THE LONG-TERM EFFECTS OF UNLOADING ON THE TRANSDIFFERENTIATION OF CONDYLAR CHONDROCYTES

A Thesis

by

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ABSTRACT

Background:

The role of chondrocyte transdifferentiation into bone cells during postnatal condylar growth remains unknown.

Purpose:

To quantify the long-term effects of masticatory forces on condylar chondrocyte transdifferentiation.

Research Design:

Chondrocyte tracing was performed in 12 Aggrecan-Cre^{ERT2};R26R^{TdTomato};2.3Col1a1-GFP and 12 Col10a1-Cre; R26R^{TdTomato};2.3Col1a1-GFP mice. After Tamoxifen injections at 3 weeks, they were randomly divided into soft and hard food diet groups and followed for 6 weeks. 2D and 3D μCT, H&E staining, cell proliferation, immunostaining for chondrogenic markers, and cell lineage tracing analyses were performed.

Results:

Mice on soft diet showed significantly ($p<.05$) smaller mandibular length, less chondrocyte proliferation and maturation, and fewer chondrocyte-derived bone cells in the condylar head (30% less) and mid-condylar process (28% less).

Conclusions:

Masticatory function affects condylar chondrogenesis and chondrocyte transdifferentiation, which may partially explain differences in postnatal condylar growth.

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NOMENCLATURE

Co	Condylion
Col1a1	Alpha chain of type I collagen
Col10a1	Alpha chain of type X collagen
Col II	Type II collagen
Cre	Recombinase enzyme
DAPI	4′,6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2´-deoxyuridine
GFP	Green fluorescent protein
Go	Gonion
GoT	Gonial tangent
Ic	Deepest point in notch between condylar head and coronoid process
Me	Menton
OCT	Optimal cutting temperature
Rd	Ramus depth
Sox9	Transcription factor of the SRY-related HMG box gene 9
TMJ	Temporomandibular joint
R26R	Rosa 26 Receptor
µCT	Microcomputed tomography

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1. LITERATURE REVIEW

Importance of orthopedics

Classification of a patient's mandibular morphology and direction of growth are of paramount importance in orthodontic treatment planning. Furthermore, influencing these two aspects to produce a functional and esthetic treatment result is often a treatment objective in a growing adolescent patient. Understanding the etiology of varying mandibular morphologies and more importantly how to achieve a desired orthopedic effect enables patient specific treatment, and it paves the way for future orthodontic research in improved treatment methods.

The possibility of orthopedic intervention has not always been accepted in the orthodontic community. In the early 1900s in response to discoveries by Mendel, it was believed that malocclusion was the result of inheritance, and that orthodontists had to adapt the dentition to the existing facial structures.[1; 2] However, in the 1980s that philosophy lost support, as studies on identical twins and siblings highlighted the adaptability of the jaws and heredity failed to explain most variation in malocclusions.[1; 2]

In light of this new evidence, orthopedic intervention and use of orthopedic appliances became widespread in orthodontic treatment for all malocclusion types, and research supports these appliances ability to affect condylar growth. For example, in class II malocclusions, popular functional appliances, such as the Herbst, Bionator, and Twin Block, are used and result in a significant increase in mandibular length by redirecting condylar growth in a more posterior direction.[3; 4] Studies on use of posterior intrusion appliances, such as high pull headgear or miniscrews, illustrate the ability to redirect condylar growth in a more anterior direction which

is an effective treatment in hyperdivergent class II malocclusions.[5] Use of chincup therapy in class III patients has been shown to cause significant decreases in mandibular gonial angle and mandibular length compared to controls.[6]

With the growing acceptance of the mutability of the mandible, orthodontic research has prioritized identifying how the mandible adapts to environmental changes. The macroscopic changes of the mandible have been well documented; however, how cellular changes translate to these macroscopic changes have yet to be answered. This question is essential if orthopedic intervention and its effects are to become more precise and intentional. To answer this, a clear understanding of why the mandible is adaptive to its environment must be addressed.

Mutability of the mandible

The possible sources of mandibular morphology are genetic control, epigenetic control, environmental control, or contributions from all. There is a normal distribution of phenotypes for most craniofacial traits suggesting that these features are polygenetic and have environmental influences.[7] The environment can influence the amount of contribution that these genes have on the phenotypic expression of the mandible. This concept is referred to as the norm of reaction. This phenomenon applies to the mandible and states that the same genotype can produce a variety of phenotypes across a range of environmental circumstances.[7] Orthopedics is possible because mandibular morphology is not genetically determined and abides by the norm of reaction.[7]

Features of the craniofacial complex that show greater variation either mature more slowly or are under less genetic control.[7] The mandible is the least mature bone of the craniofacial complex and is therefore readily influenced by environmental factors.[8] Mandibular modeling and the resulting morphology is hypothesized to be influenced by epigenetic factors such as mandibular posture, masticatory function, airway, or muscular strength during growth. This is evident when comparing modern day Finns with Finns from 15th and 16th centuries. This lapse in time is too quick for genetic changes to occur, yet there are marked differences in the craniofacial complex of these two samples, including larger gonial and mandibular plane angles in the modern day sample.[9] Therefore, these differences are attributed to environmental changes, particularly softer diets, since these two samples have the same genotype.[9] Decreases in masticatory strength due to softer diets has been suggested as a major culprit behind phenotypic mandibular variability, along with airway interferences and habits.[10]

The role of masticatory forces in altering malocclusion and the growth of the mandible

Historical Role

Historically, reduced masticatory forces are largely attributed to the increase in malocclusion and hyperdivergent patterns of the mandible over time. Anthropological studies support that malocclusion has increased in modern day with the advent of a more processed diet, and that malocclusion is reduced under more primitive conditions where masticatory activity is higher.[11; 12] Corruccini has compared the occlusion of many different samples all over the world, including rural vs urban India, rural vs urban Kentucky, and the occlusion of Chinese immigrants versus that of their children raised in the United Kingdom, among several others.[11]

His findings illustrate that malocclusion according to treatment priority index is significantly worse for the group consuming the modern diet. Similarly, malocclusion in the modern-day Finnish sample, including crossbites, deep bites, crowding, and buccal segments, has increased compared to the 15th and 16th century Finnish skulls.[13]

Significant findings also exist for mandibular morphology changes when comparing primitive versus modern samples. Fukase and Suma compared the craniofacial skeleton of the Jomon population against the modern Japanese population, and their findings illustrate differences between the two.[14] The Jomon population, who were hunter-gather fishers in Japan in 10,000-500 BC, had significantly larger bigonial breadth and ramus height and significantly smaller symphysis height and mandibular plane angle compared to the modern day Japanese population. Furthermore, this population had greater bone density and cortical thickness within the mandible. Analysis of Finnish skull samples compared to modern day Finns reveals similar skeletal changes. Modern day Finns have larger mandibular plane angles, larger gonial angles, smaller posterior face heights, smaller ramus heights, and narrower jaws compared to skull samples from 16th and 17th century Finns.[9; 15] Varrela attributes these differences to softer foods in the present-day diet, and hypothesizes that masticatory stress regulates the growth and shape of the mandible.

Muscle weakening related to craniofacial morphology

Hyperdivergent features have been directly related to weaker masticatory muscles. Studies assessing this relationship show that reduced muscle size, EMG activity, muscle efficiency, and bite force are related to greater skeletal hyperdivergence.[16; 17] Furthermore,

cross sectional areas of muscles of mastication are significantly smaller in hyperdivergent individuals compared to controls.[18]

This relationship between muscle function and hyperdivergence is clearly visible when assessing patients with muscular dystrophy, muscular atrophy, and Dechenne syndrome.[7] Individuals with these muscular defects develop craniofacial morphologic features consistent with the hyperdivergent phenotype: increased mandibular plane angles, gonial angles, and anterior face heights.[19-22]

Conversely, when exercises to strengthen the masticatory muscles are completed, the mandible adapts and illustrates phenotypic changes opposite those of hyperdivergent subjects. Spyropoulous illustrated that hyperdivergent patients who completed chewing exercises experienced greater forward rotation of the mandible compared to untreated hyperdivergent subjects.[23]

Animal Studies

Many animal studies have been done to assess the differences in craniofacial growth between animals fed soft versus hard food. Rat studies using differential diet consumption show decreased bone density and decreased condylar process length, width, and area in soft diet compared to hard diet fed mice[24], as well as decreased ramus height and mandibular length with soft diet.[25] Mice studies have similar results, illustrating decreases in condylar width and length in soft diet fed mice[24], as well as decreases in mandibular length, ramus height, and bone volume.[26]

Biologic basis is altered mandibular posture and position

When the mandible's biomechanical environment is altered through position or postural changes, it triggers changes in the neuromuscular activity of the orofacial muscles.[27] This explains the adaptive response of the mandible in various scenarios, such as muscle weakening, airway interferences, or habits.[7] Regardless of the etiology, the mandible is able to adapt to its environmental condition through modeling and growth changes. The mechanostat model helps explain how changes in neuromuscular activity bring about skeletal adaptions.

Mechanostat model

The mechanostat model explains the adaptability of bone in response to mechanical stresses. Bone is adaptive to loading, which regulates its structure according to the mechanical environment that it is placed.[28] Mechanical stress is introduced to bones by muscular contraction, impact loading, and gravitational forces; this stress causes mechanical strain in the bone which guides formation, regeneration, or degradation.[29-32] The components that make up the strain include magnitude, rate, frequency, distribution, number of cycles, and recovery periods. These factors all contribute to the osteogenic effect on bone.

The amount of strain applied to bone from muscular contractions or gravitational load is the most critical feature causing bone adaptation. Furthermore, muscle is the primary delivery of mechanical load to bone and the two have a synergistic relationship.[28] If these muscular contractions increase such that the strain magnitude sits higher than the minimum effective strain, then bone formation occurs to increase bone strength by adding mass and increasing cross section area. Whereas if muscular contractions decrease to below the minimum effective

strain, then mechanical degradation occurs to eliminate unnecessary mass. Essentially, bone modifies in order to meet its mechanical demands.[33-35] Strain frequency, rate, and distribution are also essential components that must be considered in the mechanostat model for determining bone adaptation.

Therefore, as the position or posture of the mandible is changed, whether by muscles or appliances, there is an introduction of mechanical signaling which alters its biochemical environment. The mechanosensory cells in the bone perceive this change as stress and respond by changing condylar growth or mandibular modeling. This entire process is termed mechanotransduction, which is the driving principle for mandibular adaptability.[33]

Condylar adaptation

While the entire mandible is affected by loading changes, most attention has been focused on the condyle and condylar adaptations. Changing the position of the condyle within the glenoid fossa and altering the masticatory muscle load through diet modification have been completed in many experimental studies and illustrate the adaptive potential of the condyle. These studies document the structural and condylar cartilage adaptations that occur in response to altered loading.

Positional changes

McNamara and Carlson in 1979 were among the first to illustrate condylar adaptation to a change in mandibular position. The authors designed a mandibular functional protrusion appliance that displaced the lower jaw of rhesus monkeys anteriorly and inferiorly. They found that in the posterior aspect of the condyle, the cartilage thickened in the experimental group

and more bone was deposited at the posterior ramus.[27] However, the authors could not conclude if mandibular length can be increased beyond what would have been expected without manipulation.[27] Similar results are seen in rat studies. Protrusion of rat mandibles compared to controls results in increase in condylar cartilage thickness and increase in chondrogenic activity within the condyle.[36; 37] When the mandibles are posteriorly displaced in rats, opposite condylar effects are seen, including a reduction in proliferative cells, cartilage width, and a flattening of the posterior condylar region.[38]

Many human studies have also looked at the effects of functional protrusion appliances. Aruajo et al. analyzed the treatment effects of bionator appliances, and illustrated that bionator use resulted in increased posterior drift of bone in the gonial region, posteriorly directed condylar growth, and less mandibular forward rotation than controls.[3] These results are consistent with other studies exploring effects of protrusion appliances, such as the Twin Block and Herbst. These studies support that condylar growth is redirected more posteriorly in response to the protrusion appliances[39]; however, it is unproven that condylar growth is "stimulated".[4]

Altered masticatory muscle load

Another way of altering the mandibular environment includes targeting muscular strength and loading, and one of the most popular experimental methods for targeting muscle function is via diet modification. The experimental literature shows a clear association between diet and condylar growth. Unfortunately, experimental results on this topic for human studies are limited, since controlling for food consistency in humans is near impossible. Animal studies, particularly rats and mice, have offered a good way of studying the effect of masticatory

loading on craniofacial morphology in a much more controlled way. Among the effects that muscular loading has on the condyle include changes to morphology, bone quality, and condylar cartilage. A systematic review and meta-analysis compiled by Scheidegger et al. summarizes the literature findings for rodents fed soft diets versus hard diets.[24]

Changes in morphology include reduction in condylar process length (14 studies), width (9 studies), area (2 studies), and ramus height (3 studies)[25; 26; 40] for soft diet fed rodents compared to hard diet fed rodents.[24] Micro CT imaging and histomorphometric analysis is used to assess differences in bone quality within the condylar process. The literature supports that for soft diet fed rodents, there is a decrease in bone mass/density (6 studies), degree of mineralization (6 studies), quantity/volume of subchondral bone (4 studies), and density and size of trabeculae (4 studies) in the condylar process.[24] There is also ample literature illustrating condylar cartilage's response to altered functional loading. The literature supports that all cartilage zones (articular, proliferative, chondroblastic, and hypertrophic) are affected in response to altered diet consistency (13 studies). Overall, the soft diet fed rodents show thinner cartilage layers, and the hard diet rodents show a greater depth of condylar cartilage (13 studies).[24]

Secondary cartilage

The literature clearly illustrates the adaptability of the mandibular condyle in response to altered environments. The condyle is responsive and adaptive because it is composed of secondary cartilage at its superior surface, the growth of which is associated with functional alterations, such as masticatory stress.

Secondary cartilage at the mandibular condyle functions in adaptive growth, endochondral bone formation, and joint articulation.[41] This type of cartilage differs from primary cartilage, in that it requires mechanical loading for its maintenance and occurs at articulations, sutures, muscle attachments, and fracture repair.[42] Primary cartilage is part of the cartilaginous skeleton, grows intrinsically, and serves as a scaffold for osteogenesis; whereas, secondary cartilage appears later in development at the margins of membranous bones and is reactive to stimuli.[41]

Histologically, the condylar cartilage is categorized into four layers. The most superior layer of the condylar cartilage is a protective fibrous layer. The proliferative layer is just inferior and consists of progenitor cells undergoing mitosis to supply cells for the lower layers. The next layer is the chondrocytic layer, which contains chondrocytes that deposit cartilage matrix. This layer has chondrocytes at various stages of maturity. The deepest layer is the hypertrophic cell layer where the cells enlarge and are surrounded by cartilaginous matrix.[43]

Development of the mandibular condyle

The mandibular condyle begins formation during the 7th week in utero with condensation of mesenchymal cells to form the condylar blastema.[44] Endochondral bone growth begins at the condylar blastema which continues vertically and posteriorly to form the mandibular condyle.[45]

In endochondral bone formation, mesenchymal cells proliferate and differentiate into chondroblasts on the surface of the condyle. These chondroblasts form hyaline cartilage which entraps the chondroblasts, thus forming chondrocytes, which hypertrophy as the cartilage

grows. It is traditionally thought that the hypertrophic chondrocytes apoptose in response to inability to get nutrients. Following apoptosis, osteoclasts from the bone marrow remove the calcified cartilage, initiating angiogenesis during which osteoprogenitor cells migrate in from the bone marrow and differentiate into osteoblasts to deposit bone to replace the cartilage.[46]

Recent evidence has supported that the traditional model for endochondral bone formation has a deficiency regarding the fate of hypertrophic chondrocytes in the condylar cartilage. New evidence supports that not all hypertrophic chondrocytes undergo apoptosis prior to bone formation as traditionally thought. Though apoptosis of hypertrophic chondrocytes is supported, the extent to which this occurs is debated.[47] The question of whether all hypertrophic chondrocytes undergo apoptosis or instead become osteoprogenitor cells goes back to the late 1800s.[48; 49] Several groups have explored this topic since the question was first proposed. Using autoradiography in 1967, Crelin and Koch demonstrated that hypertrophic chondrocytes within the interpubic symphyseal cartilage of mice fetuses transformed into osteoblasts.[50] Use of experimental cell lineage tracing has enabled researchers to explore this question further. Multiple groups have utilized fluorescent markers to trace the fate of chondrocytes in vivo, and the results support that between 60-70% of osteoblasts in the metaphysis of fetal and postnatal long bones are chondrocyte derived.[51; 52] This technique is not limited to long bones studies; it has also been applied to trace the fate of hypertrophic chondrocytes within the mandibular condylar cartilage. Jing et al. demonstrated through cell lineage tracing that up to 80% of bone cells within the superior subchondral bone during prenatal and early postnatal development are chondrocyte derived.[53] These studies

support the hypothesis that chondrocytes within the condylar cartilage contribute to the osteogenic lineage.

