division orientation

E16.5 limbs were fixed with PFA overnight, frozen embedded and sectioned at 12 or 20 μm. Sections were washed with PBS with 0.1% Triton X-100, incubated with anti-phalloidin-FITC for 1 h at RT to label the contractile ring, and mounted with DAPI to label DNA. Cells in telophase were identified by an actin-rich contractile ring between the two daughter cells. Three dimensional images were generated from a z-series of optical sections collected on Leica confocal microscope. Cell division orientation was calculated according to the model shown in Fig. S2. Line segment "b" was set parallel to the long axis of the bone, and was the distance between two nuclei in the y axis. Line segment "a" was the distance between two nuclei in the x axis. Line segments "a" and "b" were measured with the image software. Line "c" was the distance between two nuclei in the z axis, and was determined by the number of 1 μm optical sections between the centers of two cells. The angle (θ) is calculated by the formula shown in Fig. S2.

Statistical analysis

Statistical significant difference between groups was evaluated by two tailed t-test using GraphPad Prism 5. All values were expressed

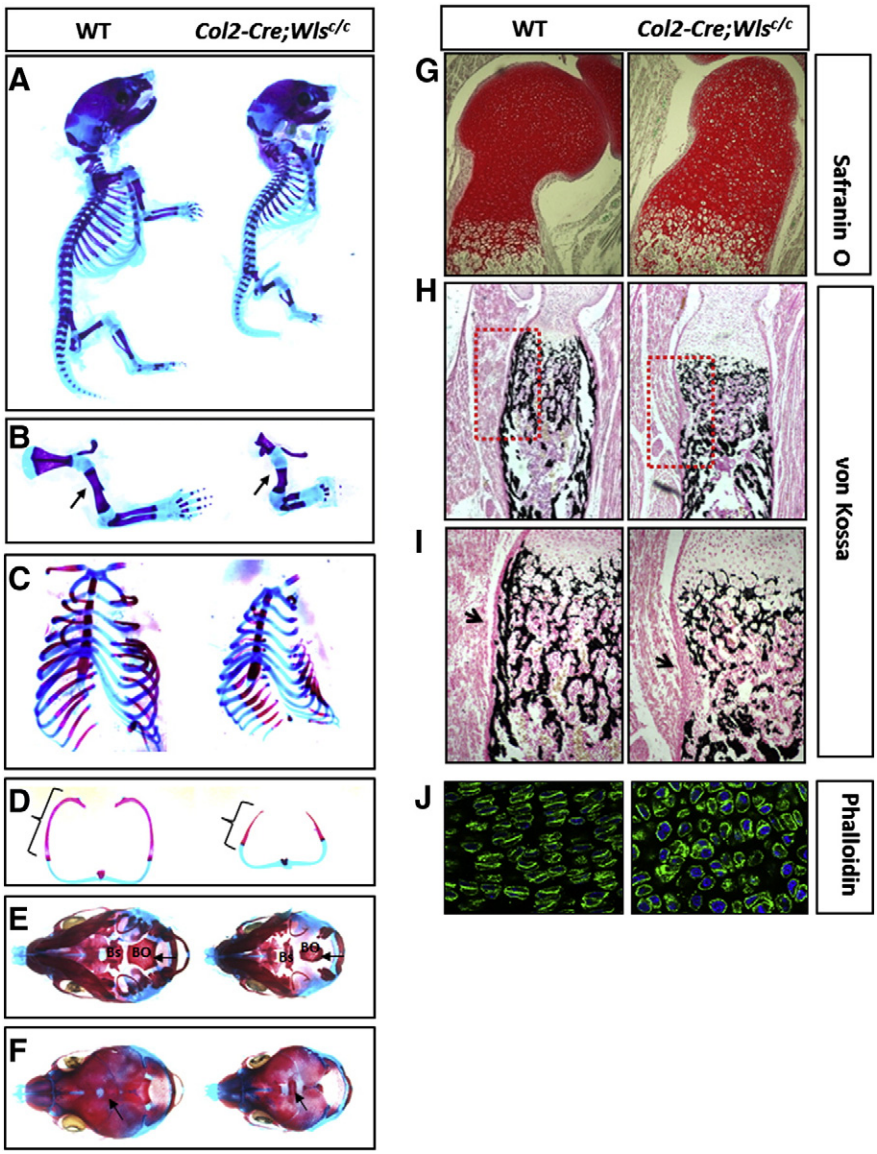

Fig. 2. Impaired skeletal ossification in the *Col2-Cre; Wls$^{c/c}$* embryos. A–F. Skeletal preparation of P0 mice. The representative images of the whole embryonic skeletons of WT and *Col2-Cre; Wls$^{c/c}$* (A). The forelimbs (B), ribs (C, D) and cranium bones (E, F) of each embryo are shown in the lower panel. Mineralized region is shortened in the long bones and rib of the mutant mouse (arrows in B and bracket in D). Morphological defects are also detected in the basioccipital (BO) and basisphenoid (BS) bone at cranium base (E). G–I. Safranin O (G) and von Kossa (H, I) staining for the humerus of P0 mice. Mineralization is reduced in the perichondrium (boxed region) of the mutant. J. Phalloidin staining for the proliferating chondrocytes in E16.5 humerus. The proliferating chondrocytes are disorganized in the mutant.

with mean ± SEM. For Figs. 1–8, *P<0.05, **P<0.01 and ***P<0.001 versus the WT or control.

