WNT LRP4 KNOCKOUT MICE SHOW SUPERNUMERARY TEETH AND FUSED MOLARS

A Thesis

by

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MASTERS IN ORAL BIOLOGY

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ABSTRACT

Several signaling mechanisms, notably the Wnt/β-catenin signaling system and its modulators, control tooth development. Lrp4 is recognized as one of these modulators that inhibits the pathway. In the absence of Lrp4, the Wnt/β-catenin signaling pathway is hyperactive, and in Lrp4-deficient mice, this leads to dental abnormalities like supernumerary teeth and fused molars. In this study, we use Wnt Lrp4$^{flox/flox}$ knockout mice to show these effects. The knockout mice were examined for supernumerary teeth, fused molars and alterations in tooth size. This demonstrates the consequences of Lrp4 deficiency on mouse tooth formation.

DEDICATION

I dedicate this to my brother Humza, who encouraged me to apply to Texas A&M Dental in the first place; my sister-in-law Momina, who always thought it was impressive that I was a post graduate student here, and my parents, who encouraged me to pursue my education and make something of myself in the world.

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I would like to also thank Dr. Feng, my first mentor, and Dr. Liu, for their guidance and enabling me to develop my lab techniques.

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Finally, thanks to my mother, father, brother and sister-in-law for their encouragement and to my pet lovebird parrot, Happy, who accompanied me at my laptop as I worked on my thesis.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Drs. Bruno Ruest and Professor Qian Wang of the Department of Biomedical Sciences and Dr. Yan Jing of the Department of Orthodontics.

All work conducted for the thesis was completed by the student independently.

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1. INTRODUCTION

The Wnt/β-catenin signaling pathway involves the interaction between the Frizzled receptors (Fz) and low-density lipoprotein receptor-related proteins 5 and 6 (Lrp5 and Lrp6) co-receptors. An important modulator in this process is Lrp4, which is a negative regulator of Wnt signaling. Lrp4 works in conjunction with Wnt modulator in surface ectoderm (Wise), a protein that is activated by BMP and binds to Lrp4, activating Lrp4 and inactivating the Wnt/β-catenin signaling pathway. Mice homozygous for a hypomorphic Lrp4 allele have a similar phenotype as the Wise-null mice and display various tooth defects, such as supernumerary teeth and molar fusion (Ahn et al., 2010; Ohazama et al., 2008).

However, the function of the gene in tooth development and the cell population involved remain elusive. It is unknown whether the full inactivation of the gene could recapitulate the phenotype of the hypomorphic allele. In this research project, we used Wnt-1Cre;Lrp4 conditional knockout mouse and investigated the function of the gene in neural crest cells. We hypothesized that the Wnt1-Cre;Lrp4$^{flox/flox}$ mice would have dental defects as well as changes in the bone density in maxilla and mandible.

2. LITERATURE REVIEW

Tooth development is the process during embryogenesis where the dental lamina and the surrounding mesenchyme develop into fully formed teeth. Along the way, the number and shape of the teeth is established (Yu T, 2020). The process of tooth development starts by the thickening of the dental lamina into the surrounding mesenchyme. This leads to the formation of a bud-shaped outgrowth of epithelium into the mesenchyme. Hence, this stage is called the bud stage. After the bud stage, the dental epithelium invaginates into the shape of a cap. The cap-shaped dental epithelium surrounds the mesenchyme, which has developed into the dental papilla. The enamel knots in the dental epithelium signify the places where future cusp tips will be present. This stage is called the cap stage. After the cap stage comes the bell stage, where the dental epithelium and dental papilla start laying down enamel and dentin matrix respectively, as the dental epithelium differentiates into ameloblasts and the dental papilla differentiates into odontoblasts. The tooth erupts as hard tissue continues to be deposited, until the tooth fully forms with a crown and root (Yu T, 2020).

One of the significant molecular cascades that controls cell fate during embryonic development is the Wnt signaling pathway (Liu J et al, 2022). The Wnt signaling pathway is further divided into the canonical signaling route that relies on the activity of β-catenin (Wnt/β-catenin pathway) and the noncanonical signaling pathways (planar cell polarity pathway and Wnt/Ca^{2+} pathway) that work independently of β-catenin. The Wnt/β-catenin signaling pathway is intricate and made up of a wide variety of receptors, inhibitors, activators, modulators, phosphatases, kinases, and other elements. A crucial, fundamental component of this route is β-catenin. This includes a minimum of three receptors: LRP 4, 5, and 6. More than twenty different Wnt ligands are known to bind to the Frizzled receptor and co-receptors LRP 5 and 6, leading to the stabilization