Cell lineage tracing

Genetic cell lineage tracing overview

Cell lineage tracing has enabled scientists to track the fate of cells and their progeny in vivo. Fluorescent reporters are commonly used in genetic cell lineage tracing experiments; if a cell with the fluorescent reporter divides, that reporter is passed on to all its progeny, enabling the researcher to efficiently and accurately trace the cell lineage.[54]

The Cre-loxP system is a commonly used means for genetic lineage tracing in the mouse model. It is created using two mouse lines. One mouse line has the tissue or cell specific promoter for the expression of Cre, a recombinase enzyme that recognizes paired loxP sites allowing for site specific recombination. The second mouse line has a genome in which two loxP sequences surround a STOP sequence that is upstream to a fluorescent reporter. The two mouse lines are bred together. In the specific cell type where Cre is active, it excises the STOP sequence between two loxP sites, thus activating a fluorescent reporter downstream. Because excision of this STOP sequence is permanent, all progeny of this cell will also express the reporter. A useful feature of the Cre-loxP system is that activation of the fluorescent transgene reporter can occur constitutively or conditionally with temporal control. If the Cre is fused to a modified form of the estrogen receptor (CreERT2), the investigator can inject tamoxifen into the mouse at a desired timepoint, causing the CreERT2 to go into the nucleus and trigger the

recombination events, thus controlling the activation of the reporter to only after this timepoint.[54; 55]

Chondrocyte cell lineage tracing in the condyle

In 2014, multiple groups utilized cell lineage tracing to show that chondrocytes can undergo cell transdifferentiation into bone cells in endochondral bone formation.[51; 52] This work illustrated that about 60% of osteoblasts in the long bones are chondrocyte derived. However, Jing et al. sought to demonstrate that this phenomenon was true in the mandibular condyle as well.

Jing et. al in 2015 utilized compound transgenic mice to trace the fate of chondrocytes in the mandibular condyle prenatally and early postnatally.[53] One mouse strain traced the fate of hypertrophic chondrocytes from embryo, Col10a1-Cre; $R26R^{TdTomato}$; 2.3Col1a1-GFP, and the other mouse strain traced all chondrocytes early postnatally (induced by tamoxifen), Aggrecan-Cre^{ERT2}; $R26R^{TdTomato}$; 2.3Col1a1-GFP.

The first mouse strain, Col10a1-Cre; $R26R^{TdTomato}$; 2.3Col1a1-GFP, contains three mutations. In cells that contain collagen type 10 (hypertrophic chondrocytes), Cre travels to the nucleus of the cell and cuts out the STOP sequence inhibiting the downstream tomato gene. This then activates the tdTomato gene which produces bright red fluorescence of the Col10 positive cell and all its progeny. The Col10-Cre is activated from embryo age 14.5, so all hypertrophic chondrocytes from this timepoint throughout adulthood will be traced. The 2.3Col1a1-GFP is a genetically modified collagen type 1 that fluoresces green. Osteoblasts and osteocytes contain collagen type 1 and will fluoresce green in this strain of mice. When all of these mutations are bred together, the red and green may superimpose leading to a yellow

fluorescence. A yellow fluorescent cell indicates the presence of both the red tomato reporter (hypertrophic chondrocyte) and green GFP reporter (bone cell), which demonstrates that the transdifferentiation of a chondrocyte into bone cell has occurred. Using this strain, Jing et al. traced the fate of hypertrophic chondrocytes in the mandibular condyle prenatally and counted the number of red, yellow, and green cells to determine the contribution of transdifferentiated chondrocytes to the condylar process. Jing divided the condylar process into 3 vertical levels: superior, middle, and inferior. In the superior level, about 80% of bone cells were either red or yellow, indicating chondrocyte derivation. In the middle portion, this contribution reduced to 70%, and in the inferior portion, about 40% of bone cells were chondrocyte derived.[53]

The second mouse strain, Aggrecan-Cre^{ERT2}; R26R^{TdTomato}; 2.3Col1a1-GFP, also contains three mutations and was used to trace chondrocytes in early postnatal condylar development. This mouse strain is different from the first in that tracing of chondrocytes can be induced by tamoxifen injections. In this strain, Cre is bound to Aggrecan, a chondrocyte specific protein. When tamoxifen is injected into the mouse it interrupts the interaction between Aggrecan and Cre, enabling Cre to enter the nucleus. In the nucleus, Cre cuts out the STOP sequence inhibiting the downstream tomato gene, which then activates the tdTomato gene. This produces bright red fluorescence of the Aggrecan positive chondrocyte and all its progeny following injection. Similar to the first strain, osteoblasts and osteocytes made from development until time of sacrifice will fluoresce green due to the 2.3Col1a1-GFP mutation. When the 3 mutations are superimposed, presence of a yellow cell indicates that a chondrocyte has undergone transdifferentiation into a bone cell during the time between injection of tamoxifen and sacrifice. Jing et al. injected this strain of mice at postnatal 14 days with

tamoxifen to begin the tracing of chondrocytes within the mandibular condyle. Sacrifices were completed at 2, 8, and 14 days following tamoxifen injections. As days post-injection increased, a gradual increase in red bone cells was found in the condylar subchondral bone, supporting the presence of chondrocyte transdifferentiation into bone postnatally.[53]

The work by Jing et al. illustrates that bone in the superior portion of the condylar process is mostly chondrocyte derived, and that transdifferentiated chondrocytes play a critical role in condylar bone formation during natal and early postnatal growth. However, these results do not explain if transdifferentiated chondrocytes have a role in condylar modeling and late postnatal growth. Furthermore, it is unknown if transdifferentiated chondrocytes and their contribution to condylar growth are influenced by external environmental changes to the mandible.

Study Aims

How mechanical strain regulates mandibular condylar growth and remodeling remains unclear. Current research shows that up to 70% of chondrocytes transdifferentiate into bone within the mandibular condyle. Whether this mechanism is responsive to loading of the condyle via mechanical strain has not been established. The primary aim of this research was to explore this question using a mouse model and masticatory force modification (hard vs soft diet), and to quantify the number of transdifferentiated chondrocytes in the condyle in response to altered loading.

2. MATERIALS AND METHODS

Samples

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M College of Dentistry (IACUC 2018-0138-CD, Reference Number 116605). Twenty-four 3-week-old transgenic mice (twelve Aggrecan-Cre^{ERT2}; R26R^{TdTomato}; 2.3Col1a1-GFP and twelve Col10a1-Cre; R26R^{TdTomato}; 2.3Col1a1-GFP) were divided into experimental (soft food diet, n=6) and control (hard food diet, n=6) groups (Figure 1). Three-week-old mice are equivalent to pre-pubescent humans, approximately 6-8 years of age.[56] Aggrecan-Cre^{ERT2}; R26R^{TdTomato}; 2.3Col1a1-GFP genotype traced the fate of chondrocyte cells beginning at post-natal 3 weeks, whereas Col10a1-Cre; R26R^{TdTomato}; 2.3Col1a1-GFP genotype traced the fate of hypertrophic chondrocytes beginning at embryo age 14.5 days. The benefit to using two different genotypes for tracing chondrocyte cells was the ability to verify and confirm the cell lineage tracing results. Sample size was based on published estimates assuming a power of 0.95 and alpha of 0.05, with a 10% difference and effect size of 2.4.[57]

Experimental Procedures

The 12 Aggrecan-Cre^{ERT2}; R26R^{TdTomato}; 2.3Col1a1-GFP mice were anesthetized with a Xylazine/Ketaset combination (Xylazine 1mg/mL; Ketaset 10mg/mL; 30 μL/g) and injected with 75 mg/kg of Tamoxifen intraperitoneally to induce the Cre event as a fluorescent label. The Aggrecan-Cre^{ERT2}; R26R^{TdTomato}; 2.3Col1a1-GFP and 12 Col10a1-Cre; R26R^{TdTomato}; 2.3Col1a1-GFP mice were then randomly divided into soft diet and hard diet fed groups (n=6 per dietary group per transgenic genotype). Soft diet food consisted of prepackaged “DietGel 76A Purified Soft

Diet" mouse food (ClearH_20, Westbrook, ME). The bedding of the soft diet group consisted of soft paper to minimize extraneous chewing. The hard diet consisted of ordinary laboratory hard pellet mouse food (Figure 2). Mice were monitored daily, and food and water were provided ad libitum. Weight of each mouse was measured weekly. At 9 weeks of age (after 6 weeks of dietary modification), all of the mice were injected with EdU (10μg/ml, Invitrogen A10044) and sacrificed three hours later by cervical dislocation.

Tissue Preparation

The mice craniums were fixated using 4% paraformaldehyde for 2 days at 4°C, and the mandibles were dissected to remove most of the muscle tissue.

μCT Analysis to Assess Mandibular Morphology and Bone Quality

The mandibles were separated in half along the mid-symphysis. μCTs (μCT 35 Scanco Medical, Switzerland; 55 kV, 0.8 s, 0.145 mA, 10 um) were taken of one mandibular half for linear and angular measurements. The μCT 3D reconstruction of the hemimandibles were similarly oriented (to visualize all cusps of the first molar equally from the lingual view), and 2D images were captured of that orientation (Figure 3). The 2D images were exported into Image J software, which was used to measure total mandibular length, mandibular base length, ramus height, condylar length, ramus angle, and gonial angle by one examiner according to published protocols (Table 1, Figure 3).[25; 57; 58] Measurements were repeated by the same examiner for reliability.

Separate μCTs, scanning in the sagittal plane, were taken of the hemimandibles to quantify the bone quality of the condylar region superior to the inferior alveolar nerve (55 kV, 0.8 s, 0.145 mA, 10 um) (Figure 4). Tracing was completed by one examiner and repeated for reliability. Trabecular number, trabecular thickness, trabecular spacing, total volume, bone volume, BV/TV, mean/density of TV, and mean/density of BV were estimated using the μCT software.

Histologic and Molecular Analysis

The rami were sectioned from the mandibles and decalcified in a 4°C 10% EDTA bath for 5 days, dehydrated for 1 day in a 4°C 30% sucrose bath, and then embedded in OCT for frozen sectioning. Using a cryosection machine, the samples were sectioned (10 μm thick) along the frontal plane parallel with the length of the ramus. The sections for analysis were selected by one examiner where the mediolateral width of the condyle was largest. A SP5 Leica confocal microscope was used to capture fluorescent images for cell proliferation and cell transdifferentiation analysis. All images were captured at wavelengths ranging from 488 (green)-561 (red) μm. Multiple stacked images were taken at 200Hz and dimension of 1024x1024 using 10x, 20x, or 63X glycerol objective lenses.

The sections were used to assess qualitative changes with H&E and Toluidine blue staining, chondrocyte cell proliferation using EdU staining[59], cartilaginous molecular changes using immunohistochemistry stains[59-61], and cell transdifferentiation using fluorescent imaging.[55] Edu identifies cells actively undergoing DNA replication and mitosis and was selected as the indicator for chondrocyte cell proliferation. Three antibody stains were used, including

Sox9, Aggrecan, and Col10a1. Sox9 is a transcription factor in the nuclei of chondrocytes that is crucial for chondrogenesis, and its expression is characteristic in the chondroblast layer.[61; 62] Aggrecan, the major proteoglycan in articular cartilage (Abcam; 1:400), is a marker for chondrocyte differentiation and is expressed in all cartilage layers.[59; 61] The deepest cartilage zone is the hypertrophic zone, and chondrocytes in this zone are characterized by expression of Col10a1 (collagen type 10a1, Abcam; 1:400).[59; 61; 63]

Cell proliferation for each animal was quantified based on the average Edu^{+}chondrocytes/condylar cartilage area (Figure 5) of 4 contiguous histologic sections. Chondrogenic activity and maturation was quantified based on the ratios $Sox9^{+}Tomato^{+}$(yellow)/$Sox9^{+}$(green) cells in condylar cartilage (Figure 6), $Aggrecan^{+}$area/condylar cartilage area, and $Col10a1^{+}$area/condylar cartilage area using Image J software[59] (Figure 7). This process was repeated by one examiner for reliability.

Chondrocyte cell transdifferentiation was evaluated in the superior and inferior portions of the subchondral bone separately. The inferior portion was 250 microns thick immediately inferior to the widest portion of the condyle as viewed in the frontal orientation (Figure 8). The ratios of (Aggrecan-Cre^{+}2.3Col1a1-GFP^{+} + Aggrecan-Cre^{+}) cells/all subchondral bone cells and (Col10a1-Cre^{+}2.3Col1a1-GFP^{+} + Col10a1-Cre^{+}) cells/all subchondral bone cells in each region were quantified using Image J software[60]. In summary, the number of yellow cells plus red cells divided by all bone cells in each region equals the contribution of chondrocyte derived bone cells in the subchondral bone. The values for each animal were based on the average of 4 contiguous histologic sections.

Statistical Evaluation

Based on the skewness and kurtosis statistics, the data were normally distributed. Parametric means and standard deviations were used to describe central tendency and dispersion. Between-group comparisons were made using independent samples T-test for analyses that controlled for condylar cartilage size. For 2D and 3D μCT, sox9 staining, and cell lineage tracing data, between-group comparisons were made using ANCOVA, controlling for final mouse weight differences.

3. RESULTS

Weight

There were no statistically significant between-group differences in weight prior to diet modification. After one week, the soft food diet mice were significantly lighter (2-3 grams) than the hard food diet mice. This difference was maintained throughout the course of the study (Table 2).

Long-term Soft Diet Demonstrated Shorter Mandibular Morphology Measurements

The ANCOVA results demonstrate that total mandibular length (Co-Me) (p=.004) and mandibular base length (Go-Me) (p=.006) were significantly shorter in the soft food compared to hard food diet group (Table 3). The difference in ramus height (Co-GoT) approached statistical significance, favoring the hard food group. The ramus angle comparison also approached statistical significance, with the soft food diet group angle being more acute. The remaining 2D linear and angular measurements were not statistically different between the two groups.

The 3D μCT results showed no significant differences in the bone quality of the condylar process between groups. Bone volume (p= .113), total volume (p=0.178), and BV/TV (p=.122) of the condylar process approached significance, with the soft diet group being lower for those quantities. Trabecular thickness, trabecular spacing, trabecular number, apparent density, and material density were not significantly different between the two groups (Table 4).

Qualitative assessment of the condylar cartilage using Toluidine blue staining showed a smaller cartilage matrix and a thinner cartilage depth in the soft diet samples compared to the hard diet samples (Figure 9).

In summary, the μCT data demonstrate shorter mandibles among mice fed softer diets.

Long-term Soft Diet Demonstrated Less Chondrocyte Cell Proliferation

The confocal images for EdU staining showed fewer Edu^+ dividing cells in soft diet than hard diet fed mice (Figure 10a). The T-test showed the number of EdU^+ chondrocytes per unit condylar cartilage area was significantly ($p=.017$) lower in the soft diet group (Figure 10b, Table 5). These results demonstrate that cell proliferation within the condylar cartilage was significantly lower in mice eating softer diet.