Results

Multiple Wnts are expressed in differentiating chondrocytes

A variety of Wnts have been revealed to be expressed in different populations of chondrocytes in growth plate. During embryonic cartilage development, Wnt4, Wnt14 and Wnt16 are expressed at various joint regions, whereas Wnt5b and Wnt5a are differentially detected in prehypertrophic and hypertrophic chondrocytes, respectively [7,15,27,30–32]. In postnatal growth plate, *Wnt2b*, *Wnt4*, *Wnt10b*, *Wnt5a*, *Wnt5b* and *Wnt11* are observed in proliferating and hypertrophic chondrocytes [33]. These examinations are performed through in situ hybridization (ISH) [7,15] or quantitative RT-PCR [33]. To systemically validate the Wnt protein expression in growth plate chondrocytes, immunohistochemistry (IHC) analyses for multiple Wnts were conducted in the sections of wild-type (WT) humerus at E16.5. As shown in Fig. 1, Wnt5a, Wnt5b, Wnt10b and Wnt11 were highly expressed in the hypertrophic chondrocytes, while their expression in the resting and proliferating chondrocytes was comparatively lower or not detectable. In addition, the expression of Wnt5a and Wnt10b was observed in the perichondrium. Wnt5a was also expressed in perichondrium and osteoblasts in spongiosa (Fig. S1). In line with previous reports by ISH [32], these findings confirmed that a variety of canonical and non-canonical Wnts, including Wnt10b, Wnt5a/5b and Wnt11, were preferentially expressed in the differentiating chondrocytes.

Wls deletion in chondrocytes and perichondrium impairs endochondral ossification

To investigate the paracrine effect of Wnt proteins on cartilage development, we generated the *Col2-Cre; Wls$^{c/c}$* mouse. The *Wls* conditional knockout mouse *Wls$^{c/c}$* was crossed with *Col2-Cre* transgenic mouse, in which Cre activity is detected in the chondrocytes and

