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ABSTRACT

Osterix (*Osx*) is a transcription factor essential for osteoblast differentiation and bone mineralization. Although there are indications that *Osx* also plays a regulatory role in cartilage, this has not been well-studied. The goal of this study was to define the function of *Osx* in the post-natal growth of the secondary cartilage at the mandibular condyle. Conditional *Osx* knockout (cKO) mice that were missing *Osx* only in cartilage were generated by crossing *Osx*-loxP mice to Aggrecan-Cre mice. Cre activity was induced by tamoxifen injection twice a week from day 12 to 1 mo of age, and specimens were collected at 1 and 5 mo of age. At 1 mo of age, the condylar hypertrophic chondrocyte zone in the cKO-mice was > three-fold thicker than that in the age-matched control, with little sign of endochondral bone formation. Immunohistochemistry and analysis of histological data revealed a defect in the coupling of chondrogenesis and osteogenesis in the cKO mice. In five-month-old mice examined to address whether late-stage removal of the Cre-deletion event would alleviate the phenotype, the hypertrophic chondrocyte zone in the cKO condyles was considerably larger than in wild-type mice. There were large discrete areas of calcified cartilage in the hypertrophic zone, few signs of endochondral bone formation, and large regions of disorganized intramembranous bone. Analysis of these data further strengthens the notion that Osterix is essential for the coupling of terminal cartilage differentiation and endochondral ossification in mandibular condylar cartilage.

KEY WORDS: temporomandibular joint, mandibular condyle, fibrocartilage, conditional knockout, cartilage, endochondral ossification.

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Osterix Couples Chondrogenesis and Osteogenesis in Post-natal Condylar Growth

INTRODUCTION

The transcription factor Osterix (*Osx*) has long been known to act downstream of Runx2 as an essential mediator of osteoblast differentiation, such that mice lacking Osterix demonstrate a complete lack of bone formation (Nakashima *et al.*, 2002). However, several lines of evidence suggest that Osterix may also play a regulatory role in cartilage. Two recent studies (Omoteyama and Tagaki, 2010; Park and Kim, 2013) have demonstrated that *Osx* silencing in chondrogenic ATDC5 cells results in down-regulation of markers of chondrogenic differentiation such as Col 2 and Col 10, and that Osterix is up-regulated by BMP-2 in chondrocyte cultures (Yagi *et al.*, 2003). In addition, developing long bones from mice with a chondrocyte-specific *Osx* conditional knockout with Col2a1-Cre exhibit reduced chondrocyte differentiation and endochondral ossification that impairs their skeletal growth (Oh *et al.*, 2012). While this study provides persuasive evidence of the importance of *Osx* as a positive regulator of chondrocyte differentiation in the growth plate of developing long bones, its possible role in cartilage during post-natal growth is largely unknown. Whether this model can be extrapolated to so-called secondary cartilages, in which Osterix is also expressed both pre- and post-natally (Shibata and Yokohama-Tamaki, 2008; Ochiai *et al.*, 2010; Zhang *et al.*, 2013), is similarly unclear. The most well-known and important secondary cartilage is the mandibular condylar cartilage (MCC), which caps the surface of the mandibular condyle and serves as a site of articulation with the skull as well as a locus for endochondral ossification that contributes to growth of the mandible in length and height (Hinton and Carlson, 2005). The MCC develops adjacent to the periosteum of the intramembranous bone of the mandibular ramus from alkaline phosphatase-positive progenitor cells (Shibata and Yokohama-Tamaki, 2008) and grows by proliferation of pre-chondrocytes (rather than chondrocytes as in the growth plate) situated in a perichondrium overlying the cartilage proper (Luder *et al.*, 1988; Mizoguchi *et al.*, 1990).

The goal of this study was to investigate whether Osterix plays a role in the regulation of post-natal MCC growth. To this end, we created a conditional knockout of Osterix in cartilage using Aggrecan-Cre and evaluated the morphology and gene expression in the MCC of cKO mice compared with that in wild-type mice.