Long-term Soft Diet Demonstrated Less Expression of Chondrocyte Maturation Markers within the Condylar Cartilage

The expression of $Sox9^+$ (green) and $Sox9^+Tomato^+$ (yellow) within the condylar cartilage was less evident in the soft diet than hard diet samples (Figure 11a). The ANCOVA results show that number of $Sox9^+Tomato^+$ cells ($p<.001$), the number of $Sox9^+$ cells ($p=.012$), and the ratio of $Sox9^+Tomato^+$ to $Sox9^+$ cells ($p=.004$) within the condylar cartilage were all significantly less in the soft diet sample (Figure11b, Table 6).

The confocal images showed that the Aggrecan expression (green) was substantially less evident in the condylar cartilage of the soft diet sample (Figure 12a). The $Aggrecan^+$ area

(p=.002) was significantly smaller, and the ratio of Aggrecan^{+} area to condylar cartilage area (p<.001) was significantly less in the soft diet group (Figure 12b, Table 7).

Similarly, Col10a1 expression (green) was much less evident in the soft diet compared to hard diet sample (Figure 13a). The Col10a1^{+} area (p=.010) and the ratio of Col10a1^{+} area to condylar cartilage area (p=.005) were significantly smaller in the soft diet group (Figure 13b, Table 7).

These results demonstrate that there are fewer mature chondrocytes within the condylar cartilage of mice fed softer diets as shown by less expression of Sox9, Aggrecan, and Col10a1 markers.

Long-term Soft Diet Demonstrated Fewer Transdifferentiated Chondrocytes into Bone Cells within the Subchondral Bone

The fluorescent confocal images of both genotypes, Aggrecan-Cre^{ERT2}; R26R^{TdTomato}; 2.3Col1a1-GFP and Col10a1-Cre; R26R^{TdTomato}; 2.3Col1a1-GFP, illustrated similar qualitative results (Figure 14a, Figure 15a). There were fewer yellow and red cells (transdifferentiated chondrocytes) within the subchondral bone of the soft diet than hard diet condyles. Furthermore, the number of yellow and red cells were greater in the superior than inferior regions of the subchondral bone of both food groups.

The quantitative results confirm the qualitative results. Within the Aggrecan-Cre^{ERT2}; R26R^{TdTomato}; 2.3Col1a1-GFP genotype, the number of Acan-Cre^{+}2.3Col1a1-GFP^{+} cells (p=.036) and total bone cells (p=.025) in the superior subchondral bone region were significantly less in the soft diet than hard diet group (Table 8). However, the number of non-chondrocyte derived

bone cells in the superior region showed no statistically significant between-group difference (p=.645). The ratio of Acan-Cre^{+}2.3Col1a1-GFP^{+} cells to total bone cells in the superior region of the subchondral bone approached statistical significance (p=.104), with the ratio being smaller in the soft diet group.

The trend was similar although less striking within the inferior region of the subchondral bone (Figure 14c, Table 9). The number of Acan-Cre^{+}2.3Col1a1-GFP^{+} cells in the inferior region was smaller and contributed a smaller proportion to the total bone cells compared to in the superior region. Furthermore, the number of Acan-Cre^{+}2.3Col1a1-GFP^{+} cells was significantly (p=.028) less in the soft than hard food group, and the number of total bone cells was smaller in the soft diet group and approached statistical significance (p=.105). The number of non-chondrocyte derived bone cells within the inferior region was not significantly different between groups (p=.807), and the ratio of Acan-Cre^{+}2.3Col1a1-GFP^{+} cells to total bone cells in that region was not statistically significant (p=.226) but suggests the ratio is smaller in the soft diet group.

The quantitative results for cell lineage tracing in the Col10a1-Cre; R26R^{TdTomato}; 2.3Col1a1-GFP genotype were similar to the Aggrecan-Cre^{ERT2}; R26R^{TdTomato}; 2.3Col1a1-GFP genotype. The number of Col10a1-Cre^{+}2.3Col1a1-GFP^{+} cells (p=.002) and the number of total bone cells (p=.001) in the superior subchondral bone region were significantly less in the soft than hard diet condyles (Figure 15b, Table 10), whereas the number of non-chondrocyte derived bone cells was not significantly different (p=.078). The ratio of Col10a1-Cre^{+}2.3Col1a1-GFP^{+} cells to total bone cells within the superior region (p=.814) was also not significantly different between groups.

In the inferior subchondral bone region, there were significantly fewer Col10a1-Cre^{+}2.3Col1a1-GFP^{+} cells ($p=.006$), non-chondrocyte derived bone cells ($p=.032$), and total bone cells ($p<.001$) in the soft diet than hard diet group (Figure 15c, Table 11). The ratio of Col10a1-Cre^{+}2.3Col1a1-GFP^{+} cells to total bone cells in the inferior region was not significantly different between groups ($p=.633$).

In summary, the number of chondrocyte derived bone cells in the condylar process was significantly less in the soft diet than hard diet group, while the number of non-chondrocyte derived bone cells did not differ. Furthermore, the number of total bone cells in the condylar process was significantly less in mice fed the softer diet. The chondrocyte derived bone cells in the superior region of the condylar process make up a greater proportion of total bone cells (65-80%) than in the inferior region (45-70%).

4. DISCUSSION

The hard/soft diet animal model has been widely used to study the effects of altered mechanical strain on the craniofacial complex. It has been shown that there is a direct causal relationship between soft diet consistency and reduced masticatory muscle loading.[24; 64; 65] A variety of animal models have shown that long-term maintenance on a soft diet produces smaller muscle fiber diameters, smaller masticatory muscles, reduced total masticatory muscle weight, reduced masticatory muscle strength, lower duty time of the masseter muscle at high activity levels, and changes in muscle fiber composition.[24; 57; 66-69] Subsequent changes in quality, size, and shape of the bone and cartilage has led to the conclusion that masticatory muscle loading plays a significant role in the development of the craniofacial complex, especially the growth and morphology of the mandibular condyle.[65] Soft diet is accepted as the classic model to mimic the situation of decreased muscle loading to study its effects on craniofacial growth. Though the macroscopic changes that occur in the condyle and condylar cartilage in response to loading are well documented, the microscopic mechanisms responsible remain unanswered. Yet, it is critical to understand these mechanisms if the orthodontic field wants to target and influence condylar growth. Using cell lineage tracing, the present study demonstrated for the first time that reduced muscle loading through soft diet is linked to less chondrocyte derived osteogenesis within the mandibular condyle, with less chondrocyte cell proliferation, maturation, and transdifferentiation into bone cells resulting in smaller mandibles.

It has been established that chondrogenesis within the condylar cartilage is altered in response to muscular loading. The present study showed 47% fewer EdU^+ cells per unit cartilage area among the soft than hard-diet mice, indicating less chondrocyte cell proliferation in the condylar cartilage. These results are consistent with the existing literature. Qualitative differences among rats fed soft diets include significant thinning of all condylar cartilage zones (articular, proliferative, chondrocytic/blastic, and hypertrophic)[57; 70-72] along with a 28% thinner condylar cartilage compared to hard diet fed rats.[70] Using a radiolabeled thymidine proliferative marker, a 48% reduction in condylar chondrocyte cell proliferation has been reported in rats in response to reduced loading through incisor trimming and soft diet.[73] A study utilizing an intraoral 0.5 N spring for forced mouth opening 1 hr/day showed a 65.5% increase in EdU^+ cells in the loaded group compared to an unloaded group that received incisor trimming.[74]

These qualitative and quantitative differences in the condylar cartilage can be attributed to the adaptable nature of secondary cartilage and its relation to functional activity. Secondary cartilage, which is present on the surface of the mandibular condyle, has been shown to respond to altered loading conditions produced by diet modification, paralysis of skeletal muscles[42; 75], and blockage of mechanotransduction signaling molecules,[76] and its induction requires mechanical stimulation, such as compressive loading.[77] The present study supports this quality of secondary cartilage and shows that chondrocyte cell proliferation within the condylar cartilage is significantly downregulated when muscle function is reduced with a softer diet.

In addition to reduced proliferation, the present study demonstrated fewer mature chondrocytes within the condylar cartilage of mice fed soft diets. This was demonstrated by significantly less expression of extracellular chondrogenesis markers Sox9 (50% less), $Aggrecan^+$

per condylar cartilage area (31% less), and Col10a1^{+} per condylar cartilage area (30% less) in the soft diet group. These markers identify molecular changes that chondrocytes undergo during maturation and migration through the cartilage zones. The present study is consistent with the literature regarding loading and expression of chondrogenic markers. In the study utilizing incisor trimming to simulate unloading, there was significantly less expression of Sox9 and Col10a1 compared to the loaded group with a 0.5 N intraoral spring.[74] Using a combination of soft diet and incisor trimming in mice to simulate unloading conditions, significant reductions in extracellular expression of Sox9 (69% less) and Col II (a marker for chondrocyte differentiation similar to Aggrecan) (50% less) have been reported after 4 weeks, which also corresponded to a decrease in mRNA expression of Sox9 (43% less) and Col II (64% less).[62] Together, the results of the present study and others suggest decelerated chondrocyte maturation in response to unloading. Less expression of these markers in the soft diet group demonstrates that masticatory muscle loading is also an essential regulator for chondrocyte maturation and migration of the chondrocytes from the proliferative layer to the hypertrophic layer within the condylar cartilage.

The chondrogenic changes in response to loading have been well documented; however, the literature does not associate these changes with condylar bone changes, as chondrogenesis and osteogenesis were considered two separate processes. Through cell lineage tracing, the present study is the first to explore if and how chondrogenic changes in response to altered loading directly affect osteogenesis. The present study is novel in that no existing literature has evaluated if chondrocyte transdifferentiation into bone cells: 1) is active during late postnatal adolescent growth 2) is regulated by loading and 3) helps explain

osteogenic changes in response to loading. Using cell lineage tracing, it was confirmed that chondrocyte transdifferentiation is an active process during late postnatal growth in older mice, and the number of transdifferentiated chondrocytes changed significantly in response to altered loading. The Aggrecan-Cre^{ERT2} soft diet mice had 30% fewer transdifferentiated chondrocyte cells in the superior region and 27% fewer in the inferior subchondral bone region, and in the Col10a1-Cre genotype, there were 44% and 35% fewer transdifferentiated cells, respectively.

Furthermore, there were fewer total bone cells in the condyles of mice fed soft diets. In the Aggrecan-Cre^{ERT2} genotype, there were 21% and 16% fewer bone cells in the superior and inferior portions, respectively. In the Col10a1-Cre genotype, there were 37% and 36% fewer bone cells in those respective portions. The differences in total bone cells between groups can be attributed to the contribution of transdifferentiated chondrocytes, because the number of non-chondrocyte derived bone cells was not significantly affected. This implies that the chondrocyte derived osteogenesis pathway is responsible for the fewer total bone cells during masticatory muscle unloading. The percent differences in number of transdifferentiated bone cells are very similar to the percent differences in chondrocyte cell proliferation (47% less) and maturation (30-50% less), highlighting the continuous nature of this chondrocyte-to-bone-cell pathway.

Chondrocyte-derived bone cells make up the majority of subchondral bone and are the major cell source of condyles during adolescent development. In the present study, chondrocyte-derived bone cells of mice fed hard diets made up 74-81% of the cells in the superior and 51-66% in the inferior portions of the condylar head. Though no study has

previously quantified the percent contribution in late postnatal condylar modeling, the results of this study align with the findings that hypertrophic chondrocytes contribute ~80% of bone cells in the superior subchondral bone, ~70% in the more inferior region, and ~40% in the most inferior region of the condylar neck for early postnatal condylar development.[53] The smaller percent makeup in the inferior portion of the condylar process can be explained by bone remodeling that is occurring throughout the condylar process. The transdifferentiated bone cells present in the inferior portion are thought to be older and thus more likely to have been replaced by remodeling throughout the course of the study.

The percent of transdifferentiated chondrocytes to total bone cells within the condylar process is also probably less among mice fed soft diets. Numerically, there are approximately 30% fewer chondrocyte derived bone cells within the condylar process of soft than hard diet mice. However, the differences in percent of transdifferentiated chondrocytes to total bone cells between groups were not statistically significant, but there was a clear pattern, and the differences approached the significance level. Within the superior portion, the percent of Acan-Cre^{+}2.3Col1a1-GFP^{+} cells to total bone cells was 74% in the hard diet group versus 64% in the soft diet group. For the Col10a1-Cre genotype, these percents were 81% and 72% respectively. More studies with likely larger sample size are needed to confirm that the rate of chondrocyte cell transdifferentiation decreased with decreased masticatory loading. The present results imply that masticatory unloading leads to less chondrocyte proliferation and maturation within the condylar cartilage, thus creating a smaller pool of chondrocyte cells to undergo transdifferentiation.

If unloading results in significantly fewer transdifferentiated chondrocytes within the subchondral bone, this probably contributes to the observed differences in bone morphology and quality. The present study demonstrated that long-term masticatory force modification with soft diet produces significantly shorter mandibles. The soft diet mandibles were 3.1% shorter in total length and 3.0% shorter in base length. Though not statistically significant, ramus height was 2.8% shorter and ramus angle was 3.1% smaller. Hichijo et al., who used diet modification for 11 weeks in rats, showed 4% shorter mandibular lengths, 4% shorter base lengths, and 13% shorter ramus heights (using different ramus height landmarks compared to present study) in the soft diet group.[25] In a separate study using rats, the same authors reported negligible differences in total length and base length, but a 7% reduction in ramus height for the soft diet group.[57] Compared to rats fed hard pellet diets, those fed a powdered diet for 6 weeks showed shorter (2.1%) mandibular lengths, smaller (3.3%) base lengths, and shorter (8%) ramus heights.[40] Though not evaluated in the present study, studies measuring condylar head dimensions found 5%-18% smaller condylar head lengths and 20%-24% smaller condylar head widths among soft diet fed mice.[26; 66; 70; 72] Percentage discrepancies between studies could be due to the different dietary consistencies used or small sample sizes with increased variability.

The masticatory force modification studies, including the present study, found small but significant changes of 2-8% in mandibular dimensions. Mouse mandible shape and size is expected to reach 95% maturity by age 35 days and 24 days, respectively.[78] This means that 95% of the mouse mandible size reached maturity by the start of the present study and others. Therefore, it is expected to see very small percent differences in mandibular dimensions, as

minimal growth occurs throughout the course of these studies. In the present study, there was not a significant difference in vertical measurements, such as ramus height. It is accepted that mice mandibular growth occurs differently than humans. Mice mandibles grow in a more posterior direction, as opposed to humans whose condyles grow vertically.[78] It is expected that growth amount differences should be expressed in horizontal measurements, as opposed to vertical, as illustrated by the results of this study. This is also supported in the present study by the ramus angle approaching statistical significance with the hard food diet group being more obtuse, indicating posterior growth.

No significant differences in bone quality of the condylar process were observed in the present study; however, multiple values approached significance indicating less bone volume and total volume for the soft diet mice. Soft diet fed mice had condylar processes with 24% less bone volume, 20% less total volume, a 4% smaller BV/TV ratio, 3% less apparent total density, and <1% less material bone density. Kufley et al. used rats and diet modification to look at differences in condylar bone density at various time points. At 20 days of diet modification, there was a significant difference in all the measured values.[79] However, at 40 days of modification (approximately the length of the present study), the soft diet group showed 21% less bone volume, 22% less total volume, 5% less bone density, and 3% less apparent density, none of which were statistically significant. These percent differences approximate the present study. The lack of significant differences could be due to greater variation during highly active growth periods in the animals, whereas at a more mature age, less growth is occurring.[79] Other studies support a significant change in condylar bone quality in response to long-term soft diet: 18% reduction in bone volume after 4 weeks of soft diet[26], 24% reduction in bone volume and

5% reduction in BV/TV at the condyle after 11 weeks with soft diet[25], and 10% reduction in degree of mineralization of condylar trabecular bone after 9 weeks with soft diet.[80] The percent differences in bone quality of the present study are very similar to studies that identify these changes as statistically significant. It is likely that the present study did not have a large enough sample size to reach statistical significance. Another possible explanation for the variability among studies is the difference in μCT tracing location. In the previous studies, the examiners assessed the bone quality of just the condylar head. In the present study, bone tracing was completed from the condylar head inferiorly to the inferior alveolar nerve. It is likely that bone at such an inferior level was already present at the start of the present study and that this tracing parameter is too inferior. However, more studies need to be completed to confirm this assumption.