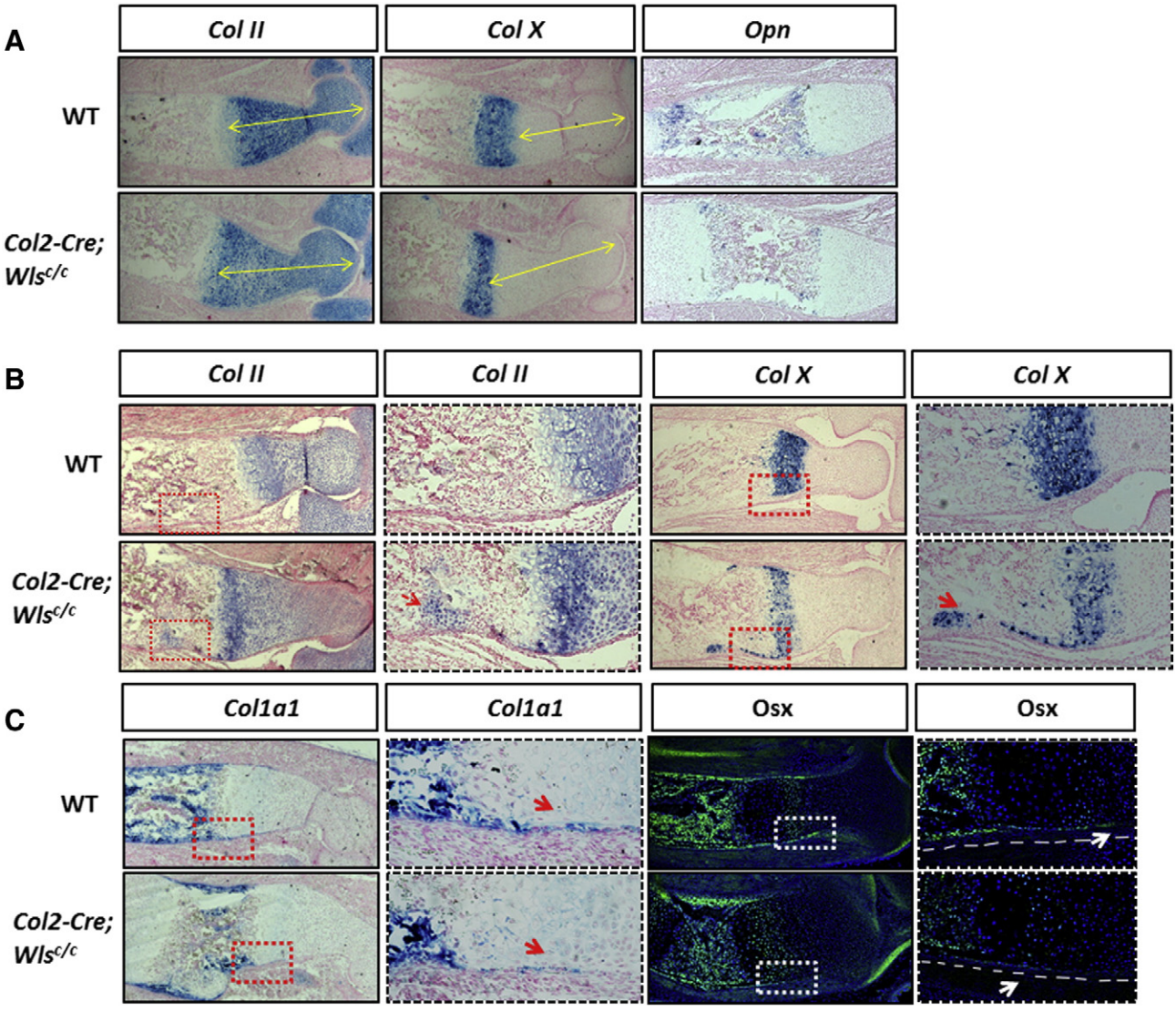

Fig. 3. Delayed chondrocyte differentiation and perichondrial osteogenesis in the *Col2-Cre; Wls$^{c/c}$* embryos. A. Chondrocyte hypertrophic differentiation and maturation are delayed in the growth plate of the *Col2-cre; Wls$^{c/c}$* mutant, revealed by the expanded *Col2*-expression and late onset of *Col X* and *Opn* expression in E16.5 humerus. B. Ectopic expression of *Col2* and *Col X* is detected in the periosteum region of the E18.5 humerus in mutant embryos. The red boxed regions are enlarged in the right panel. C. Delayed onset of osteogenic differentiation in the perichondrial region is shown by the weaker expression of *Col1a1* and Osterix (Osx) in the E16.5 humerus of mutant embryos. The boxed regions are enlarged in the right panel. Osx expression is examined by IHC. The other markers are examined by in situ hybridization with the indicated DIG-labeled probes.

perichondrium/periosteum [34]. The *Col2-Cre; Wls$^{c/c}$* mutants were born with Mendelian ratio, but died at birth of respiratory failure. qRT-PCR analysis of *Wls* in the E15.5 rib cartilage and IHC examination in E15.5 humerus sections with *Wls* antibody showed the efficient depletion of *Wls* in the mutants (Figs. 1E, F and G). The existence of patchy residual *Wls*-expression chondrocytes was still detected in the mutant (Fig. 1F), indicating that the inactivation of *Wls* by *Col2-Cre* was not complete. Skeletal preparation at P0 showed that the *Col2-Cre; Wls$^{c/c}$* mutant displayed dwarfism and morphological defects in most of the bones formed by endochondral ossification, including shorter long bones and rib cage (arrows in Figs. 2A–D), as well as agenesis of the cranium base bones of basioccipital bone (BO) and basisphenoid bone (BS) (arrows in Fig. 2E). Moreover, the suture fusion in the frontal bones was relatively delayed compared to WT controls (arrows in Fig. 2F). Mineralized region was relatively downsized in the long bones and ribs of the mutant mice compared to the WT (arrows in Fig. 2C and brackets in Fig. 2D). In addition, histological analyses based on Safranin O staining and phalloidin staining demonstrated an absolute loss of columnar structure in the growth plate chondrocytes when compared with controls (Figs. 2G, J). Von Kossa staining revealed that the mineralization in the bone collar was decreased in the mutant compared to the WT littermates (arrows in Figs. 2H–I). These observations indicate that loss of *Wls* in chondrocytes and neighboring perichondrium impairs the growth plate chondrocyte assembly, perichondrial mineralization and endochondral bone formation.

Delayed chondrocyte hypertrophy and perichondrial osteogenesis in the Col2-Cre; Wls$^{c/c}$ mutant

To molecularly characterize the defects of skeletal development in the *Col2-Cre; Wls$^{c/c}$* mutant, ISH was performed in sections of embryonic cartilage with Dig-labeled probes. The *Col2*-expressing non-hypertrophic domain was expanded, whereas the *ColX*-expressing hypertrophic domain was more distant from the articular end in the humerus of *Col2-Cre; Wls$^{c/c}$* mice compared with controls at E16.5 (Fig. 3A), suggesting that the onset of hypertrophic differentiation was delayed. Moreover, the *Opn* expression, which is a marker of mature hypertrophic chondrocytes, was much lower in the corresponding region of mutant (Fig. 3A). These data suggests that chondrocyte and perichondrium-derived Wnts are indispensable for the chondrocyte differentiation and maturation.

Meanwhile, a decrease of *Col1a1* and Osterix (Osx) expression was detected in the perichondrium and periosteum of the mutants (arrows in Fig. 3C). In addition, in some sections of the mutants, ectopic expression of *Col2* and *ColX* were found in the periosteum region of the developing humerus cartilage (Fig. 3B), where they were not detected in the WT embryos. These observations indicated that the onset of osteogenic differentiation was delayed and ectopic chondrocyte differentiation occurred at the expense of osteogenic commitment from the mesenchymal progenitor cells in the mutant perichondrium. The defective perichondral ossification in the mutant was consistent with the histological analysis by von Kossa staining (Figs. 2H, I). These features mimic the defects that were observed in mice with inactivation of β-catenin in chondrocytes by *Col2-Cre* [8].

Reduced proliferation and enhanced apoptosis in the growth plate of Col2-Cre; Wls$^{c/c}$ mutant

To examine the mechanism underlying the skeletal abnormality in the *Col2-Cre; Wls$^{c/c}$* mutant, we examined the proliferation and apoptosis of the chondrocytes in the growth plate. Analysis of BrdU incorporation after 2-hour labeling showed that the proliferation rate of chondrocytes was decreased in growth plate (Fig. 4A), which was quantified in Fig. 4B. On the other hand, TUNEL staining indicated that severe apoptosis occurred in the resting chondrocytes of proximal femur, where very few apoptotic cells were detected in the controls at this stage (Fig. 4C). Thus, chondrocyte and perichondrium-derived Wnt proteins are important for chondrocyte proliferation and survival in the cartilage growth plate.