of β-catenin (He et al, 2004). When β-catenin is no longer being degraded, it activates the TCF/LEF family of transcription factors which leads to gene expression. A number of inhibitors, including sclerostin, dickkopf, and secreted frizzled-related protein, all prevent the activation of β-catenin (MacDonald BT, 2009). The destruction complex forms to prevent the activation of β-catenin, made up of Axin, APC, GSK3 and CK1. The destruction complex inactivates β-catenin by phosphorylating β-catenin. GSK3 and CK1 strengthen the bond between β-catenin, Axin and APC, which causes further stabilization of the destruction complex. Another inhibitor of the Wnt signaling mechanism is LRP4, the focus of this project. These modulators/regulators direct β-catenin either to the nucleus to control gene expression or to the proteasome for destruction (Duan P, 2016).

The main target and crucial element of the Wnt/β-catenin signaling pathway is the protein β-catenin. From cell fate determination, polarity, and differentiation to migration, proliferation, and function, this pathway is implicated in many aspects of growth and development in many organs and tissues (Moon et al., 2002; Visweswaran et al., 2015). For instance, Wnt/β-catenin plays a crucial role in the creation of the body axis and the control of tissue and organ development throughout embryonic development.

Wnt signaling plays key roles in the differentiation, proliferation, and synthesis of bone matrix by osteoblasts as well as the differentiation and function of osteoclasts during development (Bonewald and Johnson, 2008; Glass II and Karsenty, 2006).

Numerous components of this signaling system have been linked to bone mineral density and fracture susceptibility in genome-wide association studies, or GWAS, according to Hsu and Kiel (2012). Research has linked poor bone mineral density, osteoporosis, and fracture vulnerability to LRP5 and the gene that code for sclerostin, SOST. Sclerostin an inhibitor of bone

growth. Additionally, the genes Lrp4, DKK1, Wnt4, Wnt16, and ctnnb1 (the gene encoding β-catenin) are strongly linked to the formation of bones. As the global deletion of β-catenin is fatal, targeted deletion has been carried out in bone cells to ascertain if β-catenin is essential for osteoblast, osteoclast, or the function of both cells. By controlling the activity of the bone-forming osteoblasts and, indirectly, the bone-resorbing osteoclasts, the Wnt/β-catenin signaling pathway is crucial for bone mass maintenance in the postnatal and adult skeleton (Glass II and Karsenty, 2006). During early odontogenesis, Wnt/β-catenin signaling predominantly controls the odontogenic fate and also partially controls cell proliferation in the dental epithelium (Yuan G, Yang G, Zheng Y, et al., 2015).

A crucial element of the Wnt/β-catenin signaling pathway has identified as Lrp4. Its extracellular domain has structural and sequence similarities with LRP5 and LRP6. Lrp4 was suggested to be a negative regulator of Wnt signaling because it lacked several of the motifs in Lrp5 and Lrp6 that are known to be necessary for Wnt co-receptor activity (Herz and Bock, 2002; Johnson et al., 2005; Weatherbee et al., 2006; Willnow et al., 2012). In cultured cells, overexpressing Lrp4 reduces the activity of the Wnt/β-catenin signaling pathway, which is consistent with this theory (Johnson et al., 2005; Li et al., 2010; Ohazama et al., 2008). The extracellular domain of Lrp4 may directly interact with Sost and Wise in in vitro binding tests, indicating that Lrp4's ability to suppress Wnt signaling may be dependent on its ability to interact with Wnt antagonists (Choi et al., 2009; Karner et al., 2010; Ohazama et al., 2008).

Currently, it is not understood to what degree cell signaling is integrated outside of the cell. Ohazama et al demonstrated that the low-density receptor-related protein Lrp4 interacts with the secreted Bmp antagonist protein Wise to control and integrate Bmp and canonical Wnt signaling during tooth morphogenesis. Lrp4 hypomorphic and Wise null mouse mutants have the same

dental morphology, such as extra incisors and molars, as well as fused molars (Ohazama et al, 2008).