It has been suggested that increasing bone mass is an adaptation to better withstand higher functional demands, and that a larger condyle in response to higher loads is in agreement with this.[72; 81] The 2D and 3D μCT and histology results in the present study demonstrate more bone formation in the condylar process among mice fed a hard diet. Through use of cell lineage tracing, this study was able to attribute those differences to chondrocyte derived osteogenesis. Therefore, we can assume that chondrocyte derived bone formation is the pathway responsible for adaptions to functional changes.

In summary, this is the first study to explain why muscle activity during adolescent growth is so important for mandibular morphology. Masticatory muscle loading regulates chondrocyte cell proliferation, maturation, and transdifferentiation into bone. Because

transdifferentiated chondrocytes are the major cell source of bone in the condyle, masticatory loading may be used in the future in controlling orthodontic treatments.

Clinical Implications

The hyperdivergent, retrognathic patient is among the most challenging phenotypes to orthodontically treat. This patient has functional concerns, related to weak musculature and poor masticatory performances, esthetic concerns, and this phenotype will not self-correct, and some even worsen over time.[16-18; 82-85] A known etiology for this phenotype is weak muscles. This contributes to altered mandible posture, through posturing down and back, and it affects condylar growth, as demonstrated by the present study. The altered posture then impacts mandibular divergence. This leads to poor muscle biomechanics that is unable to deliver a normal force output, which affects condylar growth and the strength of the muscle itself. This formation of a positive feedback loop could explain why this phenotype commonly worsens with time. Therefore, if this positive feedback loop is to be stopped, then muscle force or biomechanics of the system must be targeted during treatment.

Many treatment options have been proposed, such as Baylor intrusion protocol through use of miniscrews, vertical-pull chin cup, and high-pull headgear, which create a more biomechanically sound system through targeting rotation. In light of the present study, would it be more efficacious during treatment to also target muscle force output? Few clinical studies have explored the effects of targeting muscles in orthodontic therapy for open bite treatment.[23; 86] Spyropoulos et al. compared the treatment effects of a hard chewing gum with that of vertical chip cup only and a combination of vertical chin cup and the hard chewing gum in skeletal open bite patients.[23] Gum chewing was prescribed for at least 45 minutes per day

and vertical pull chin cup for 14 hours per day. The most significant treatment effects were seen in the combination group followed closely behind by the hard chewing gum only group. A similar study looked at the effects of targeting masticatory muscles in long-face children through use of a tough resin chewing material 2 hours per day.[86] After one year, there was a significant increase in bite force and muscle activity of the masseter and temporalis muscles during maximal bite in the subjects (more than expected with growth). Also, considerably greater forward mandibular rotation (2.5 degrees) than expected during normal growth was seen in 9 of 12 cases, with 3.0-3.5 degrees in 5 of the cases. Buschang et al. showed that forward mandibular rotation is highly correlated with amount of condylar growth[87] The present study may help explain why the adolescent patients undergoing masticatory muscle training experienced greater forward mandibular rotation and an improvement in their skeletal open bites. Targeting the chondrocyte derived osteogenesis pathway through masticatory muscle loading opens up therapeutic possibilities, and orthodontists may have the ability to manipulate condylar growth through muscle strengthening.

Regarding condylar growth, there are two likely players: one is the mechanical advantage of the system which is impacted by rotation, and the other is the size or strength of the muscle itself. This may explain the high correlation between amount of condylar growth and mandibular rotation for both hypo and hyperdivergent patients, and it may help explain why hypodivergent patients have greater condylar growth: they have larger muscles and better biomechanics.

Throughout the course of a patient's orthodontic treatment, the entire orofacial system is put through a lot of changes. There's a change in the patient's occlusion and ability to

function, the number of contacts and near contacts are temporarily lowered, the patient is sometimes propped open with bite turbos, there's pain involved, and the patient is told to only eat soft foods. All of these changes lead to lowered masticatory muscle activity. One study actually looked at the change in masseter size over the course of orthodontic treatment and found significant thinning of the masseter muscle.[88] In adult patients or hypodivergent adolescent patients, this is likely not a concern. However, in hyperdivergent retrognathic patients, it may be detrimental to lower the masticatory muscle activity throughout the course of treatment, and it may be beneficial to offset the inevitable change in masticatory muscle function with strengthening exercises or hard gum chewing.

There is ample support that muscle activity during adolescent development is very important for craniofacial morphology.[25] The present study helps illustrate why masticatory loading plays a role in mandibular, particularly condylar development. Chondrocytes are the major cell contributor to condylar bone during adolescent growth and are shown to be regulated by masticatory loading. Therefore, emphasis should be placed on maintaining masticatory muscle loads during adolescent orthodontic treatment.

Limitations

One limitation of this study is that we used a mouse model. Mouse condyles grow in a posterior direction, which is different from humans'; this makes it challenging to relate morphology changes seen in the mice to what would be expected in humans. Also mice have different masticatory patterns, with mostly a vertical chewing pattern and no lateral excusive movements. The next limitation was the sample size. The results produced very comparable percent changes to the literature, yet some of our results were not statistically significant. We

also did not regionally analyze the condyle to see if the changes were localized to one area. However, this offers a potential future research topic worth exploring. Also this study does not directly measure the force on the condylar cartilage, so we do not have any insight into the relationship between direct loading and transdifferentation.

5. CONCLUSIONS

Three-week old mice maintained on soft and hard diets for six weeks demonstrate that long-term maintenance on soft food diet results in:

a. Less chondrocyte cell proliferation within the condylar cartilage;
b. Less chondrocyte maturation within the condylar cartilage;
c. Fewer transdifferentiated chondrocytes and total bone cells within the subchondral bone of the condyle;
d. Shorter mandibular length

REFERENCES

1 Cakan, D. G., Ulkur, F., & Taner, T. U. (2012). The genetic basis of facial skeletal characteristics and its relation with orthodontics. *Eur J Dent*, *6*(3), 340-345. https://www.ncbi.nlm.nih.gov/pubmed/22904665

2 Proffit, W. R., Fields, H.W., & Sarver, D.M. (2013). *Contemporary orthodontics* (Sixth ed.). Elsevier/Mosby.

3 Araujo, A. M., Buschang, P. H., & Melo, A. C. (2004). Adaptive condylar growth and mandibular remodelling changes with bionator therapy--an implant study. *Eur J Orthod*, *26*(5), 515-522. https://doi.org/10.1093/ejo/26.5.515

4 Meikle, M. C. (2007). Remodeling the dentofacial skeleton: the biological basis of orthodontics and dentofacial orthopedics. *J Dent Res*, *86*(1), 12-24. https://doi.org/10.1177/154405910708600103

5 Buschang, P. H., Carrillo, R., & Rossouw, P. E. (2011). Orthopedic correction of growing hyperdivergent, retrognathic patients with miniscrew implants. *J Oral Maxillofac Surg*, *69*(3), 754-762. https://doi.org/10.1016/j.joms.2010.11.013

6 Deguchi, T., Kuroda, T., Hunt, N. P., & Graber, T. M. (1999). Long-term application of chincup force alters the morphology of the dolichofacial Class III mandible. *Am J Orthod Dentofacial Orthop*, *116*(6), 610-615. https://doi.org/10.1016/s0889-5406(99)70194-0

7 Buschang, P. H., Jacob, H., & Carrillo, R. (2013). The Morphological Characteristics, Growth, and Etiology of the Hyperdivergent Phenotype. *Seminars in Orthodontics*, *19*(4), 212-226. https://doi.org/10.1053/j.sodo.2013.07.002

8 Buschang, P. H., Baume, R. M., & Nass, G. G. (1983). A Craniofacial Growth Maturity Gradient for Males and Females between 4 and 16 Years of Age. *American Journal of Physical Anthropology*, *61*(3), 373-381. https://doi.org/DOI 10.1002/ajpa.1330610312

9 Varrela, J. (1990). Effects of attritive diet on craniofacial morphology: a cephalometric analysis of a Finnish skull sample. *Eur J Orthod*, *12*(2), 219-223. https://doi.org/10.1093/ejo/12.2.219

10 Varrela, J., & Alanen, P. (1995). Prevention and early treatment in orthodontics: a perspective. *J Dent Res*, *74*(8), 1436-1438. https://doi.org/10.1177/00220345950740080101

11 Corruccini, R. S. (1984). An epidemiologic transition in dental occlusion in world populations. *Am J Orthod*, *86*(5), 419-426. https://doi.org/10.1016/s0002-9416(84)90035-6

12 Corruccini, R. S. (1999). *How anthropology informs the orthodontic diagnosis of malocclusion's causes*. Edwin Mellen Press.

13 Varrela, J. (1990). Occurrence of malocclusion in attritive environment: a study of a skull sample from southwest Finland. *Scand J Dent Res*, *98*(3), 242-247. https://doi.org/10.1111/j.1600-0722.1990.tb00968.x

14 Fukase, H., & Suwa, G. (2008). Growth-related changes in prehistoric Jomon and modern Japanese mandibles with emphasis on cortical bone distribution. *American Journal of Physical Anthropology*, *136*(4), 441-454. https://doi.org/10.1002/ajpa.20828

15 Varrela, J. (1992). Dimensional variation of craniofacial structures in relation to changing masticatory-functional demands. *Eur J Orthod*, *14*(1), 31-36. https://doi.org/10.1093/ejo/14.1.31

16 Ueda, H. M., Ishizuka, Y., Miyamoto, K., Morimoto, N., & Tanne, K. (1998). Relationship between masticatory muscle activity and vertical craniofacial morphology. *Angle Orthodontist*, *68*(3), 233-238. <Go to ISI>://WOS:000073985700012

17 Garcia-Morales, P., Buschang, P. H., Throckmorton, G. S., & English, J. D. (2003). Maximum bite force, muscle efficiency and mechanical advantage in children with vertical growth patterns. *European Journal of Orthodontics*, *25*(3), 265-272. https://doi.org/DOI 10.1093/ejo/25.3.265

18 Vanspronsen, P. H., Weijs, W. A., Valk, J., Prahlandersen, B., & Vanginkel, F. C. (1992). A Comparison of Jaw Muscle Cross-Sections of Long-Face and Normal Adults. *Journal of Dental Research*, *71*(6), 1279-1285. https://doi.org/Doi 10.1177/00220345920710060301

19 Kreiborg, S., Jensen, B. L., Moller, E., & Bjork, A. (1978). Craniofacial Growth in a Case of Congenital Muscular-Dystrophy - Roentgencephalometric and Electromyographic Investigation. *American Journal of Orthodontics and Dentofacial Orthopedics*, *74*(2), 207-215. https://doi.org/Doi 10.1016/0002-9416(78)90086-6

20 Granger, M. W., Buschang, P. H., Throckmorton, G. S., & Iannaccone, S. T. (1999). Masticatory muscle function in patients with spinal muscular atrophy. *American Journal of Orthodontics and Dentofacial Orthopedics*, *115*(6), 697-702. https://doi.org/Doi 10.1016/S0889-5406(99)70296-9

21 Hamada, T., Kobayashi, M., & Kawazoe, Y. (1981). Electromyographic activity of masticatory muscles in patients with progressive muscular dystrophy (Duchenne type): relation between integrated electromyographic activity and biting force. *Spec Care Dentist*, *1*(1), 37-38. https://doi.org/10.1111/j.1754-4505.1981.tb01219.x

22 Houston, K., Buschang, P. H., Iannaccone, S. T., & Seale, N. S. (1994). Craniofacial Morphology of Spinal Muscular-Atrophy. *Pediatric Research*, *36*(2), 265-269. https://doi.org/Doi 10.1203/00006450-199408000-00020

23 Spyropoulous, M. N. (1985). An early approach for the interception of skeletal open bites: a preliminary report. *J Pedod*(9), 200-209.

24 Scheidegger, R., Koletsi, D., & Eliades, T. (2018). The impact of dietary consistency on structural craniofacial components: Temporomandibular joint/condyle, condylar cartilage, alveolar bone and periodontal ligament. A systematic review and meta-analysis in experimental in vivo research. *Arch Oral Biol*, *94*, 33-47. https://doi.org/10.1016/j.archoralbio.2018.06.016

25 Hichijo, N., Tanaka, E., Kawai, N., van Ruijven, L. J., & Langenbach, G. E. (2015). Effects of Decreased Occlusal Loading during Growth on the Mandibular Bone Characteristics. *PLoS One*, *10*(6), e0129290. https://doi.org/10.1371/journal.pone.0129290

26 Enomoto, A., Watahiki, J., Yamaguchi, T., Irie, T., Tachikawa, T., & Maki, K. (2010). Effects of mastication on mandibular growth evaluated by microcomputed tomography. *Eur J Orthod*, *32*(1), 66-70. https://doi.org/10.1093/ejo/cjp060

27 McNamara, J. A., Jr., & Carlson, D. S. (1979). Quantitative analysis of temporomandibular joint adaptations to protrusive function. *Am J Orthod*, *76*(6), 593-611. https://doi.org/10.1016/0002-9416(79)90206-9

28 Hart, N. H., Nimphius, S., Rantalainen, T., Ireland, A., Siafarikas, A., & Newton, R. U. (2017). Mechanical basis of bone strength: influence of bone material, bone structure and muscle action. *J Musculoskelet Neuronal Interact*, *17*(3), 114-139. https://www.ncbi.nlm.nih.gov/pubmed/28860414

29 Kohrt, W. M., Barry, D. W., & Schwartz, R. S. (2009). Muscle forces or gravity: what predominates mechanical loading on bone? *Med Sci Sports Exerc*, *41*(11), 2050-2055. https://doi.org/10.1249/MSS.0b013e3181a8c717

30 Turner, C. H. (1998). Three rules for bone adaptation to mechanical stimuli. *Bone*, *23*(5), 399-407. https://doi.org/10.1016/s8756-3282(98)00118-5

31 Bergmann, P., Body, J. J., Boonen, S., Boutsen, Y., Devogelaer, J. P., Goemaere, S., Kaufman, J., Reginster, J. Y., & Rozenberg, S. (2010). Loading and skeletal development and maintenance. *J Osteoporos*, *2011*, 786752. https://doi.org/10.4061/2011/786752

32 Ehrlich, P. J., & Lanyon, L. E. (2002). Mechanical strain and bone cell function: a review. *Osteoporos Int*, *13*(9), 688-700. https://doi.org/10.1007/s001980200095

33 Turner, C. H., & Pavalko, F. M. (1998). Mechanotransduction and functional response of the skeleton to physical stress: the mechanisms and mechanics of bone adaptation. *J Orthop Sci*, *3*(6), 346-355. https://doi.org/10.1007/s007760050064

34 Turner, C. H. (1991). Homeostatic control of bone structure: an application of feedback theory. *Bone*, *12*(3), 203-217. https://doi.org/10.1016/8756-3282(91)90043-i

35 Frost, H. M. (1983). A determinant of bone architecture. The minimum effective strain. *Clin Orthop Relat Res*(175), 286-292. https://www.ncbi.nlm.nih.gov/pubmed/6839601

36 Rabie, A. B., She, T. T., & Hagg, U. (2003). Functional appliance therapy accelerates and enhances condylar growth. *Am J Orthod Dentofacial Orthop*, *123*(1), 40-48. https://doi.org/10.1067/mod.2003.45