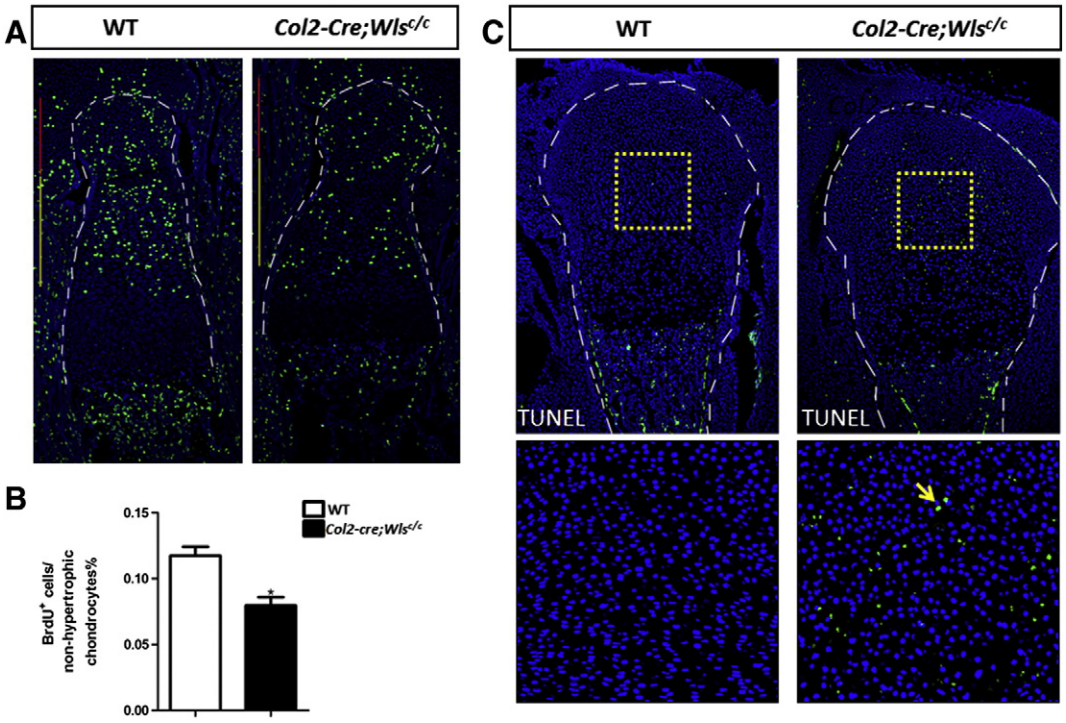

Fig. 4. Decreased chondrocyte proliferation and enhanced apoptosis in the *Col2-Cre; Wls$^{c/c}$* embryos. A–B. Representative images of BrdU labeling in the chondrocytes of distal humerus at E16.5 (A). Quantification of BrdU labeling is shown in B (n = 3). C. TUNEL assay on sections of developing femur at E18.5. The boxed region is enlarged in the lower panel. Ectopic apoptosis is detected in the resting chondrocytes of the mutant (yellow arrows) whereas few TUNEL-positive cells are observed in the corresponding region of WT.

Oriented cell divisions in proliferating chondrocytes is disrupted in the Col2-Cre; Wls$^{c/c}$ mutant

Chondrocytes in the cartilage growth plate display distinct morphologies while they undergo a sequential differentiation program. The cellular morphology in the proliferating chondrocytes in the *Col2-Cre; Wls$^{c/c}$* mutant was quite distinct from WT controls (Figs. 2J, 5A and B). The proliferating chondrocytes in the mutant remained round and big, while those in the wild types were typically discoid. Similar alterations were detected in *Wnt5a$^{-/-}$* mutants (Figs. 5A and B). In contrast to the resting and proliferating chondrocytes, the morphology of the hypertrophic chondrocytes in the *Col2-Cre; Wls$^{c/c}$* mutants was largely intact (Figs. 5A and B).

Cell division in proliferating chondrocytes is correlated with their polarity and is oriented in an angle perpendicular to the long axis of the cartilage, followed by intercalation of the two daughter cells [17]. The orientation of cell division can be indicated by the angle of telophase θ, which is defined by the intersection of the line connecting the two daughter cells in the telophase and a line drawn parallel to the long axis of the cartilage. Cells in telophase were identified by a strong staining of phalloidin (Fig. 5C). Telophase θ exhibited a strong bias for nearly 90° in the WT proliferating chondrocytes, but was irregularly distributed from 0 degree to 90 degree in the *Col2-Cre; Wls$^{c/c}$* and *Wnt5a$^{-/-}$* mutants (Figs. 5C and D). These observations suggest that oriented cell division in proliferating chondrocytes was disrupted in the chondrocyte-specific *Wls* null mutants. The alterations of the cellular morphology and division in the mutant indicate that Wnts from chondrocytes and perichondrium, including the Wnt5a, regulate the chondrocyte polarity and division in the growth plate.

