Introduction. It is well accepted that Wnt/b-catenin signaling plays important roles in skeletal development. Published work demonstrates its critical functions in regulating the balance between osteoblast and chondrocyte differentiation, in supporting chondrocyte proliferation, and in promoting cartilage maturation. In vivo studies have highlighted these important roles for b-catenin signaling but have been limited by the embryonic and peri-natal lethality of existing b-catenin gain- and loss-of-function mouse models (referred to as GOF and LOF models hereafter). An additional limit to these studies has been the lack of cell-specificity in targeting gene deletion. In order to study the specific role of chondrocyte-expressed b-catenin in embryonic and postnatal skeletal development, we used tamoxifen-inducible Col2a1-CreERT2;\(\text{loxP}^{\text{ex3/3}}/\text{wildtype}\) and Col2a1-CreERT2;\(\text{loxP}^{\text{ex3/3}}/\text{null}\) mouse models. Our findings reveal that cartilage-specific b-catenin expression is critical in initiating secondary ossification center (SOC) formation, with specific roles in vascularization signals involved in SOC formation. Furthermore, our findings confirm that chondrocyte-specific b-catenin expression regulates the progression of chondrocyte maturation and endochondral bone development.

Methods. Col2a1-CreERT2;\(\text{loxP}^{\text{ex3/3}}/\text{wildtype}\) mice were bred with b-catenin\(\text{loxP}^{\text{ex3/3}}/\text{wildtype}\) and Col2a1-CreERT2;\(\text{loxP}^{\text{ex3/3}}/\text{null}\) mice were bred with b-catenin\(\text{loxP}^{\text{ex3/3}}/\text{null}\) mice to study b-catenin GOF and LOF, respectively. In order to achieve gene recombination in embryos, pregnant female mice were injected with tamoxifen (TM, 1mg/10g body weight, IP) between 12.5 and 13.5 dpc. To study post-natal animals, lactating female mice were injected with TM at the above dose at P1. Embryos were collected at E14.5, E16.5 and E18.5; pups were sacrificed at P3, P5, P7, and P10. All specimens were skinned, fixed (10% NBF for 24-48h), decalcified as appropriate, and processed for paraffin histology. Sections of forelimbs from mutant animals and their Cre-negative littermates were studied using H&E staining, immunohistochemistry (IHC) for b-catenin and VEGF protein, and radioactive in situ hybridization (ISH) for chondrocyte markers (COL2A1, COL1A2, COL9A1, HYAL2, MMP13, bone markers (BSP, OC, COL), angiogenic markers (VEGF, Angiopoietin), and secondary ossification center markers (CTGF, MMP14). All studies conducted were in compliance with the regulations of the University Committee on Animal Resources at the University of Rochester.

Results. b-catenin Gain of Function: On gross observation, E18.5 b-catenin GOF embryos displayed shorter long bones in the fore- and hind-limbs than their Cre-negative littermates. H&E staining reveals disrupted growth plates with loss of columnar organization of proliferating, pre-hypertrophic, and hypertrophic chondrocytes. There are fewer hypertrophic chondrocytes, which form a conical hypertrophic zone with a corresponding thickened bone collar. Most notably, there is the appearance of an early secondary ossification center with a pool of hypertrophic chondrocytes centered in the proximal humeral epiphysis (fig. 1a., fig. 1b. littermate control). Examination of earlier time points reveals earlier appearance of the primary center of ossification. These observations are supported by ISH findings revealing diffuse chondrocyte maturation markers in the growth plate, enhanced bone marker expression in the bone collar, and hypertrophic chondrocyte (COL5A1 and bone (BSP) markers in the presumed secondary ossification center. ISH for VEGF and CD-31 IHC reveal evidence of early angiogenesis in the humeral epiphyses. b-galactosidase staining of Col2a1-CreERT2;\(\text{loxP}^{\text{ex3/3}}/\text{null}\), R26\(\text{R}\) embryos reveals that only chondrocytes exhibit gene recombination and that the observed phenotype results from chondrocyte-specific activation of b-catenin. b-catenin Loss of Function: As with the GOF embryos, on gross observation, E18.5 b-catenin LOF embryos displayed shorter long bones in the fore- and hind-limbs than their littermate controls. H&E staining of E16.5 humerus reveals lack of a primary ossification center with only a small hypertrophic zone (fig. 2a., fig. 2b. littermate control); however the primary ossification center is present by E18.5, suggesting delayed progression of chondrocyte maturation and endochondral ossification. These findings are supported by ISH findings that reveal decreased chondrocyte maturation and bone markers. The appearance of the secondary ossification center is delayed in postnatal b-catenin LOF animals; no hypertrophic cells are present in the proximal humerus at P5, the normal time of SOC onset in wildtype animals.