37 Ghafari, J., & Degroote, C. (1986). Condylar cartilage response to continuous mandibular displacement in the rat. *Angle Orthodontist*, *56*(1), 49-57. https://doi.org/10.1043/0003-3219(1986)056<0049:CCRTCM>2.0.CO;2

38 Cholasueksa, P., Warita, H., & Soma, K. (2004). Alterations of the rat temporomandibular joint in functional posterior displacement of the mandible. *Angle Orthodontist*, *74*(5), 677-683. https://doi.org/10.1043/0003-3219(2004)074<0677:AOTRTJ>2.0.CO;2

39 Croft, R. S., Buschang, P. H., English, J. D., & Meyer, R. (1999). A cephalometric and tomographic evaluation of Herbst treatment in the mixed dentition. *Am J Orthod Dentofacial Orthop*, *116*(4), 435-443. https://doi.org/10.1016/s0889-5406(99)70230-1

40 Maki, K., Nishioka, T., Shioiri, E., Takahashi, T., & Kimura, M. (2002). Effects of dietary consistency on the mandible of rats at the growth stage: computed X-ray densitometric and cephalometric analysis. *Angle Orthodontist*, *72*(5), 468-475. https://doi.org/10.1043/0003-3219(2002)072<0468:EODCOT>2.0.CO;2

41 Delatte, M., Von den Hoff, J. W., van Rheden, R. E., & Kuijpers-Jagtman, A. M. (2004). Primary and secondary cartilages of the neonatal rat: the femoral head and the mandibular condyle. *Eur J Oral Sci*, *112*(2), 156-162. https://doi.org/10.1111/j.0909-8836.2004.00108.x

42 Solem, R. C., Eames, B. F., Tokita, M., & Schneider, R. A. (2011). Mesenchymal and mechanical mechanisms of secondary cartilage induction. *Dev Biol*, *356*(1), 28-39. https://doi.org/10.1016/j.ydbio.2011.05.003

43 Luder, H. U., Leblond, C. P., & von der Mark, K. (1988). Cellular stages in cartilage formation as revealed by morphometry, radioautography and type II collagen

immunostaining of the mandibular condyle from weanling rats. *Am J Anat*, *182*(3), 197-214. https://doi.org/10.1002/aja.1001820302

44 Purcell, P., Joo, B. W., Hu, J. K., Tran, P. V., Calicchio, M. L., O'Connell, D. J., Maas, R. L., & Tabin, C. J. (2009). Temporomandibular joint formation requires two distinct hedgehog-dependent steps. *Proc Natl Acad Sci U S A*, *106*(43), 18297-18302. https://doi.org/10.1073/pnas.0908836106

45 Lee, S. K., Kim, Y. S., Oh, H. S., Yang, K. H., Kim, E. C., & Chi, J. G. (2001). Prenatal development of the human mandible. *Anat Rec*, *263*(3), 314-325. https://doi.org/10.1002/ar.1110

46 Hall, B. K. (2015). *Bones and cartilage : developmental and evolutionary skeletal biology* (Second edition. ed.). Elsevier/AP, Academic Press is an imprint of Elsevier. Publisher description http://www.loc.gov/catdir/enhancements/fy1606/2015458896-d.html

47 Hinton, R. J., Jing, Y., Jing, J., & Feng, J. Q. (2017). Roles of Chondrocytes in Endochondral Bone Formation and Fracture Repair. *Journal of Dental Research*, *96*(1), 23-30. https://doi.org/10.1177/0022034516668321

48 Van Der Stricht, O. (1890). Recherches sur le cartilage articulaire des oiseaux. *Archives of Biological Sciences*, *10*, 1-41.

49 Brachet, A. (1983). Etude sur la resoprtion des cartilages et la developpement des os longs.chez les oiseaux. *Int. Monatsschr. Anat. Physiol*, 391-417.

50 Crelin, E. S., & Koch, W. E. (1967). An autoradiographic study of chondrocyte transformation into chondroclasts and osteocytes during bone formation in vitro. *Anat Rec*, *158*(4), 473-483. https://doi.org/10.1002/ar.1091580410

51 Zhou, X., von der Mark, K., Henry, S., Norton, W., Adams, H., & de Crombrugghe, B. (2014). Chondrocytes transdifferentiate into osteoblasts in endochondral bone during development, postnatal growth and fracture healing in mice. *PLoS Genet*, *10*(12), e1004820. https://doi.org/10.1371/journal.pgen.1004820

52 Yang, L., Tsang, K. Y., Tang, H. C., Chan, D., & Cheah, K. S. (2014). Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. *Proc Natl Acad Sci U S A*, *111*(33), 12097-12102. https://doi.org/10.1073/pnas.1302703111

53 Jing, Y., Zhou, X., Han, X., Jing, J., von der Mark, K., Wang, J., de Crombrugghe, B., Hinton, R. J., & Feng, J. Q. (2015). Chondrocytes Directly Transform into Bone Cells in Mandibular Condyle Growth. *J Dent Res*, *94*(12), 1668-1675. https://doi.org/10.1177/0022034515598135

54 Humphreys, B. D., & DiRocco, D. P. (2014). Lineage-tracing methods and the kidney. *Kidney Int*, *86*(3), 481-488. https://doi.org/10.1038/ki.2013.368

55 Jing, Y., Simmer, P., & Feng, J. Q. (2021). Cell Lineage Tracing: Colocalization of Cell Lineage Markers with a Fluorescent Reporter. *Skeletal Development and Repair, 2 Edition*, *2230*, 325-335. https://doi.org/10.1007/978-1-0716-1028-2_18

56 Dutta, S., & Sengupta, P. (2016). Men and mice: Relating their ages. *Life Sci*, *152*, 244-248. https://doi.org/10.1016/j.lfs.2015.10.025

57 Hichijo, N., Kawai, N., Mori, H., Sano, R., Ohnuki, Y., Okumura, S., Langenbach, G. E., & Tanaka, E. (2014). Effects of the masticatory demand on the rat mandibular development. *J Oral Rehabil*, *41*(8), 581-587. https://doi.org/10.1111/joor.12171

58 Guerreiro, F., et al. (2013). Effects of masticatory hypofunction on mandibular morphology, mineral density and basal bone area. *Brazilian Journal of Oral Sciences*, *12*, 205-211. https://doi.org/10.1590/S1677-32252013000300010

59 Ma, C., Jing, Y., Li, H., Wang, K., Wang, Z., Xu, C., Sun, X., Kaji, D., Han, X., Huang, A., & Feng, J. (2021). Scx(Lin) cells directly form a subset of chondrocytes in temporomandibular joint that are sharply increased in Dmp1-null mice. *Bone*, *142*, 115687. https://doi.org/10.1016/j.bone.2020.115687

60 Jing, Y., Hinton, R. J., Chan, K. S., & Feng, J. Q. (2016). Co-localization of Cell Lineage Markers and the Tomato Signal. *J Vis Exp*(118). https://doi.org/10.3791/54982

61 Li, J., & Dong, S. (2016). The Signaling Pathways Involved in Chondrocyte Differentiation and Hypertrophic Differentiation. *Stem Cells Int*, *2016*, 2470351. https://doi.org/10.1155/2016/2470351

62 Chen, J., Sorensen, K. P., Gupta, T., Kilts, T., Young, M., & Wadhwa, S. (2009). Altered functional loading causes differential effects in the subchondral bone and condylar cartilage in the temporomandibular joint from young mice. *Osteoarthritis Cartilage*, *17*(3), 354-361. https://doi.org/10.1016/j.joca.2008.05.021

63 Shibukawa, Y., Young, B., Wu, C., Yamada, S., Long, F., Pacifici, M., & Koyama, E. (2007). Temporomandibular joint formation and condyle growth require Indian hedgehog signaling. *Dev Dyn*, *236*(2), 426-434. https://doi.org/10.1002/dvdy.21036

64 Liu, Z. J., Ikeda, K., Harada, S., Kasahara, Y., & Ito, G. (1998). Functional properties of jaw and tongue muscles in rats fed a liquid diet after being weaned. *J Dent Res*, *77*(2), 366-376. https://doi.org/10.1177/00220345980770020501

65 Kono, K., Tanikawa, C., Yanagita, T., Kamioka, H., & Yamashiro, T. (2017). A Novel Method to Detect 3D Mandibular Changes Related to Soft-Diet Feeding. *Front Physiol*, *8*, 567. https://doi.org/10.3389/fphys.2017.00567

66 Beecher, R. M., & Corruccini, R. S. (1981). Effects of dietary consistency on craniofacial and occlusal development in the rat. *Angle Orthodontist*, *51*(1), 61-69. https://doi.org/10.1043/0003-3219(1981)051<0061:EODCOC>2.0.CO;2

67 Kiliaridis, S., & Shyu, B. C. (1988). Isometric muscle tension generated by masseter stimulation after prolonged alteration of the consistency of the diet fed to growing rats. *Arch Oral Biol*, *33*(7), 467-472. https://doi.org/10.1016/0003-9969(88)90026-x

68 Kiliaridis, S., Engstrom, C., & Thilander, B. (1988). Histochemical analysis of masticatory muscle in the growing rat after prolonged alteration in the consistency of the diet. *Arch Oral Biol*, *33*(3), 187-193. https://doi.org/10.1016/0003-9969(88)90044-1

69 Moore, W. J. (1967). Masticatory Function and Skull Growth. *Journal of Anatomy*, *101*, 609-&. <Go to ISI>://WOS:A19679715300038

70 Bouvier, M., & Hylander, W. L. (1984). The effect of dietary consistency on gross and histologic morphology in the craniofacial region of young rats. *Am J Anat*, *170*(1), 117-126. https://doi.org/10.1002/aja.1001700109

71 Bouvier, M. (1988). Effects of age on the ability of the rat temporomandibular joint to respond to changing functional demands. *J Dent Res*, *67*(9), 1206-1212. https://doi.org/10.1177/00220345880670091101

72 Kiliaridis, S., Thilander, B., Kjellberg, H., Topouzelis, N., & Zafiriadis, A. (1999). Effect of low masticatory function on condylar growth: a morphometric study in the rat. *Am J Orthod Dentofacial Orthop*, *116*(2), 121-125. https://doi.org/10.1016/s0889-5406(99)70207-6

73 Pirttiniemi, P., Kantomaa, T., & Sorsa, T. (2004). Effect of decreased loading on the metabolic activity of the mandibular condylar cartilage in the rat. *Eur J Orthod*, *26*(1), 1-5. https://doi.org/10.1093/ejo/26.1.1

74 Kaul, R., O'Brien, M. H., Dutra, E., Lima, A., Utreja, A., & Yadav, S. (2016). The Effect of Altered Loading on Mandibular Condylar Cartilage. *PLoS One*, *11*(7), e0160121. https://doi.org/10.1371/journal.pone.0160121

75 Fang, J., & Hall, B. K. (1995). Differential expression of neural cell adhesion molecule (NCAM) during osteogenesis and secondary chondrogenesis in the embryonic chick. *Int J Dev Biol*, *39*(3), 519-528. https://www.ncbi.nlm.nih.gov/pubmed/7577443

76 Wu, Q., Zhang, Y., & Chen, Q. (2001). Indian hedgehog is an essential component of mechanotransduction complex to stimulate chondrocyte proliferation. *J Biol Chem*, *276*(38), 35290-35296. https://doi.org/10.1074/jbc.M101055200

77 Asano, T. (1986). The effects of mandibular retractive force on the growing rat mandible. *Am J Orthod Dentofacial Orthop*, *90*(6), 464-474. https://doi.org/10.1016/0889-5406(86)90106-x

78 Swiderski, D. L., & Zelditch, M. L. (2013). The complex ontogenetic trajectory of mandibular shape in a laboratory mouse. *J Anat*, *223*(6), 568-580. https://doi.org/10.1111/joa.12118

79 Kufley, S., Scott, J. E., & Ramirez-Yanez, G. (2017). The effect of the physical consistency of the diet on the bone quality of the mandibular condyle in rats. *Arch Oral Biol*, *77*, 23-26. https://doi.org/10.1016/j.archoralbio.2017.01.015

80 Tanaka, E., Sano, R., Kawai, N., Langenbach, G. E., Brugman, P., Tanne, K., & van Eijden, T. M. (2007). Effect of food consistency on the degree of mineralization in the rat mandible. *Ann Biomed Eng*, *35*(9), 1617-1621. https://doi.org/10.1007/s10439-007-9330-x

81 Kiliaridis, S., Bresin, A., Holm, J., & Strid, K. G. (1996). Effects of masticatory muscle function on bone mass in the mandible of the growing rat. *Acta Anat (Basel)*, *155*(3), 200-205. https://doi.org/10.1159/000147805

82 Buschang, P. H., & Martins, J. (1998). Childhood and adolescent changes of skeletal relationships. *Angle Orthodontist*, *68*(3), 199-206; discussion 207-198. https://doi.org/10.1043/0003-3219(1998)068<0199:CAACOS>2.3.CO;2

83 Naini, F. B., Donaldson, A. N., McDonald, F., & Cobourne, M. T. (2012). Influence of chin height on perceived attractiveness in the orthognathic patient, layperson, and clinician. *Angle Orthod*, *82*(1), 88-95. https://doi.org/10.2319/050111-311.1

84 Czarnecki, S. T., Nanda, R. S., & Currier, G. F. (1993). Perceptions of a balanced facial profile. *Am J Orthod Dentofacial Orthop*, *104*(2), 180-187. https://doi.org/10.1016/S0889-5406(05)81008-X

85 Bishara, S. E., & Jakobsen, J. R. (1985). Longitudinal changes in three normal facial types. *Am J Orthod*, *88*(6), 466-502. https://doi.org/10.1016/s0002-9416(85)80046-4

86 Ingervall, B., & Bitsanis, E. (1987). A pilot study of the effect of masticatory muscle training on facial growth in long-face children. *Eur J Orthod*, *9*(1), 15-23. https://doi.org/10.1093/ejo/9.1.15

87 Buschang, P. H., & Gandini Junior, L. G. (2002). Mandibular skeletal growth and modelling between 10 and 15 years of age. *Eur J Orthod*, *24*(1), 69-79. https://doi.org/10.1093/ejo/24.1.69

88 Pan, Y., Chen, S., Shen, L., Pei, Y., Zhang, Y., & Xu, T. (2020). Thickness change of masseter muscles and the surrounding soft tissues in female patients during orthodontic treatment: a retrospective study. *BMC Oral Health*, *20*(1), 181. https://doi.org/10.1186/s12903-020-01168-6

APPENDIX A: FIGURES

Figure 1

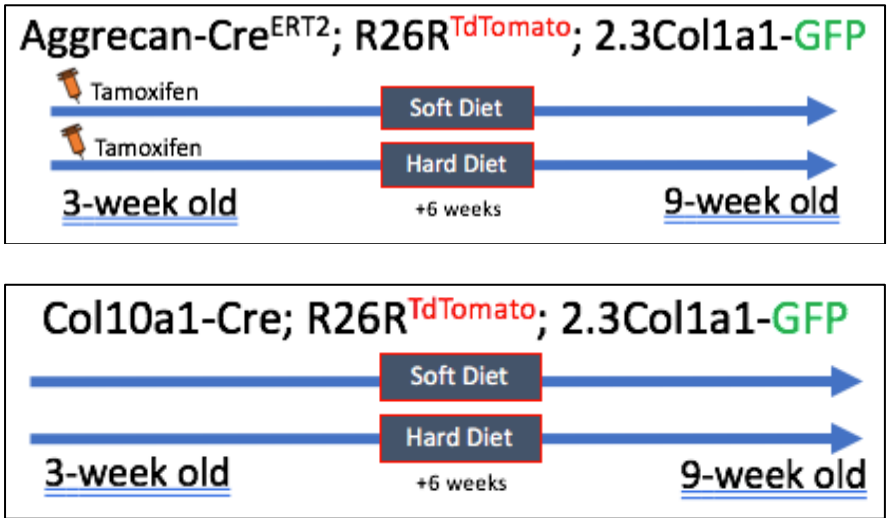

Figure 1: Twelve Aggrecan-Cre^{ERT2}; R26R^{TdTomato}; 2.3Col1a1-GFP mice were injected with tamoxifen at 3 weeks to permanently label Aggrecan^{+} cartilage cells and their progeny. Six of these mice were fed a soft diet only, and six were fed a hard diet only for 6 weeks, followed by sacrifice of both groups at 9 weeks of age. Twelve Col10a1-Cre; R26R^{TdTomato}; 2.3Col1a1-GFP mice were weaned at 3 weeks old. Six were fed a soft diet only, and six were fed a hard diet only for 6 weeks, followed by sacrifice of both groups at 9 weeks of age.