MATERIALS & METHODS

Generation of Osterix Conditional Mutant Mice

Osx^{flax/flax} mice were mated with aggrecan -CreER mice, which express CreER under the control of the cartilage-specific aggrecan gene regulatory

sequences (Henry *et al.*, 2009). Conditional *Osx* knockout mice were obtained by crossing homozygous *Osx* floxed mice (*Osx*^{flax/flax}) and *Osx*^{flax/+}; aggrecan -Cre mice. To induce Cre activity, we administered tamoxifen twice a week from day 12 to 1 mo of age, 5 times in total. To detect Cre activity, we crossed aggrecan-CreER mice with ROSA26R mice. The recombination was detected in the perichondrium but was more active in chondrocytes and hypertrophic chondrocytes. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M Baylor College of Dentistry.

Osterix-lacZ Knock-in Mice

Heterozygous Osterix mice (*Osx*^{+/-}), widely used for tracing the *Osx* expression pattern (Nakashima *et al.*, 2002), were kindly provided by Dr. Benoit de Crombrughe. In these six-week-old mice, one *Osx* allele was inactive and replaced by LacZ expressed under the regulatory sequences that normally control the *Osx* gene. The mandible was dissected from the mice and fixed with 4% paraformaldehyde (PFA) for 1 hr with shaking, followed by washing with PBS for 5 min $\times$ 3. Condyles and adjacent bone were dissected free and placed for 24 to 36 hr in X-gal solution until it turned blue at 37° in the dark, then washed with PBS for 5 min $\times$ 3. Specimens were fixed in 4% PFA overnight at 4° and washed again with PBS for 5 min $\times$ 3. Finally, specimens were decalcified, dehydrated, and embedded in paraffin. Sections were cut, stained for β -galactosidase by standard protocols (Madison *et al.*, 2002), and counterstained with nuclear fast red.

Radiography and Micro-computed Tomography (Micro-CT)

Mice were sedated with Xylazine/Ketaset injection and sacrificed at 1 or 5 mo of age by cervical dislocation, then fixed in 4% PFA at 4° overnight. Both the wild-type (WT) and *Osx* cKO mandibles were radiographed with a Faxitron model MX-20 System (Faxitron X-Ray LLC, Lincolnshire, IL, USA) and then subjected to micro-computed tomography (μ CT) with a CT40 SCANCO Medical System (Southeastern, PA, USA) with a 36-mm holder at 45 kV of energy, 12- μ m scanning thickness, and medium resolution. Two-dimensional slice images were selected and used to generate three-dimensional reconstructions with the following parameters: filter width sigma = 0.8, support level = 1.0, and threshold = 173. The same values were used to analyze wild-type and mutant samples at each specified time point. Three-dimensional images were rotated at specific angles to generate a lateral view of the mandibles and condyles.

Histology and Histomorphometric Analysis

The mandibular condyles were decalcified, embedded in paraffin, sectioned at 3 to 4 μ m, and stained with hematoxylin and eosin (H&E), Toluidine blue, and Safranin O. For immunohistochemical analyses, the following antibodies were used: anti-Osterix (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:400), rabbit anti-aggrecan antibody (a kind gift from Dr. Koji Kimata, Institute for Molecular Science of Medicine, Aichi Medical University, Japan; 1:200), rabbit anti-vascular endothelial growth factor (VEGF) polyclonal antibody (Abcam,

Cambridge, MA, USA; 1:1,000), rabbit anti-PCNA polyclonal antibody (Abcam; 1:100), rabbit anti-collagen II monoclonal antibody (Santa Cruz Biotechnology; 1:50), rabbit anti-collagen X monoclonal antibody (Santa Cruz Biotechnology; 1:25), rabbit anti-SOX9 polyclonal antibody (Santa Cruz Biotechnology; 1:100), or rabbit anti-osteopontin polyclonal antibody (Santa Cruz Biotechnology; 1:100). All immunohistochemistry experiments were detected with a 3,3'-diaminobenzidine kit (Vector Laboratories, Burlingame, CA, USA) according to the instructions of the manufacturer. Bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO, USA; 10 μ L/g body wt) was injected into the mice 2 times; the second injection was 24 hr later than the first, and the mice were sacrificed 2 hr after the second injection. Histomorphometric analysis was performed (4 samples in each group) by Image J software (NIH, Bethesda, MD, USA).