Mice mutant for Lrp4 or Wise exhibit comparable developmental abnormalities in ectodermal tissues, such as teeth, hair, and mammary glands, supporting the connection between Lrp4 and Wise (Ahn et al., 2013; Narhi et al., 2012; Ohazama et al., 2008). Wnt signaling, along with other major signaling pathways, has diverse roles in the control of patterning and morphogenesis at different stages (Ahn, 2015; Balic and Thesleff, 2015; Biggs and Mikkola, 2014). Early development of these tissues requires reciprocal interactions between the epithelium and underlying mesenchyme. Wise is expressed in the surrounding epithelial and mesenchymal cells whereas Lrp4 is found in the epithelial signaling centers of the tooth germ (Ahn et al., 2010; Laurikkala et al., 2003; Ohazama et al., 2008). According to Ahn et al. (2010) and Ohazama et al. (2008), mice that are homozygous for a hypomorphic Lrp4 allele have the same phenotype as Wise-null animals and exhibit a variety of dental abnormalities, including extra teeth and molar fusion. It is probable that Lrp4 interacts with Wise as a crucial molecular mechanism for controlling Wnt/β-catenin signaling in teeth and other settings since Wise-null animals have tooth abnormalities brought on by increased Wnt/β-catenin signaling (Ahn et al., 2010).

Through innovative and varied pathways, Lrp4 modulates Wnt/β-catenin signaling throughout development (Ahn et al., 2017). In mice hypomorphic for Lrp4, enamel knot indicators' and their downstream target genes' poorer and more restricted expression patterns suggest improper tooth formation. The aforementioned comparisons show that Lrp4 or Wise mutant mice have different dental morphologies than wild-type mice do, as well as some similarities. This suggests that Lrp4 and Wise may have separate and overlapping processes that regulate tooth development. Regarding overlapping pathways, it's probable that Lrp4 or Wise deficiencies have

a comparable impact on tooth development because of their participation in the control of a similar signaling cascade. Reduced Wise dose worsens Lrp4 gain-of-function traits, and Wise and Lrp4 overexpression together suppresses tooth growth more strongly.

In both Wise-deficient and Lrp4-deficient animals, studies found that molar abnormalities are more severe in the maxilla than in the mandible, causing overgrowth and fusion of the distal teeth, in accordance with prior findings (Ahn et al., 2010; Ohazama et al., 2008). As considerable recovery of the deficiency requires removal of at least two copies of Lrp5 and Lrp6, this less variable fusion phenotype is linked to elevation of Wnt/β-catenin signaling to a considerably higher level in the maxilla of the mutants (Ahn et al., 2010).

Loss of Lrp4 in osteoblasts increases bone mass in postnatal bone, resembling bone morphology seen in animals lacking Sost (Chang et al., 2014a; Collette et al., 2012; Li et al., 2008; Xiong et al., 2015).

Each quadrant of the jaw has only one incisor in mice. Lrp4 mutants had supernumerary incisors in the mandible and maxilla (Ohazama et al., 2008). In each jaw quadrant of mice, there is just one incisor and three molars, and this space is known as the diastema. The first molar is the biggest and most anterior molar in a quadrant, and it is followed subsequently by the second and third molars. None of the eleven Lrp4 mutants that the study looked at (44 quadrants) had a normal phenotype in the maxilla when they looked at their molars. Out of the 22 quadrants of the maxilla, 18 had unusually big teeth in the first molar position. With varied degrees of penetrance, second and third molar occurrence as well as the existence of extra teeth anterior (mesial) to the first molars were also seen. The first molar and a supernumerary tooth in the fourth quadrant were not visible in the other four quadrants. Only three quadrants of the mandible had a large molar, while another three quadrants displayed an excessive number of teeth (Ohazama et al., 2008).

Micro CT examination was carried out to determine if the unusually big molars grew from a single tooth germ or from the fusion of many molar tooth germs. Each maxillary molar contains numerous roots in wild-type jaws; the first molar has three roots, the second has three roots, and the third has one or two roots. In Lrp4 mutants, the big maxillary molars often possessed seven roots that may be divided into three or four distinct groups. Typically, the tooth's most anterior part had one root, then two sets of three roots each. The supernumerary teeth discovered in the quadrants lacking the major molars were all shown to have a single root by micro CT scanning. This shows that the first and second molars fused together with an anterior extra tooth to generate the huge molars. This huge molar root pattern was produced from a fusion of first and second molars or first, second, and third molars, according to other instances where quadrants had a large molar and a distinct anterior supernumerary tooth (Ohazama et al., 2008).

After demonstrating how Wise binds to Lrp4 and how their complementary expression patterns coincide throughout tooth development, the study next evaluated the phenotype of molar teeth in Wise mutants and Lrp4 mutants. Having unusually big maxillary molars and root patterns that resembled those of Lrp4 mutants, Wise mutants may have developed their huge molars by a mechanism akin to Lrp4 mutants (Ohazama et al., 2008).