Bone-specific Wls ablation results in a shorter hypertrophic zone and increased TRAP positive cells in the chondro-osseous junction of growth plate

To investigate the impact of osteoblastic Wnts on cartilage and bone development, we generated the bone-specific *Wls* deficient mice *Col1-Cre; Wls$^{c/c}$*. The *Col1-Cre* line we used is driven by the 3.6 kb promoter of *Col1a1* gene, which has *Cre* activity in osteoblast progenitors [28]. The mice with *Wls* inactivation in osteoblasts exhibited dwarfism in a dose-dependent manner (Fig. 6A). X-ray analysis and H&E staining

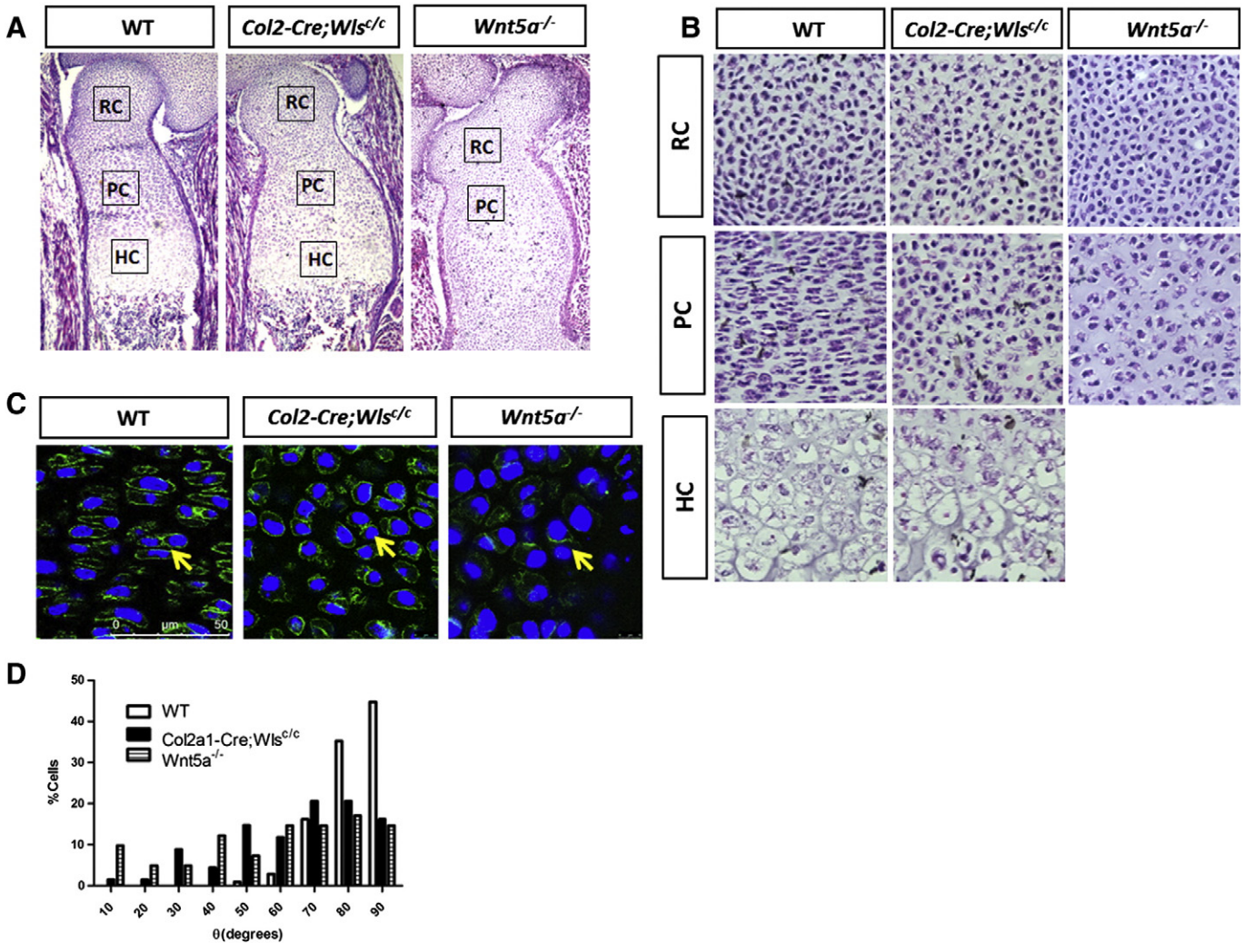

Fig. 5. Disruption of the oriented division of proliferating chondrocytes in the *Col2-Cre; Wls$^{c/c}$* and *Wnt5a$^{-/-}$* embryos. A–B. H&E staining in the sections of humerus at E16.5. RC, resting chondrocytes; PC, proliferating chondrocytes; HC, hypertrophic chondrocytes. Proliferating chondrocytes remain round and lose the columnar alignment in the *Col2-Cre; Wls$^{c/c}$* and the *Wnt5a$^{-/-}$* cartilages. C. F-actin of the proliferating chondrocytes is visualized by fluorescently labeled phalloidin staining (green) in the developing tibia at E16.5. Nuclei are counterstained with DAPI (blue). Cells in telophase are indicated with yellow arrows, marked by the phalloidin staining in the cleavage furrow or contractile ring. D. The orientation of cell division (θ) relative to the long axis of the cartilage is quantified according to the 3D images of cells in telophase in the proliferating zone. Telophase θ exhibits a strong bias for nearly 90° in the WT proliferating chondrocytes, but is uniformly distributed from 0° to 90° in the *Col2-Cre; Wls$^{c/c}$* and *Wnt5a$^{-/-}$* mutant.

indicated that the mutant mice had a decrease of bone mass accrual (Figs. 6B, C, D and G), which could be quantified by a micro-CT analysis (Wan et al., 2012, paper in revised process in Bone).

Col1-Cre; Wls$^{c/c}$ mutants had shorter hypertrophic zone in the growth plate than WT at P14 (Figs. 6Dc, Dd and F), which could also be detected at early perinatal stage and validated by the *ColX* expression in humerus (Figs. 8A–D). In contrast, the length of the proliferating zone remained intact compared with WT (Figs. 6Da, Db, 7D, E, and F).