Gene recombination in these models occurs at greater than 80% efficiency as revealed by b-galactosidase staining of humerus sections from Col2a1-CreERT2;R26\(\text{R}\) embryos and postnatal pups. Gene recombination in these models occurs at greater than 80% efficiency as revealed by b-galactosidase staining of humerus sections from Col2a1-CreERT2;R26\(\text{R}\) embryos and postnatal pups. Gene recombination in these models occurs at greater than 80% efficiency as revealed by b-galactosidase staining of humerus sections from Col2a1-CreERT2;R26\(\text{R}\) embryos and postnatal pups.

Discussion. The results presented here confirm previous findings from other groups that in vivo, b-catenin GOF and LOF function results in profound cartilage and skeletal defects and in vitro, b-catenin modulates chondrocyte maturation. To add to these findings, these results reveal several novel insights. Use of a tamoxifen inducible model allowed for specific timing of gene recombination; by initiating gene recombination after E12.5, we were able to target b-catenin activation or inactivation specifically in chondrocytes. Before this time point, both chondrocytes and perichondrial cells express the Col2a1 promoter but chondrocytes primarily express this promoter after this time point. Initiating gene recombination after birth allowed us to bypass the embryonic and peri-natal lethality of non-inducible b-catenin GOF/LOF models and to therefore study post-natal animals. The temporal specificity afforded in this model is a capability unavailable in previous models.

An important insight is that b-catenin acts in a cell non-autonomous manner such that chondrocyte-specific expression of b-catenin modulates both cartilage development and bone collar formation. Understanding the source of the b-catenin signal that regulates development can influence future developments to more specifically target cells and signals that influence processes important in situations such as fracture repair. That chondrocyte-specific b-catenin signaling can have profound effects on not only chondrocytes but also on other cell types that communicate via the b-catenin signal. These intercellular signaling pathways must be further studied.

A most intriguing finding of these studies is the influence of b-catenin on the initiation of the secondary ossification center. In humans, SOC formation is important in multiple clinical scenarios. Because the articular cartilage exists on the SOC, it stands to reason that a properly formed SOC is essential for proper articular function and that an improperly formed SOC can result in pathology. In adults, this might translate to osteoarthritis. In children, this translates to epiphyseal dysplasia, developmental dysplasia of the hip, and perhaps even more poorly understood diseases such as Legg Calve Perthes disease.

SOC development is poorly understood but is thought to occur by mechanisms unique from growth plate development. Signals that initiate SOC formation are not fully known although several markers of SOC formation have been identified. Our findings suggest that b-catenin is an important initiator of SOC formation. Past research suggests that vascularization patterns in the SOC are distinct from those in the growth plate such that new vessels may appear before the appearance of hypertrophic chondrocytes rather than afterwards. Our findings suggest that b-catenin may regulate vascularization signals in the epiphysis and chondrocyte hypertrophy signals to form the SOC.

The work presented here fine-tunes our understanding of cell-specific b-catenin signaling in skeletal development and opens new questions as to how different cell types, such as chondrocytes and osteoblasts, might communicate via the b-catenin signal. Importantly, these results shed light on the previously unknown effects of b-catenin on post-natal skeletal development, specifically on secondary ossification center development and vascularization signals involved in SOC formation.