Lrp4 and Wise interact to control how individual teeth are separated, according to the fusion phenotype (between the supernumerary tooth and first molar, as well as between the first molar and second molar) seen in mutants of these two proteins. The study looked at the gene expression in the anterior or posterior regions of the first molar tooth germ and the anterior parts of the second molar tooth germ in order to explore the function of Lrp4 and Wise in molar development. At E14.5, the anterior and posterior portions of the first molar epithelium showed relatively modest expression of Lrp4, in contrast to primary enamel knots. Wise was expressed

throughout the mesenchyme of these regions. At this point, Shh expression was significantly downregulated in the first molar epithelium of the Lrp4 and Wise mutants, indicating a connection between the loss of Shh signaling and the molar fusion process (Ohazama et al, 2008).

Largely expanded molar teeth are the most noticeable dental phenotype in Lrp4 mutants. During development, many distinct molar tooth germs fuse to form these massive molars. Although the prevalence of the various molar fusions varies from person to person, the maxilla typically experiences greater penetration than the mandible. The study found a virtually same spectrum of molar fusions and supernumerary teeth in Lrp4 mutants compared to mutants of the Bmp/Wnt antagonist Wise (Ohazama et al., 2008).

Molar teeth fuse as a consequence of increased Wnt and Bmp signaling and decreased Shh signaling brought on by the deletion of either Wise or Lrp4. When junctional epithelial cells develop into inner enamel epithelial cells, this fusion takes place. Since conditional deletion of Shh in the dental epithelium also results in comparable molar teeth fusions, the reduction in Shh signaling that occurs along with the rise in Bmp/Wnt activity is functionally significant (Gritli-Linde A et al., 2002; Dassule HR et al., 2000). Since Lrp4 mutants exhibit polysyndactyly with digit fusions and molecular changes that include reduction in Shh signalling, this common morphogenetic pathway may also include limb development (Johnson EB et al., 2005). The development of supernumerary teeth (mesial) anterior to the first molars, in the position of a premolar, has been described in several different mice with mutations that affect Fgf, Eda, Bmp and Shh signaling (Kassai Y et al, 2005, Mustonen T et al, 2003, Klein OD et al, 2006). An ectopic patch of Shh expression in the diastema at E14.5 may be used to initially detect the emergence of the extra teeth in Lrp4 mutants, which closely mimic those identified in Wise mutants. It's interesting to note that at this same time, Shh expression is noticeably downregulated in the nearby

developing molars of Lrp4 mutant embryos. The context-dependent function of Wise in Wnt signaling, which may either promote or antagonize Wnt signaling as observed in Xenopus, is suggested by this, according to Itasaki N et al. (2003).

In the mandible and maxilla of Lrp4 mutants that phenocopy the Wise mutants, extra incisors have been seen (Murashima-Suginami A et al., 2007). The presence of extra teeth in the incisor and molar areas supports shared pathways controlling the number of teeth in these two regions, and Lrp4 is necessary for the proper regulation and integration of various pathways controlling the shape and patterning of the teeth (Ohazama, 2008).

However, it is unknown what exact cell population Lrp4 affects with regards to number and shape of teeth. We know the dental abnormalities that arise as a result of Lrp4 mutants but we do not know which cell populations are affected.