Back-scattered SEM and Acid-etch SEM

To acquire images of hypertrophic chondrocytes and bone cells, we performed scanning electron microscopy (SEM) of resin-cast bone samples. Bone tissues were fixed in 70% ethanol and embedded in methyl methacrylate (MMA) (Buehler, Lake Bluff, IL, USA). The surface of the MMA-embedded bone was polished, then acid-etched with 37% phosphoric acid for 2 to 10 sec, 5% sodium hypochlorite for 5 min, and coated with gold and palladium. Samples were examined by an FEI/Philips XL30 field emission environmental scanning electron microscope (Phillips, Hillsboro, OR, USA). For back-scattered electron microscopy imaging, we used a method described previously (Feng *et al.*, 2006).

Statistical Analysis

Statistical significance was determined by an independent-sample *t* test with SPSS 12.0. A *p* value of < .05 was considered statistically significant.

RESULTS

Morphological and Molecular Characterization of Post-natal Mandibular Condyles

At 1 mo of age, the condylar cartilage in both WT and knockout mice displayed a polymorphic (pm) or articular/pre-chondroblastic cell layer, a flattened chondrocyte (fc) zone, and a hypertrophic chondrocyte (hc) zone. Use of Osterix reporter mice and X-gal staining showed that Osterix is highly expressed in all different layers of the condyle (Appendix Fig. 1A). To analyze the Aggrecan-cre activity, we carried out X-Gal staining, and the image showed that positive X-gal cells were detected predominantly in the flattened chondrocyte and hypertrophic chondrocyte layers of the MCC but not in the underlying bone (Appendix Fig. 1B). Immunohistochemical staining confirmed that *Osx* was deleted in the knockout mice (Appendix Fig. 1C).

One-month-old Conditional *Osx*-deficient Mice

Micro-CT analysis revealed that the bony condyles in the one-month-old knockout mice were somewhat flattened and the cartilage-bone interface was slightly concave relative to that in

Figure 1. Morphological changes and alterations in cartilage-specific markers in one-month-old Osterix knockout (Ox-cKO) condyles. **(A)** Micro-CT images of the posterior mandibles of wild-type and cKO mice, showing the aberrant morphology of the mandibular condyle in cKO mice. **(B)** Safranin O-staining demonstrated the much greater thickness of the mandibular condylar cartilage (MCC) in cKO mice and the irregular invaginations of the cartilage deep into the subchondral bone in cKO mice. **(C)** Safranin O-staining showed that cartilage residues in the subchondral bone in WT mice are virtually absent in the cKO condyles. **(D-G)** Sox 9, aggrecan, Col II, and Col X immunoreactivity in the MCC were increased in extent in the cKO mice. **(H)** Thickness of the hypertrophic chondrocyte layer was significantly greater in cKO MCC than in WT MCC.

the condyles in WT mice (Fig. 1A). Safranin O staining (Fig. 1B) revealed that the MCC of mutant mice was considerably thickened compared with that of WT mice (Fig. 1C). Moreover, the subchondral bone was much more porous with fewer bony trabeculae in the Osterix-deficient mice compared with that in the WT mice. Multiple invaginations of the hypertrophic chondrocytes into the underlying bone (Fig. 1C) resulted in an extremely irregular cartilage-bone interface that differed considerably from that in the WT. In addition, cartilage residues within the subchondral bone, frequently seen by Safranin O staining in WT mice, were absent in the cKO mice (Fig. 1C). A greater extent of immunoreactivity for Sox9, aggrecan, and type II collagen was evident in mutant mice (Figs. 1D-1F). The extent of immunoreactivity for Col X was particularly expanded in the mutant mice (Fig. 1G), and the hypertrophic layer was more than three-fold thicker than in the WT (Fig. 1H).

The total number of BrdU-labeled cells in the polymorphic layer was significantly greater in the WT MCC than in the cKO MCC (Fig. 2A). However, more BrdU-labeled chondrocytes

were evident in the MCC of cKO mice. Apoptotic cells revealed by TUNEL staining were present in WT mice, mostly in the deeper layers of the cartilage; in the mutant condyles, these were considerably less numerous (Fig. 2B). TRAP stain (Fig. 2C) and immunoreactivity for VEGF (Fig. 2D), confined to the cartilage-bone interface in WT mice, was almost non-existent in mutant MCC. Immunostaining for DMP1 was greatly attenuated in the bone underlying the MCC in mutant mice (Fig. 2E). Moreover, immunoreactivity for MMP13 was strongly reduced in the mutant condyles (Fig. 2F).