Figure 2

Figure 2: Soft diet food (as seen on the left) consisted of prepackaged "DietGel Purified Soft Diet" mouse food. The bedding of the soft diet group consisted of soft paper. Hard diet food consisted of ordinary laboratory hard pellet mice food.

Figure 3

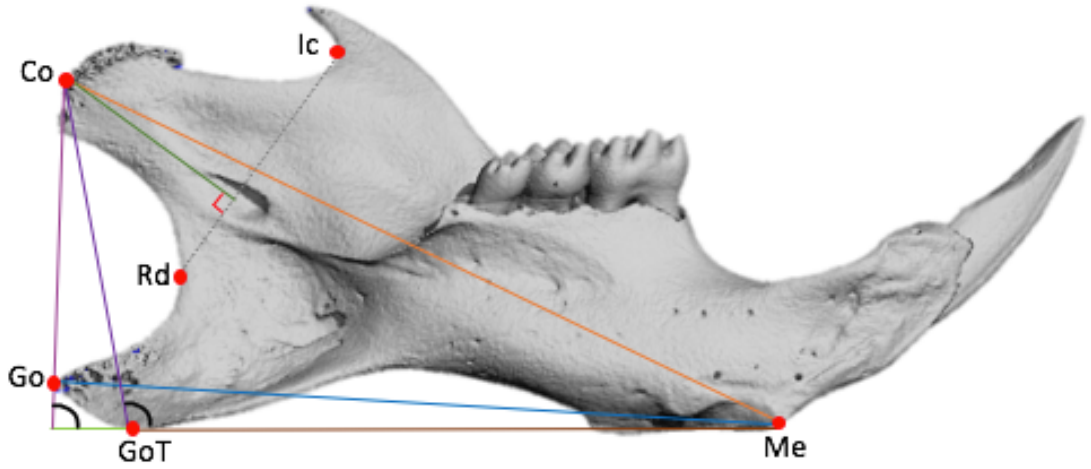

Figure 3: The 3D μCT image of the hemimandible was oriented so that the cusps of the first molar could be visualized with equal heights. A 2D image was captured and exported into Image J software for quantitation of linear and angular measurements. The following measurements were obtained: Total Mandibular Length (Co-Me); Mandibular Base Length (Me-GoT); Mandibular Base Length (Me-Go); Ramus Height (Co-GoT); Condylar Length (Co- ⊥Ic-Rd); Gonial Angle (CoGo/GoTMe); Ramus Angle (CoGoT/GoTMe)

Figure 4

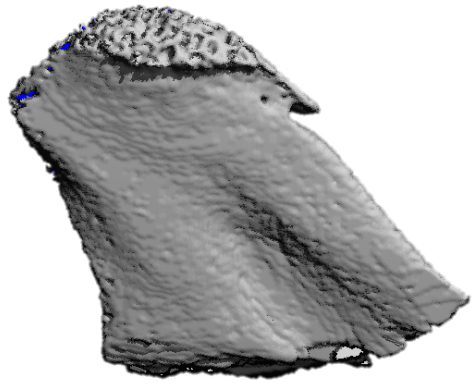

Figure 4: μCT tracing of the condylar process superior to the inferior alveolar nerve was completed for bone quality assessment. Trabecular number, trabecular thickness, trabecular spacing, tissue volume, bone volume, BV/TV, mean/density of TV, and mean/density of BV were evaluated with measurements obtained from the μCT software.

Figure 5

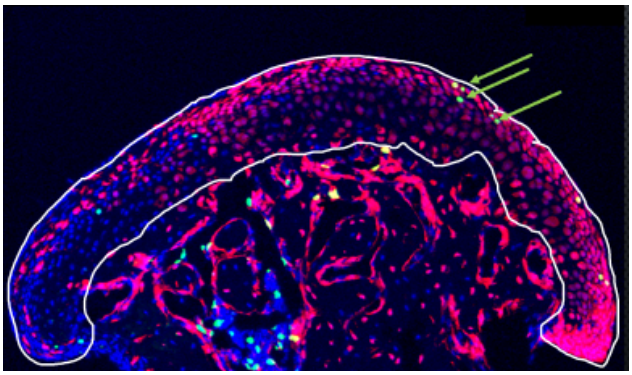

Figure 5: Cell proliferation was evaluated by quantifying Edu^{+}chondrocytes (green cells) / condylar cartilage area (outlined in white).

Figure 6

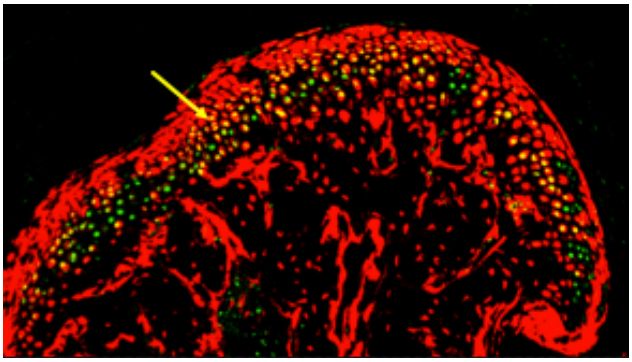

Figure 6: Chondrogenic activity was evaluated by quantifying $Sox9^{+}Tomato^{+}$ (yellow cells) / $Sox9^{+}$ (green cells) in condylar cartilage.

Figure 7

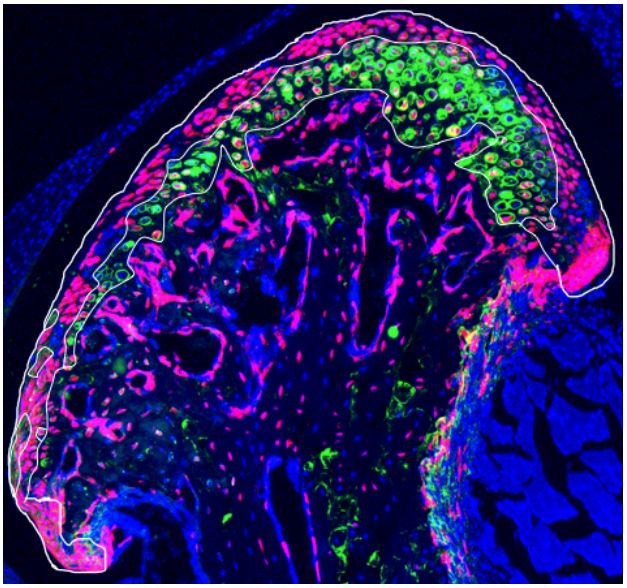

Figure 7: Chondrogenic activity was evaluated by quantifying Aggrecan^{+}area (green area) / condylar cartilage area and Col10a1^{+}area (green area) / condylar cartilage area. Shown here is Aggrecan stain.

Figure 8

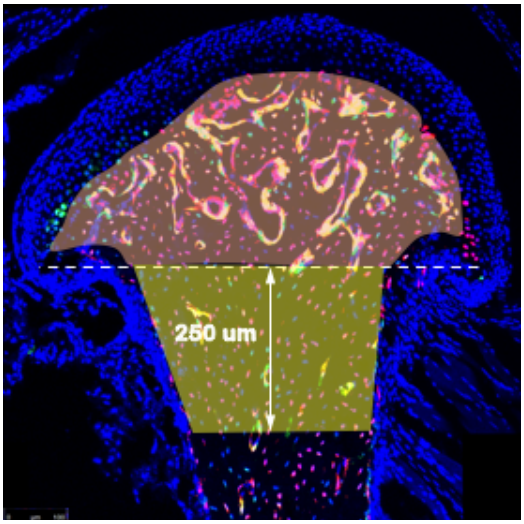

Figure 8: Demarcation of superior and inferior regions of subchondral bone evaluated for cell transdifferentiation quantification.

Figure 9

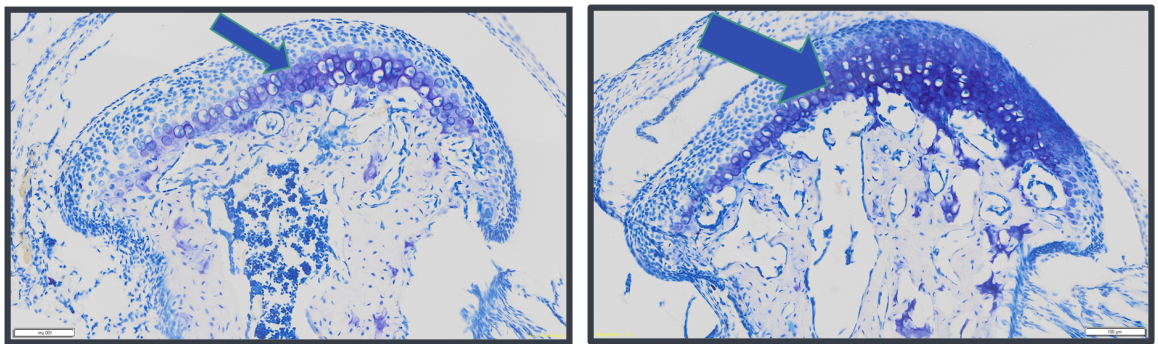

Figure 9: Qualitative assessment of the condylar cartilage using Toluidine blue staining showed a smaller cartilage matrix (purple color) and a thinner cartilage depth in the soft diet samples on the left compared to the hard diet samples on the right.

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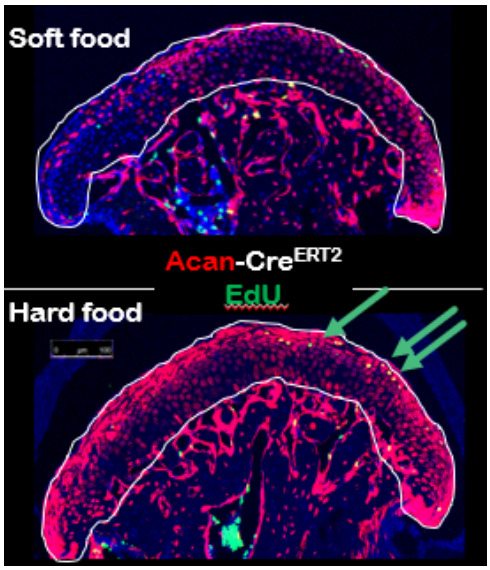
Figure 10

a)



b)

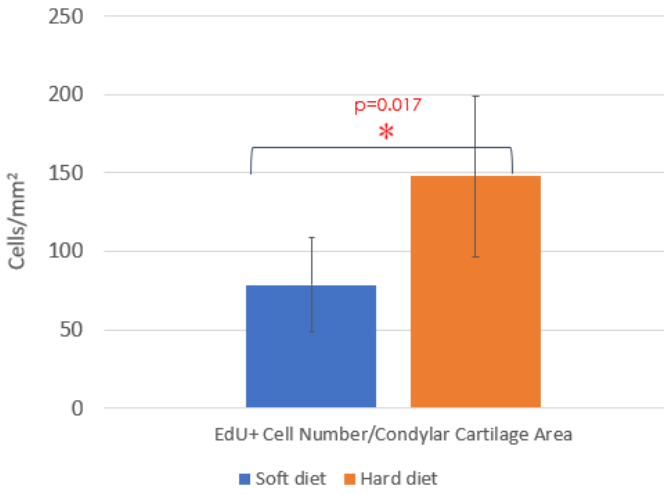
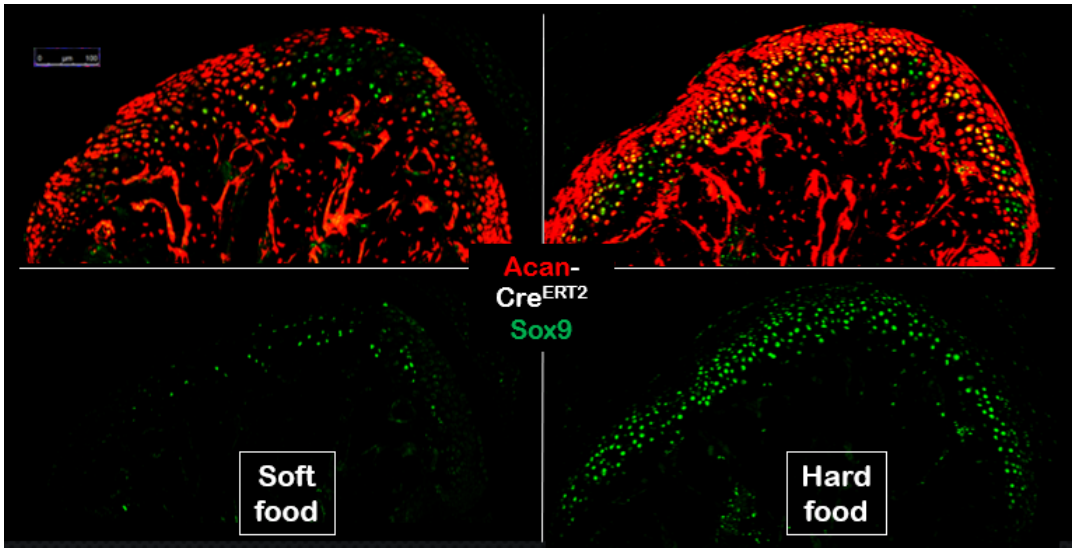
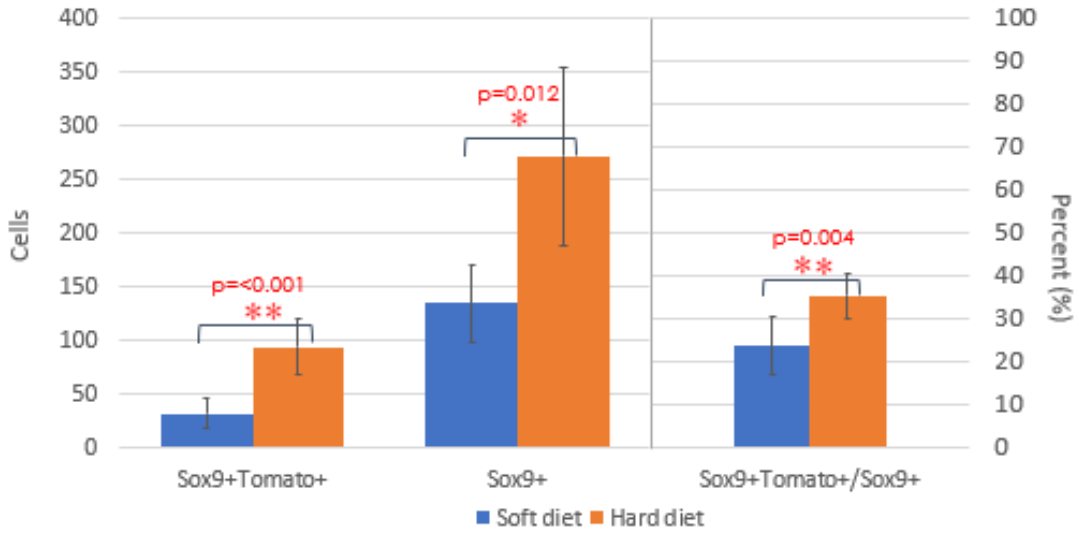


Figure 10: Reduction in masticatory muscle loading decreased chondrocyte proliferation in the condylar cartilage. a) Confocal images for EdU staining showed fewer dividing cells in the soft diet group. b) Means and standard deviations (SD) of EdU^+ cell number over condylar cartilage area (cells/mm^2) confirmed the significant decrease of EdU^+ cell number/condylar cartilage area in soft diet group.