We then investigated the cause for the shorter hypertrophic zone in the mutants. BrdU labeling assay showed that the proliferation of non-hypertrophic chondrocytes in the growth plate of *Col1-Cre; Wls*$^{c/c}$ mutants was relatively normal (Figs. 7D–F). TUNEL assay showed that the apoptosis in the chondro-osseous junction was also not significantly altered (Figs. 7G, H). Meanwhile, the terminal maturation of the hypertrophic chondrocytes was not accelerated in the *Col1-Cre; Wls*$^{c/c}$ mutants, indicated by the relatively normal expression of *Mmp13* in the chondro-osseous junction of growth plate (Figs. 8E, F). Instead, more TRAP positive cells were detected in the chondro-osseous junction in the *Col1-Cre; Wls*$^{c/c}$ mutants compared with WT (Figs. 7A–C). Those TRAP positive cells could be chondroclasts or osteoclasts that were responsible for the cartilage and bone resorption. Thus, the shorter hypertrophic zone was probably caused by the enhancement of osteoclast-mediated hypertrophic chondrocyte degradation, but not due to alterations in chondrocyte proliferation, terminal differentiation and apoptosis.

Discussion

Wls is a conserved chaperon protein dedicated to the Wnt secretion. It is also important for the regulation of bone mineral density and bone mass [25,26]. Here we generated mice with cartilage- or bone-specific *Wls* deficiency to study the function of Wnt proteins in the cartilage and bone development. Loss of *Wls* in cartilage resulted in delayed chondrocyte differentiation, disorganized chondrocyte arrangement, and impaired endochondral bone formation. On the other hand, depletion of *Wls* in bone have not affected the chondrocyte differentiation and proliferation, but resulted in a decreased bone mass and shorter hypertrophic zone in the growth plate, probably through regulating chondroclast/osteoclast-mediated cartilage and bone resorption.

Multiple Wnts have been detected to be expressed in the growth plate cartilage [7,15,27,30,31]. According to the previous studies and our own investigation, at least at an embryonic stage, a variety of Wnts are preferentially observed in differentiating chondrocytes, especially in prehypertrophic and hypertrophic chondrocytes. These Wnts included canonical Wnt10b and non-canonical Wnt5a/Wnt5b/Wnt11. We have not detected Wnt2b and Wnt4 expression in the growth plate, which is consistent with the previous report by ISH [32]. Of note, we could not exclude the existence of *Wnt2b* and *Wnt4* in the postnatal growth plate. Neither have we detected the expression of all potential Wnt proteins due to the limited availability of antibody. Interestingly, few canonical Wnt are detected in resting and proliferating