Five-month-old Conditional *Ox*-deficient Mice

Both the radiographic and micro-CT images showed that the mandibular condyle is longer and the condylar neck shorter in mutant mice (Fig. 3A). Most strikingly, these radiographic and micro-CT images of the cKO condyles also demonstrate a pronounced radiopaque region separated by a radiolucent gap from the bone of the ramus (Fig. 3A, right). In addition, the cartilaginous

Figure 2. Abnormal expression of non-cartilage markers in one-month-old *Osx*-cKO condyles. **(A)** BrdU staining revealed that cell proliferation was significantly reduced in the polymorphic cell layer of the MCC in cKO mice. **(B)** TUNEL staining indicated no detectable apoptosis in hypertrophic chondrocytes of MCC in mutant mice. **(C-F)** Dramatic reductions in osteoclasts (TRAP), VEGF immunostaining, and MMP-13 immunostaining characterized the MCC in cKO mice.

areas of the MCC were noticeably more cellular than the MCC of WT condyles, and the amount of cartilage at the MCC of mutant mice was several times greater than in WT MCC. Hypertrophic chondrocytes, virtually absent in WT condyles, were noticeable in cKO mice, and clustering of chondrocytes appeared more frequently in the mutant MCC (Fig. 3B). Despite the overall larger size of the condyle, the mutant mandibles were significantly ($p < .05$) reduced in length relative to the WT mandibles (Fig. 3C, right).

Staining with Goldner's trichrome indicated that substantial areas of the MCC stained like bone, not cartilage (Fig. 3D). Histological examination showed that the radiolucent "gap" reflected large discrete areas of cartilage that were positive for von Kossa staining, indicating calcification (Fig. 3D). Non-mineralized cartilage was present superficial to these calcified areas as well as deep to them, accounting for the "gap" seen in the radiographic and micro-CT images. This appearance of the condyle was characteristic of all five-month-old condyles examined. Back-scattered SEM and acid-etch SEM showed that the calcified areas in the mutant MCC contained hypertrophic chondrocytes (Fig. 3E). In these large areas of the mutant MCC that were identified as calcified by von Kossa and Goldner staining, Col II immunoreactivity was also present (not shown), and the cells resembled chondrocytes. Moreover, immunoreactivity for osteopontin was pronounced in this area and in the bone, whereas it was not present in the uncalcified margins of the mutant MCC or in the uncalcified cartilage of the WT animals (Fig. 3F). In WT mice, osteopontin immunoreactivity was

confined to the deepest 2 to 3 cell layers of the cartilage and to the underlying bone.

BrdU incorporation was evident in a few cells within the WT MCC, but no labeled cells were apparent in the mutant mice (Fig. 4A). TUNEL immunostaining was evident in several cells in the MCC of WT mice, but was reduced in mutant MCC (Fig. 4B). Immunoreactivity for Sox9 was present in more cells in the mutant MCC (Fig. 4C, right) compared with the wild-type MCC. Immunoreactivity for aggrecan and Col II was also abundant in the MCC of mutant condyles, but there were regions, sometimes coinciding with the calcified areas, in which this immunoreactivity was minimal or absent (Figs. 4D, 4E, right side). Col X immunostaining was minimal in WT condyles; while scattered in the knockout mice, it was noticeable in the pericellular matrix around several clusters of chondrocytes (Fig. 4F).

DISCUSSION

Only about 2 wk after Osterix expression was knocked out by tamoxifen injection, the MCC in the one-month-old cKO mice was noticeably thickened, primarily due to increased thickness of the Col X-immunoreactive part of the cartilage (Fig. 1G). A more modest decrease in proliferation (BrdU-labeled polymorphic cells) was also observed in cKO mice, coupled with an increase in BrdU-labeled chondrocytes. The increased number of BrdU-positive chondrocytes in the mutant condyle may result from an enhanced rate of differentiation from the polymorphic layer. However, it should be recalled that the primary locus of