Figure 11

a)



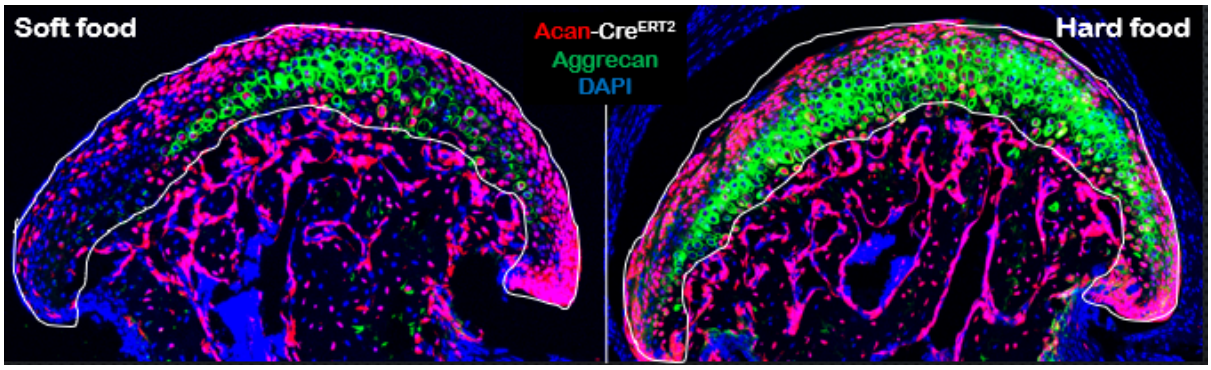


b)

Figure 11: Reduction in masticatory muscle loading reduced chondrogenic activity expressed by Sox9 marker in the condylar cartilage. a) Confocal images for Sox9 staining showed fewer $Sox9^{+}$ (green) cells and fewer $Sox9^{+}Tomato^{+}$ (yellow) cells in the condylar cartilage of the soft diet group. b) Means and standard deviations (SD) of the number of $Sox9^{+}Tomato^{+}$ cells, $Sox9^{+}$ cells, and the ratio of $Sox9^{+}Tomato^{+}/Sox9^{+}$ cells within the condylar cartilage confirmed a significant decrease in chondrogenic activity within the condylar cartilage in the soft diet group compared to hard diet group.

Figure 12

a)



b)

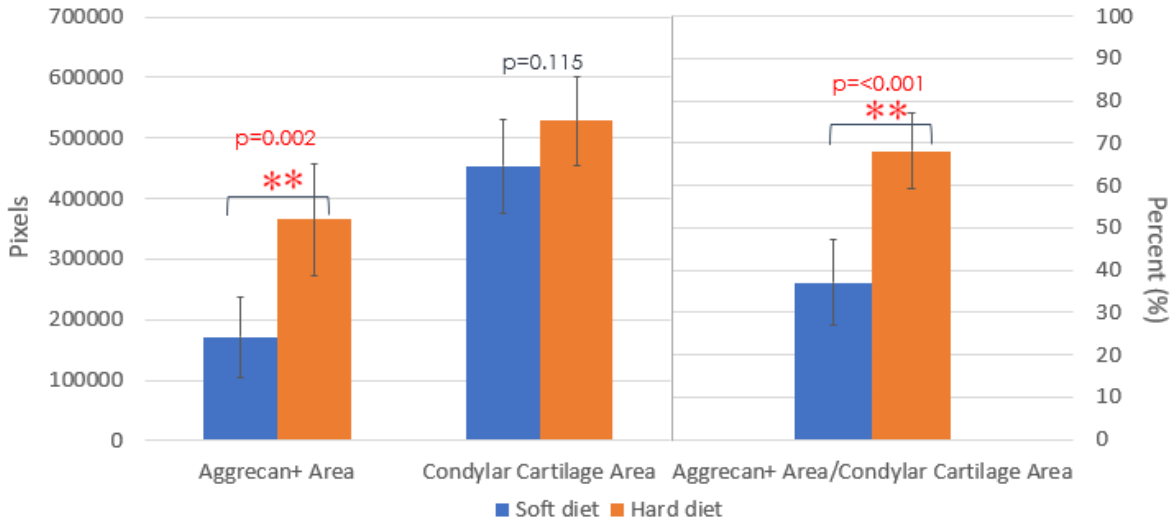
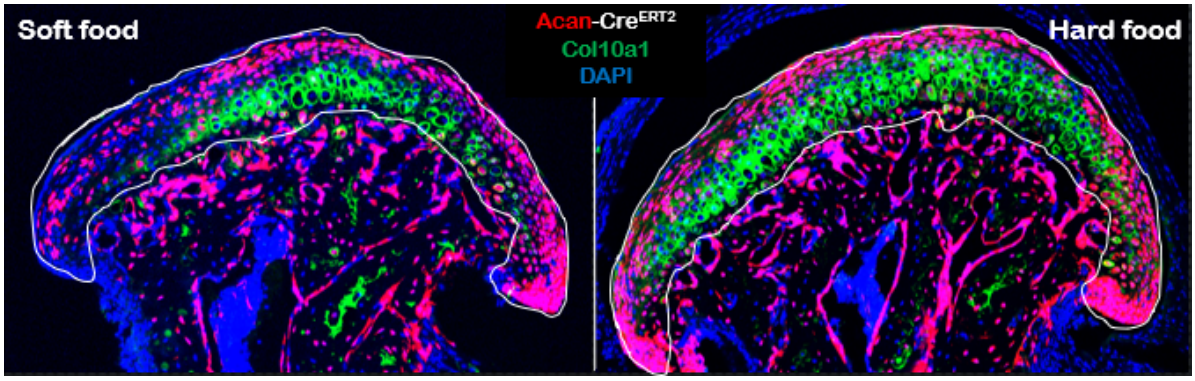


Figure 12: Reduction in masticatory muscle loading decreased chondrogenic activity expressed by Aggrecan marker in the condylar cartilage. a) Confocal images for Aggrecan staining showed reduced Aggrecan^{+} area in the condylar cartilage of the soft diet group. b) Means and standard deviations (SD) of the Aggrecan^{+} area, condylar cartilage area, and the ratio of Aggrecan^{+} area/ condylar cartilage area confirmed a significant decrease in chondrogenic activity within the condylar cartilage in the soft diet group compared to hard diet group.

Figure 13

a)



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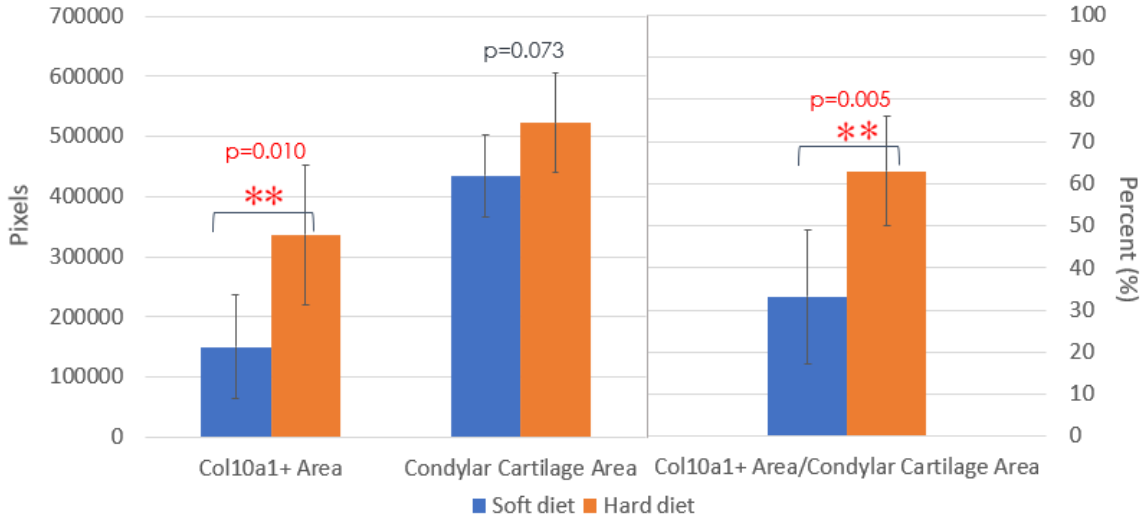
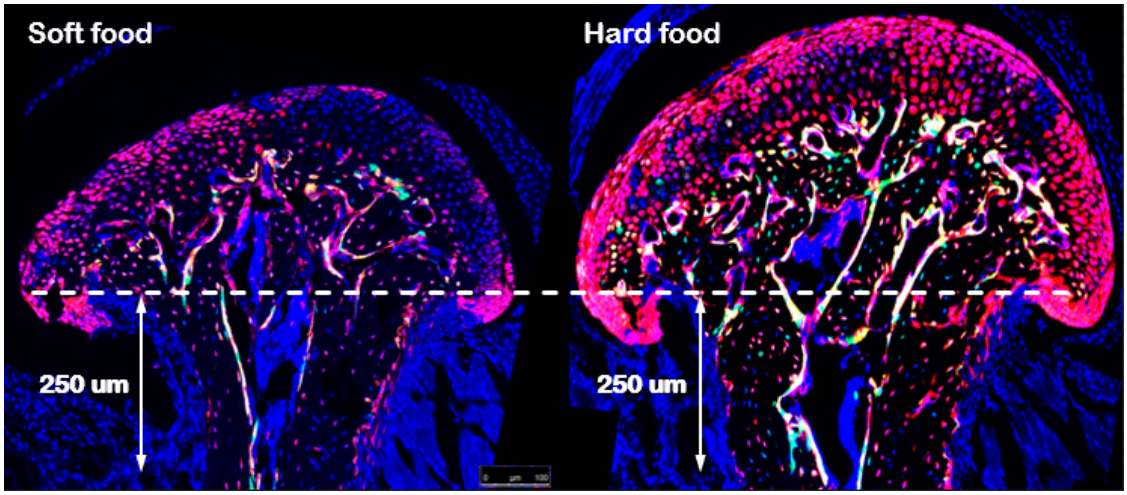


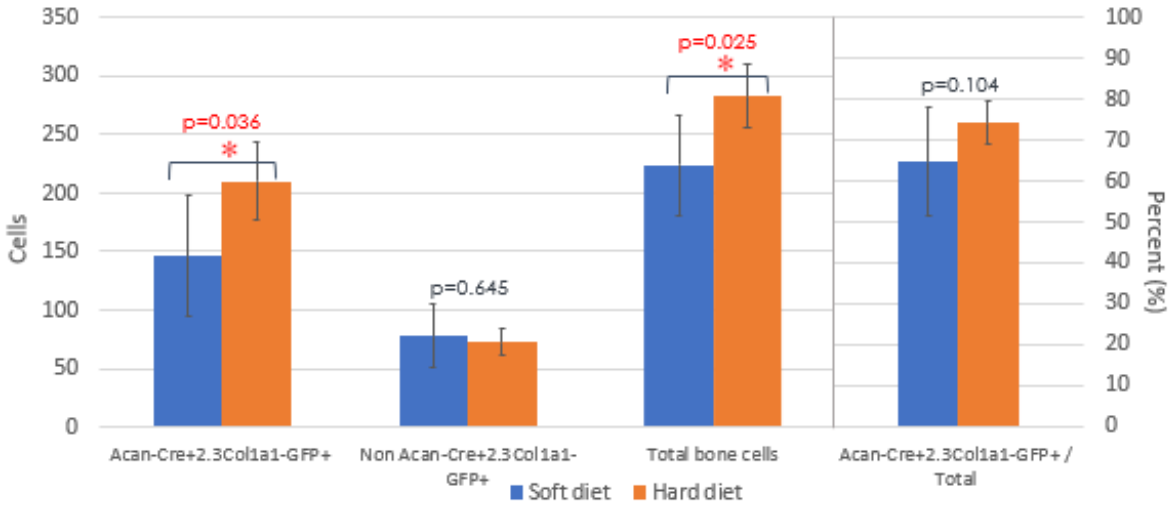
Figure 13: Reduction in masticatory muscle loading decreased chondrogenic activity expressed by Col10a1 marker in the condylar cartilage. a) Confocal images for Col10a1 staining showed reduced Col10a1^{+} area in the condylar cartilage of the soft diet group. b) Means and standard deviations (SD) of the Col10a1^{+} area, condylar cartilage area, and the ratio of Col10a1^{+} area/ condylar cartilage area confirmed a significant decrease in chondrogenic activity within the condylar cartilage in the soft diet group compared to hard diet group.

Figure 14

a)



b)



c)

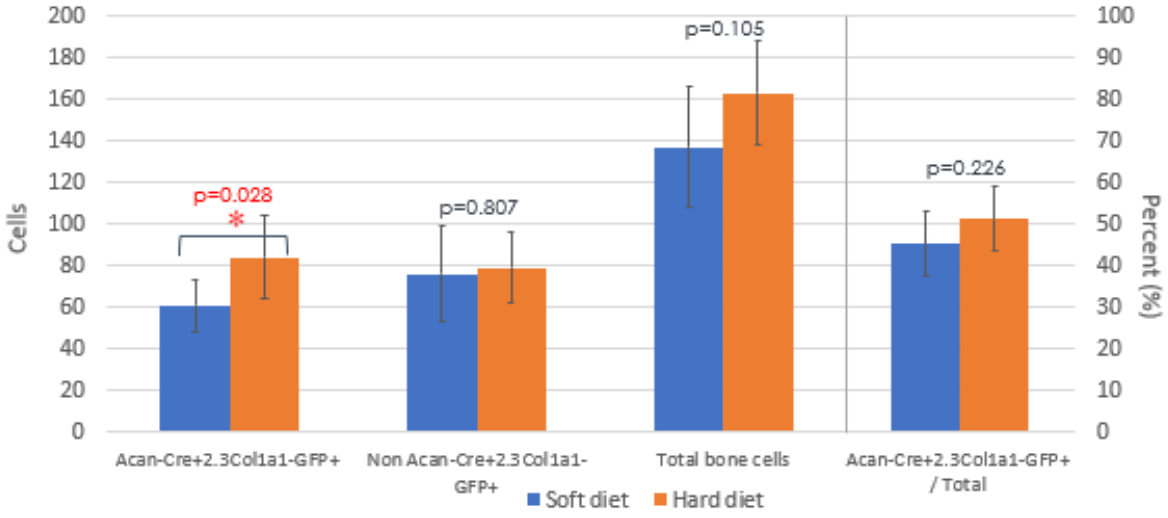
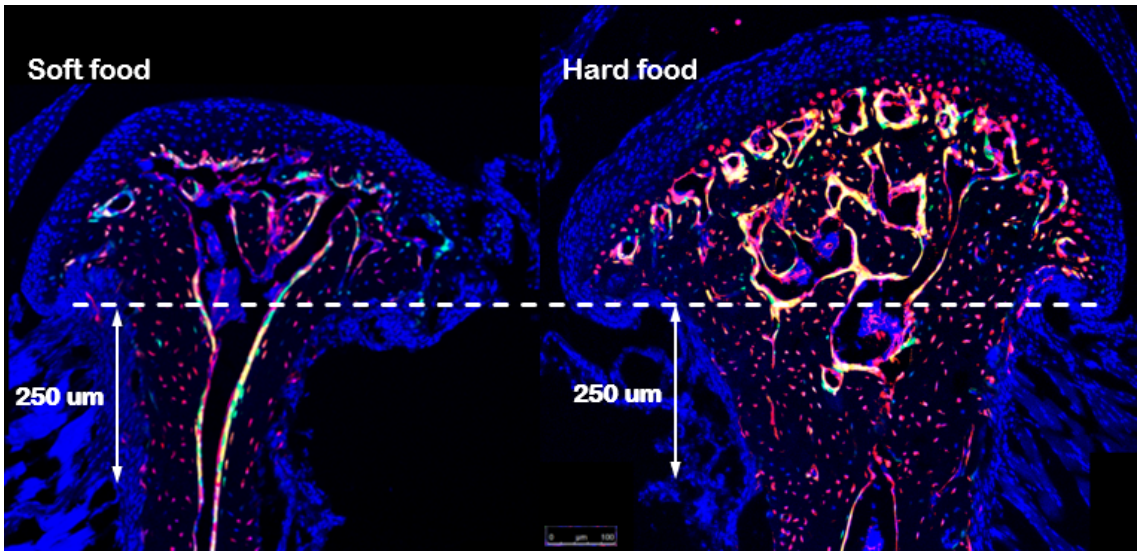