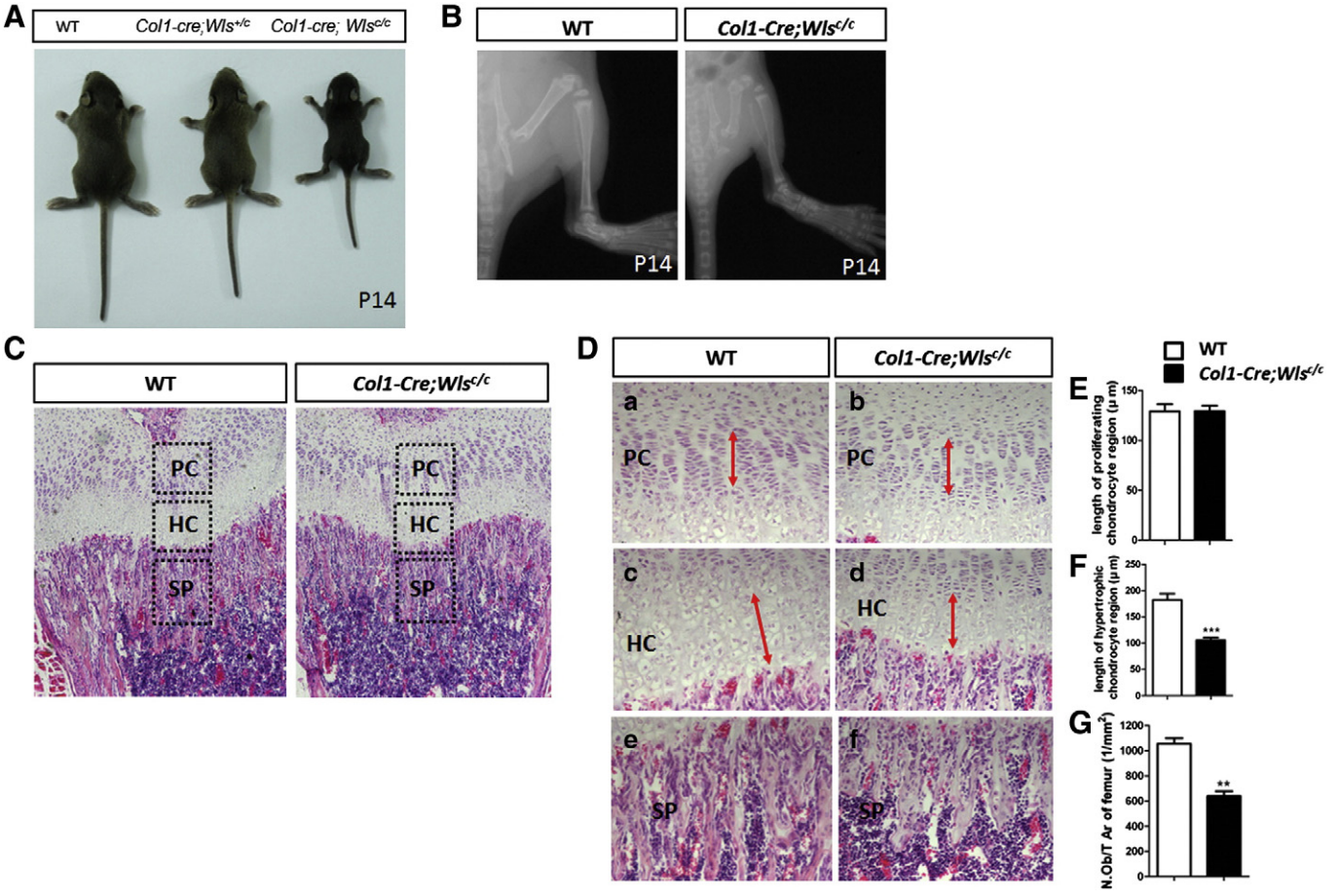

Fig. 6. Decreased bone mass and shorter hypertrophic domain in *Col1-Cre; Wls*$^{c/c}$ mutant. A. The *Col1-Cre; Wls*$^{c/c}$ mutant mice display dwarfism in a dose-dependent manner. B. Representative images of the X-ray analysis for the WT and mutant mice at P14. A significant decrease of bone mass in the hindlimb is observed in the mutant. C–D. H&E staining of the P14 femur. D (a, c, e) and D (b, d, f) were magnification of boxed region in C. PC, proliferating chondrocytes; HC, hypertrophic chondrocytes; SP, spongiosa. Proliferating chondrocyte domain is relatively normal (arrows in a, b) whereas hypertrophic domain is remarkably shorter in the mutant growth plate at P14 (arrows in c, d). E–F. Quantification of the length of proliferating and hypertrophic zones. N = 4. G. Quantification of the number of osteoblasts in spongiosa region.

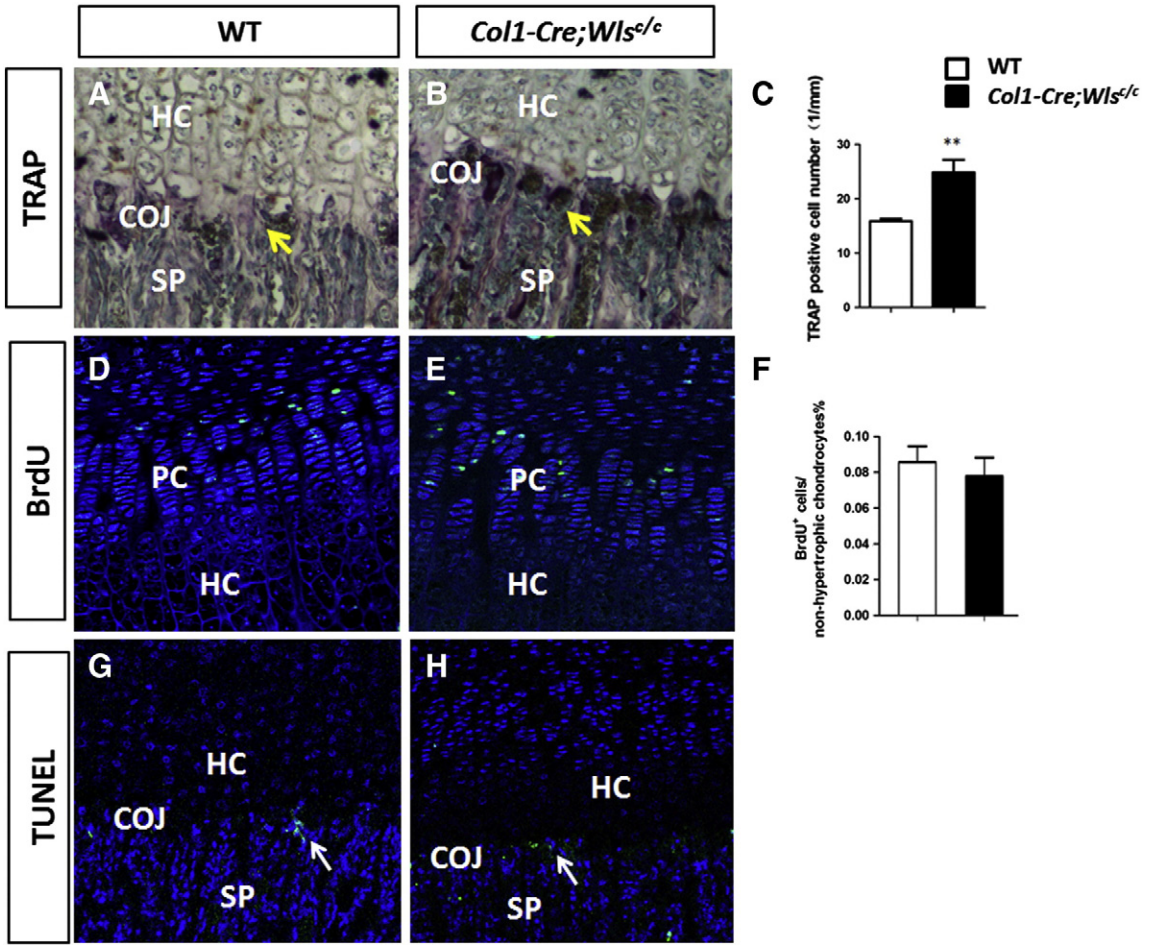

Fig. 7. TRAP positive cell number is increased in the *Col1-Cre; Wls*$^{c/c}$ mutant mice, while chondrocyte proliferating and apoptosis are not significantly affected. A–B. Representative images of TRAP staining in P14 femur. TRAP positive cells in the chondro-osseous junction are marked with yellow arrows. C. Quantification of TRAP positive cells. TRAP positive cell number is increased in the chondro-osseous junction of *Col1-Cre; Wls*$^{c/c}$ mutant mice compared to WT. N = 3. COJ, chondro-osseous junction. F. Quantification of BrdU staining in the nonhypertrophic chondrocyte region. Chondrocyte proliferation is not significantly affected. N = 3. G–H. TUNEL staining of P14 femur. Apoptotic cells in the chondro-osseous junction are marked by the white arrows. HC, hypertrophic chondrocytes; COJ, chondro-osseous junction; SP, spongiosa.