Figure 3. Large calcified cartilage masses in five-month-old *Osx*-cKO condyles. **(A)** Radiographic and μ -CT images showed that the surface of the calcified region in the mandibular condyle of cKO mice was separated from the underlying bone of the condylar neck by a radiolucent gap. **(B)** (upper images) Discrete areas of safranin-O-stained cartilage were present in the MCC of cKO mice, separated by stained regions similar to those of the underlying bone; (lower images) hypertrophic chondrocytes were more numerous in cKO condyles and sometimes clustered. **(C)** Overall mandibular length (posterior condylar border to distal incisal alveolus) was significantly reduced in cKO mice. **(D)** Goldner and von Kossa staining showed large calcified areas within the MCC in cKO mice. **(E)** Back-scattered SEM and acid-etch SEM indicated that the calcified areas in the mutant mice MCC were composed of hypertrophic chondrocytes. **(F)** Osteopontin immunoreactivity, present only in the subchondral bone and deepest layers of the MCC in the WT mice (left), was also strongly expressed in the calcified areas within the MCC in cKO mice (right). C Cartilage, calcified cartilage; Hp, hypertrophic chondrocyte.

our transgene activity was in the hypertrophic region of the MCC. In addition, indicators of cell death (TUNEL), angiogenic invasion (VEGF), and osteoclast activity (TRAP) at the cartilage-bone interface were markedly reduced in mutant condyles, and no Safranin-O-stained cartilage “remnants” were present in the bony trabeculae underlying the mutant MCC. Taken together, these observations indicate a cessation of replacement of hypertrophic chondrocytes by newly formed bone, leading to a

complete blockage of endochondral bone formation at the cartilage-bone interface. Although the MCC was not described, these conclusions are similar to those seen pre-natally in the growth plate from mice with a conditional knockout of Osterix with a Col 2a1-Cre (Oh *et al.*, 2012). The shutdown of endochondral ossification may be partly explained by evidence that Osterix directly regulates VEGF activity (Tang *et al.*, 2012). Osterix has also been shown to regulate MMP-13 produced by

Figure 4. Patterns of expression of cartilage-specific markers in five-month-old *Osx*-cKO condyles. **(A)** BrdU incorporation was present in a few cells in the polymorphic zone of WT MCC, but no BrdU-labeled cells were evident in mutant MCC. **(B)** TUNEL staining revealed a dramatic reduction in apoptosis in the MCC of cKO mice. **(C-E)** Immunoreactivity for Sox9, aggrecan, and Col II was widespread in the cKO MCC, although there were discrete areas deficient in aggrecan and Col II staining interspersed among the immunopositive matrix. **(F)** Immunostaining for Col X, virtually absent in WT MCC, was still apparent in the MCC of cKO mice and was often evident in the pericellular matrix adjacent to clusters of hypertrophic chondrocytes (arrows in lower image). **(G)** Cartoon illustrating the proposed effect of Osterix deficiency on the replacement of hypertrophic chondrocytes by endochondral bone. In the WT mice, hypertrophic chondrocytes in the deeper layers of the MCC undergo apoptosis and release autocrine factors that promote invasion by blood vessels and osteoclasts, leading to replacement of cartilage by bone via endochondral ossification. In the absence of Osterix, a severe reduction of apoptosis and invasion by blood vessels/osteoclasts leads to a virtual cessation of endochondral bone formation, which is compensated for by an increase in intramembranous bone formation in the subchondral region. This in turn results in a thickening of the hypertrophic chondrocyte layer and the MCC overall, as well as large areas of calcified cartilage that do not progress to bone formation.

hypertrophic chondrocytes (Behonick *et al.*, 2007), although other MMPs such as MMP9 are unaffected (Zhou *et al.*, 2010). In fact, the phenotype of the one-month-old mutant mice in our study has numerous similarities to that of MMP-null mice (Inada *et al.*, 2004).

The phenotype in the MCC of Osterix-null mice at 5 mo of age is considerably more dramatic: a mutant condylar cartilage that is more than 3 times larger than the WT MCC, but which encompasses large calcified areas within its borders. In light of the accumulation of hypertrophic chondrocytes and the cessation of endochondral ossification observed in the one-month-old *Osx* mutant mice, the persistence of augmented cartilaginous tissue at the head of the condyle indicates a critical role for *Osx*. Indeed, with its continued presence of hypertrophic chondrocytes, metachromasia, and isolated patches of Col X immunoreactivity, the mutant cartilage has more characteristics of a still-active chondrogenic phenotype than the WT. Because these chondrocytes cannot be transformed into bone by the usual endochondral process, the cartilaginous mass persists, and loss of normal bone formation in the condyle appears to be compensated for in part by intramembranous ossification (Fig. 4G). How and why this compensation occurs will be a future study direction.