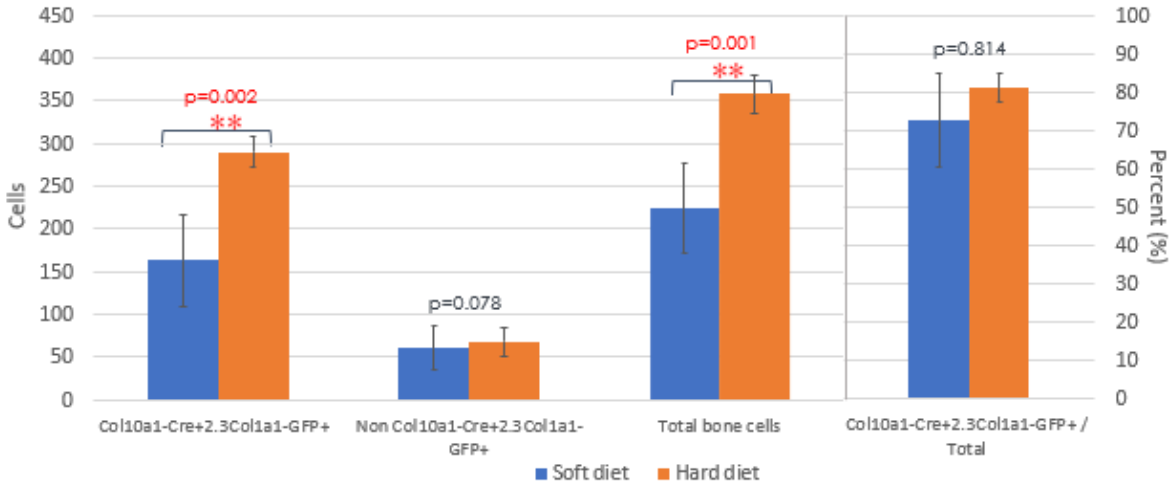
Figure 14: Reduction in masticatory muscle loading decreased the number of transdifferentiated chondrocytes in the subchondral bone. a) Confocal images from mouse genotype Acan-Cre^{ERT2}; R26R^{TdTomato}; 2.3Col1a1-GFP of the subchondral bone showed fewer transdifferentiated chondrocytes (yellow plus red cells) in the soft diet group. b) Means and standard deviations (SD) of Acan-Cre^{+}2.3Col1a1-GFP^{+} (transdifferentiated chondrocytes) cell number, non Acan-Cre^{+}2.3Col1a1-GFP^{+} cell number, and total bone cells within the superior region of the subchondral bone, and means and standard deviations (SD) of the ratio of Acan-Cre^{+}2.3Col1a1-GFP^{+} to total bone cells in the superior region of the subchondral bone. c) Means and standard deviations (SD) of Acan-Cre^{+}2.3Col1a1-GFP^{+} (transdifferentiated chondrocytes) cell number, non Acan-Cre^{+}2.3Col1a1-GFP^{+} cell number, and total bone cells within the inferior region of the subchondral bone, and means and standard deviations (SD) of the ratio of Acan-Cre^{+}2.3Col1a1-GFP^{+} to total bone cells in the inferior region of the subchondral bone. These results confirmed a significant decrease in the number of transdifferentiated chondrocytes within the subchondral bone of the soft diet group.

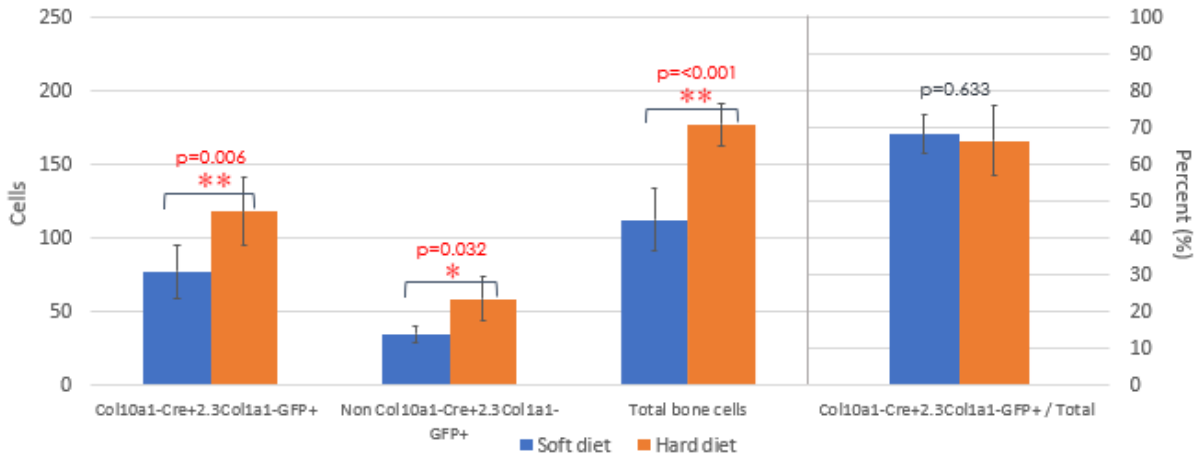
Figure 15

a)



b)





c)

Figure 15: Reduction in masticatory muscle loading decreased the number of transdifferentiated chondrocytes in the subchondral bone. a) Confocal images from mouse genotype Col10a1-Cre; R26R^{TdTomato}; 2.3Col1a1-GFP of the subchondral bone showed fewer transdifferentiated chondrocytes (yellow plus red cells) in the soft diet group. b) Means and standard deviations (SD) of Col10a1-Cre^{+}2.3Col1a1-GFP^{+} (transdifferentiated chondrocytes) cell number, non Col10a1-Cre^{+}2.3Col1a1-GFP^{+} cell number, and total bone cells within the superior region of the subchondral bone, and means and standard deviations (SD) of the ratio of Col10a1-Cre^{+}2.3Col1a1-GFP^{+} to total bone cells in the superior region of the subchondral bone. c) Means and standard deviations (SD) of Col10a1-Cre^{+}2.3Col1a1-GFP^{+} (transdifferentiated chondrocytes) cell number, non Col10a1-Cre^{+}2.3Col1a1-GFP^{+} cell number, and total bone cells within the inferior region of the subchondral bone, and means and standard deviations (SD) of the ratio of Col10a1-Cre^{+}2.3Col1a1-GFP^{+} to total bone cells in the inferior region of the subchondral bone. These results confirmed a significant decrease in the number of transdifferentiated chondrocytes within the subchondral bone of the soft diet group.

APPENDIX B: TABLES

Table 1: Landmarks and their definitions for 2D linear analysis of mouse mandible.

Measurements	**Abbreviation**	**Definition**	**Units**
Total Mandibular Length	Co-Me	Condylion (Co: most superoposterior point of mandibular condyle) to Menton (Me: most inferior point of mandibular symphysis)	mm
Mandibular Base Length	Go-Me	Gonion (Go: most everted point on angle of mandible) to Menton (Me: most inferior point of mandibular symphysis)	mm
Mandibular Base Length	GoT-Me	Gonial Tangent (GoT: lowest point of mandibular angle contour) to Menton (Me: most inferior point of mandibular symphysis)	mm
Ramus Height	Co-GoT	Condylion (Co: most superoposterior point of mandibular condyle) to Gonial Tangent (GoT: lowest point of mandibular angle contour)	mm
Condylar Length	Co- ⊥Ic-Rd	Distance from point Condylion (Co) at right angle to line connecting Ic (point located in the notch between the coronoid process and the mandibular head) and Ramus depth (Rd: the deepest point in the concavity of the mandibular ramus)	mm
Gonial Angle	CoGo/GoTMe	Intersection of lines passing through Co-Go and GoT-Me	degrees
Ramus Angle	CoGoT/GoTMe	Intersection of lines passing through Co-GoT and GoT-Me	degrees

Table 2: Means and standard deviations (SD) of weekly weights in grams along with probabilities of between-group differences.

Week	Soft diet		Hard diet		
	Mean	SD	Mean	SD	P value
0	12.55	1.12	11.88	0.87	0.276
1	15.30	2.00	17.70	1.08	**0.034**
2	19.05	1.22	21.89	0.83	**0.001**
3	23.03	1.58	25.24	1.19	**0.021**
4	24.42	2.23	27.74	1.26	**0.010**
5	25.18	2.31	28.68	1.33	**0.009**
6	25.72	2.36	29.55	1.56	**0.008**

Table 3: Means and standard deviations (SD) of two-dimensional linear and angular measurements in millimeters and degrees, respectively.

Measurements	Units	Soft diet		Hard diet		
		Mean	SD	Mean	SD	P value
Total Mandibular Length (Co-Me)	mm	9.53	0.11	9.83	0.16	**0.004**
Mandibular Base Length (Me-Go)	mm	8.65	0.26	8.92	0.21	**0.006**
Mandibular Base Length (Me-GoT)	mm	7.87	0.26	7.98	0.20	0.205
Ramus Height (Co-GoT)	mm	4.24	0.15	4.36	0.10	0.182
Condylar Length (Co- ⊥Ic-Rd)	mm	2.61	0.07	2.63	0.08	0.725
Gonial Angle (CoGo/GoTMe)	degrees	88.86	3.65	89.82	2.57	0.955
Ramus Angle (CoGoT/GoTMe)	degrees	98.81	3.26	102.00	2.89	0.167

Table 4: Means and standard deviations (SD) of microCT bone density, volume, and trabeculation of the condylar process.

Measurements	Units	Soft diet		Hard diet		
		Mean	SD	Mean	SD	P value
Trabecular Number	1/mm	10.01	0.70	9.44	0.44	0.082
Trabecular Thickness	mm	0.14	0.01	0.14	0.01	0.649
Trabecular Spacing	mm	0.07	0.00	0.06	0.01	0.474
Total Volume (TV)	mm^3	1.09	0.18	1.37	0.21	0.178
Bone Volume (BV)	mm^3	0.78	0.15	1.03	0.15	0.113
Bone Volume/Tissue Volume	1	0.72	0.03	0.75	0.02	0.122
Mean/Density of TV (apparent)	mg HA/ccm	707.76	24.18	729.84	24.73	0.332
Mean/Density of BV (material)	mg HA/ccm	865.58	18.05	871.31	22.92	0.920

Table 5: Means and standard deviations (SD) of EdU^+ cell number over condylar cartilage area ($cells/mm^2$)

Measurements	Soft diet		Hard diet		
	Mean	SD	Mean	SD	P value
EdU^+ Cell Number/Condylar Cartilage Area ($cells/mm^2$)	78.74	30.07	147.97	51.23	**0.017**

Table 6: Means and standard deviations (SD) of $Sox9^{+}Tomato^{+}$cells over $Sox9^{+}$ cells within the condylar cartilage percentage (cells/cells)

Measurements	Soft diet		Hard diet		
	Mean	SD	Mean	SD	P value
$Sox9^{+}Tomato^{+}$ (cells)	31.83	13.88	94.17	26.09	**<0.001**
$Sox9^{+}$ (cells)	134.67	35.71	271.83	83.04	**0.012**
$Sox9^{+}Tomato^{+}/Sox9^{+}$ (%)	23.66	6.72	35.19	5.03	**0.004**

Table 7: Means and standard deviations (SD) of chondrogenic activity+ area over condylar cartilage area percentage (pixels/pixels)

Measurements	Soft diet		Hard diet		
	Mean	SD	Mean	SD	P value
$Aggrecan^{+}$ Area (pixels)	170638.5	65577.3	365011.5	93065.3	**0.002**
Condylar Cartilage Area for Aggrecan Stain Sample (pixels)	453263.0	76954.1	527959.8	73033.3	0.115
$Aggrecan^{+}$ Area/Condylar Cartilage Area (%)	37.02	10.16	68.18	9.03	**<0.001**
$Col10a1^{+}$ Area (pixels)	150376.5	86668.8	337051.5	115812.1	**0.010**
Condylar Cartilage Area for Col10a1 Stain Sample (pixels)	435003.0	68546.8	522642.8	82321.2	0.073
$Col10a1^{+}$ Area/Condylar Cartilage Area (%)	33.23	15.89	62.99	12.93	**0.005**

Table 8: Means and standard deviations (SD) of Aggrecan-Cre^{+}2.3Col1a1-GFP^{+} cells (representing chondrocyte derived bone cells) out of total bone cells in superior region of condylar process

Measurements	Soft diet		Hard diet		
	Mean	SD	Mean	SD	P value
Aggrecan-Cre^{+}2.3Col1a1-GFP^{+} (cells)	146.29	51.81	209.79	33.02	**0.036**
Non Aggrecan-Cre^{+}2.3Col1a1-GFP^{+} (cells)	77.54	27.02	72.71	11.79	0.645
Total bone cells	223.83	43.16	282.50	27.01	**0.025**
Aggrecan-Cre^{+}2.3Col1a1-GFP^{+} / Total bone cells (%)	64.43	13.29	74.00	5.29	0.104

Table 9: Means and standard deviations (SD) of Aggrecan-Cre$^+$2.3Col1a1-GFP$^+$ cells (representing chondrocyte derived bone cells) out of total bone cells in inferior region of condylar process

Measurements	Soft diet		Hard diet		
	Mean	SD	Mean	SD	P value
Aggrecan-Cre$^+$2.3Col1a1-GFP$^+$ (cells)	60.83	12.77	84.04	19.98	**0.028**
Non Aggrecan-Cre$^+$2.3Col1a1-GFP$^+$ (cells)	76.00	23.11	79.04	16.81	0.807
Total bone cells	136.83	28.92	163.08	24.74	0.105
Aggrecan-Cre$^+$2.3Col1a1-GFP$^+$ / Total bone cells (%)	45.01	7.64	51.06	7.91	0.226

Table 10: Means and standard deviations (SD) of Col10a1-Cre$^+$2.3Col1a1-GFP$^+$ cells (representing hypertrophic chondrocyte derived bone cells) out of total bone cells in superior region of condylar process

Measurements	Soft diet		Hard diet		
	Mean	SD	Mean	SD	P value
Col10a1-Cre$^+$2.3Col1a1-GFP$^+$ (cells)	163.29	53.40	290.38	17.58	**0.002**
Non Col10a1-Cre$^+$2.3Col1a1-GFP$^+$ (cells)	61.04	24.91	68.00	16.01	0.078
Total bone cells	224.33	53.37	358.38	22.66	**0.001**
Col10a1-Cre$^+$2.3Col1a1-GFP$^+$ / Total bone cells (%)	72.54	12.19	81.11	3.94	0.814

Table 11: Means and standard deviations (SD) of Col10a1-Cre$^+$2.3Col1a1-GFP$^+$ cells (representing hypertrophic chondrocyte derived bone cells) out of total bone cells in inferior region of condylar process

Measurements	Soft diet		Hard diet		
	Mean	SD	Mean	SD	P value
Col10a1-Cre$^+$2.3Col1a1-GFP$^+$ (cells)	77.50	18.24	118.46	22.54	**0.006**
Non Col10a1-Cre$^+$2.3Col1a1-GFP$^+$ (cells)	35.21	5.74	58.83	14.76	**0.032**
Total bone cells	112.71	20.94	177.29	14.26	**<0.001**
Col10a1-Cre$^+$2.3Col1a1-GFP$^+$ / Total bone cells (%)	68.13	5.21	66.48	9.48	0.633