chondrocytes [7,32]. However, canonical Wnt signaling is extensively active in the growth plate chondrocytes, indicated by the TOPGAL reporter [35,36]. Therefore, chondrocytes in growth plate are responsive to canonical Wnts produced by neighboring cells, possibly from perichondrium or osteoblasts in spongiosa.

Blockage of Wnt secretion by *Wls* inactivation in chondrocytes and perichondrium delayed chondrocyte hypertrophy and impaired perichondrial ossification. Moreover, the cell proliferation and survival were affected in the resting and proliferating chondrocytes in cartilage-specific *Wls*-ablated mutant. These defects resembled phenotypes previously observed in *Col2-Cre; β-catenin*$^{c/c}$ mutants [8]. In addition, the disruption of oriented cell division in the growth plate of *Col2-Cre; Wls*$^{c/c}$ was similar to that in the *Wnt5a* null mutant mice. Collectively, these data suggest that the differentiating chondrocytes and perichondrium are important Wnt-producing cells, orchestrating the chondrocyte and osteoblast differentiation. The canonical Wnts from the differentiating chondrocytes or perichondrium, such as Wnt10b, orchestrate the growth plate chondrocytes proliferation, differentiation and perichondrial ossification. Non-canonical Wnts from the chondrocytes, such as Wnt5a and Wnt5b, majorly regulate oriented cell division and assembly of the chondrocytes in the growth plate.

On the other hand, the blockage of Wnt secretion in osteoblasts resulted in decreased bone mass and shortened hypertrophic zone in the growth plate. The canonical Wnt signaling in the osteoblasts was known to regulate the bone resorption through regulating OPG/RANKL ratio [37]. In our model, the loss of *Wls* in bone also increased the number of TRAP positive cells in the trabecular bone area, accompanied by a decrease of OPG/RANKL ratio (Wan et al., 2012, paper in revised process in Bone). Thus, the increased number of TRAP positive cells in the *Col1-Cre; Wls*$^{c/c}$ was at least partially due to a decrease of OPG/RANKL ratio. The disturbance in the length of hypertrophic zone might result from the enhanced osteoclast/chondroclast activity on cartilage resorption.

Theoretically, loss of *Wls*, which blocks the function of both canonical and non-canonical Wnts, should lead to broader and severer defects than the inactivation of *β-catenin*. However, it is not true in some cases. For instance, we postnatally blocked the chondrocyte-derived Wnts through Tamoxifen induction and observed little alteration in articular cartilage at the age up to 6-month-old (data not shown), at least not as severe as that observed in the mice with postnatal inactivation of *β-catenin* [12]. In addition, inactivation of *β-catenin* in limb mesoderm by *Prx1-Cre* or *Dermo1-Cre* results in much severer defects at limb patterning and joint integrity than the double mutant of *Wnt14/Wnt4* [27,38,39], or the limbs in *Prx1-Cre; Wls*$^{c/c}$ mutant mice [23]. The difference in the severity of phenotypes among these mutants may result from the differential timing and range of Cre-mediated recombination in these knockout mice. It may also come from some additional non-Wnt related signaling molecules that may interact with β-catenin and play important roles in cartilage and bone development. For example, β-catenin interacts with cadherin and plays an important role in cadherin-mediated cell adhesion [18,40]. These observations remind

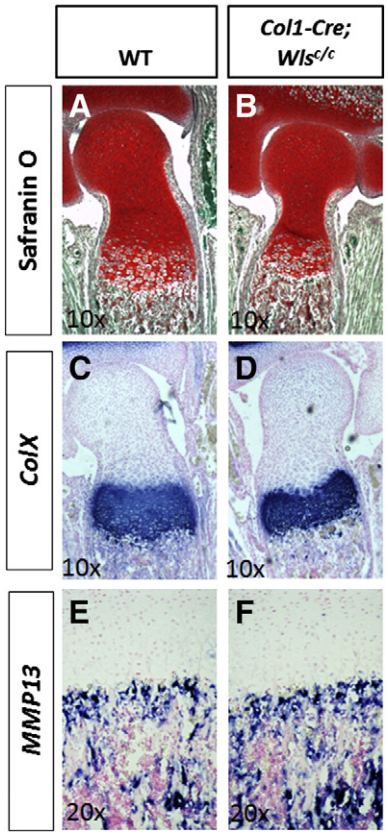

Fig. 8. Terminal chondrocyte differentiation is not significantly accelerated in the *Col1-Cre; Wls$^{c/c}$* mutant mice. A–B. Safranin O staining on sections of humerus at P0. C–D. *ColX* expression in sections of humerus at P0. E–F. *MMP13* expression in sections of humerus at P0.

us to be cautious in evaluating the canonical Wnt signaling activity in some developmental processes based on *β-catenin* manipulation.

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