The large masses of tissue within the cartilage appear calcified based on their positive von Kossa staining, and stain like bone with Goldner's Trichrome. The cells within these calcified areas resemble chondrocytes (Fig. 3D), and the matrix in this tissue displays immunoreactivity for Col II, suggesting that it represents calcified cartilage. It is interesting that Osterix inactivation with CAG-CreER (a recombinase specific to osteoblasts that was also weakly expressed in hypertrophic chondrocytes) demonstrated a very large accumulation of unresorbed calcified cartilage extending into the diaphysis inferior to the growth plate in six-week-old mice (Zhou *et al.*, 2010). Zhou and colleagues concluded that, in addition to its regulation of osteoblast differentiation and bone formation, Osterix was critical for cartilage resorption. Our results in five-month-old mice support this contention, although in our model the areas of calcified cartilage remained surrounded by unmineralized cartilage.

The marked immunoreactivity for osteopontin in the calcified areas was interesting. Osteopontin, which is found pericellularly in cartilage (Parikh *et al.*, 2003) and is increased in osteoarthritic cartilage (Pullig *et al.*, 2000), has been shown to promote mineralization in chondrocyte cultures (Rosenthal *et al.*, 2007). In addition, cartilage explants from osteopontin-deficient mice exhibited up-regulated levels of MMP-13 (Matsui *et al.*, 2009), a proteolytic enzyme important for endochondral ossification that is expressed by hypertrophic chondrocytes and osteoblasts (Behonick *et al.*, 2007). Interestingly, MMP-13 is increased in ATDC5 cells by Osterix over-expression (Nishimura *et al.*, 2012). Thus, it is possible that Osterix and osteopontin may exert reciprocal effects on MMP-13, with the former up-regulating it and the latter down-regulating it. In a model such as ours, in which Osterix is silenced and osteopontin is increased, MMP-13 levels may be too low for endochondral ossification to occur. This could account for the persistence of appreciable areas of unremodeled calcified cartilage within the MCC of five-month-old mutant mice.

To our knowledge, this is the only study to investigate the post-natal effects of Osterix silencing in cartilage *in vivo* and the only study to examine the effect of Osterix deletion on the cartilage at the temporomandibular joint. Although the effects of Osterix on osteoblast differentiation and endochondral bone formation have been well-established, its roles in the regulation of cartilage morphogenesis and growth are just now being examined. Our study demonstrates that the accumulation of hypertrophic chondrocytes and the cessation of endochondral ossification observed in the pre-natal growth plate of *Osx*-null mice continue into post-natal life. However, while the phenotype in our one-month-old mutant mice resembles that observed in the growth plate of *Osx*-null mice pre-natally, the dramatic phenotype in our five-month-old mutant mice does not resemble anything known to us in the literature. The osteopontin-rich masses of calcified cartilage embedded within still-abundant cartilage are perhaps emblematic of the total inability of the mutant MCC to transform cartilage to bone endochondrally, resulting in a significant reduction in overall length of the mandible in five-month-old mutant mice. Consequently, these results clearly demonstrate the importance of Osterix for facilitating the replacement of terminally differentiated chondrocytes by endochondrally formed bone in the condylar cartilage of the temporomandibular joint.

For many years, it was widely assumed that the cartilage at the mandibular condyle followed a cellular and molecular program similar to that in the growth plate and articular cartilage, yet few comparison studies have been performed. In this study, we observed a remarkable phenotype in the condyle of 1.5-month-old cKO mice: a three-fold increase in the hypertrophic chondrocyte zone with a sharp reduction in apoptosis, resulting in a virtual cessation of endochondral bone formation. In five-month-old cKO mice, this phenotype was much more exaggerated, with the inclusion of a large mass of calcified cartilage within the MCC. In contrast, the same cKO mice display a relatively modest change in articular and growth plate cartilage at the same tested ages, and nothing resembling the striking phenotype seen in the five-month-old cKO mice (Appendix Fig. 3). This disparity in the effects of *Osx* during post-natal growth suggests that *OSX* may be more important in the MCC for coupling chondrogenesis and osteogenesis than in limb cartilages. Conversely, there must be other molecules that are more important in the growth plate and articular cartilage but less critical in condylar formation.

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