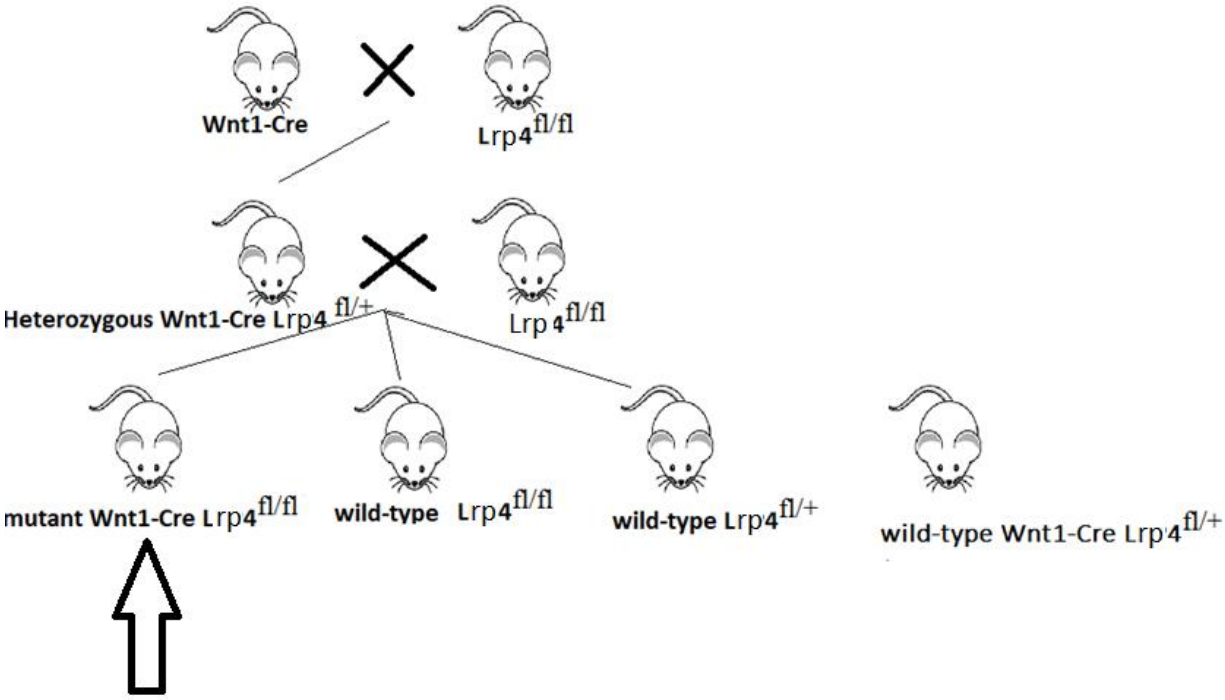


Figure 1: Generation of Wnt1-Cre;Lrp4$^{flox/flox}$ mouse.

Animal model: The conditional Wnt1-Cre Lrp4$^{flox/flox}$ knockout mice were obtained from Case Western Reserve University (Thomas PA, 2020). The Wnt1-Cre Lrp4$^{flox/flox}$ mouse was generated thus: first a Wnt1-Cre mouse (Chen G, 2017) was crossed with an Lrp4$^{flox/flox}$ mouse. This generated Lrp4$^{flox/flox}$ mouse as well as heterozygous Wnt1-Cre;Lrp4$^{flox/flox}$ mouse, which was then crossed with Lrp4$^{flox/flox}$ mouse. This led to the generation of mutant Wnt1-Cre Lrp4$^{flox/flox}$ mouse, as well as wild-type Lrp4$^{flox/flox}$, wild-type Lrp4$^{flox/+}$, and wild-type Wnt1-Cre Lrp4$^{flox/+}$ mice.

11 full samples of 1- and 2-month-old specimens were used in this project. The areas of interest for the project i.e. the upper and lower jaws were processed according to the requirements of the imaging procedures to be performed.

All animal procedures were approved by and followed the guidelines provided by the Texas A&M College of Dentistry IACUC committee.

Histology: Samples were processed for histological analysis as previously described (Wang K, 2021). Briefly, upper and lower jaw samples were collected from the mice and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH = 7.4) at 4°C for 2-4 days. Nondecalcified upper and lower jaws were dehydrated in ascending graded ethanol (EtOH) (75%, 95%, and 100% twice, 2-4 days each) followed by xylene and embedded in methyl-methacrylate (MMA, Buehler, Lake Bluff, IL). The other upper and lower jaw samples were decalcified in 15% EDTA at 4°C, embedded in OCT, and cut into 5 μm-thick sections. These sections were stained with hematoxylin and eosin and toluidine blue, then scanned in the slide scanner.

MicroCT analysis: The upper and lower jaw samples were scanned in the Scanco μCT35 imaging system. The microCT images were analyzed the software, Imaris, as previously described (Zheng J, 2018).

X ray analysis: Upper and lower jaws of Lrp4 knockout mice and wild-type mice were scanned in the Faxitron MX-20 Cabinet X-ray System as previously described (Wang K, 2021).

Scanning electron microscope (SEM) imaging: Methylmethacrylate (MMA) embedded upper and lower jaw blocks were used for SEM analyses as previously described (Wang Z, 2023). The surfaces of MMA-embedded blocks were polished using 400 grit, 800 grit, 1200 grit and 2000 grit sandpaper sheets (Buehler), followed by 1-, 0.3-, and 0.05 μm MicroPolish II alumina solutions (Buehler) on a rotating wheel covered with a soft cloth. The samples were gold coated and imaged with backscattered SEM (JEOL JSM-6010LA, Japan).

Statistical analysis: We compared the Lrp4 knockout mice to the wild-type mice using the Student t-test on Graphpad 8.0. P was < 0.05.

4. RESULTS

To investigate the role of Lrp4 in tooth development, we generate mutant mice in which the gene was inactivated during embryonic development in neural crest cells. We determined the differences in number of teeth and presence of fused molars in the Wnt1-Cre $Lrp4^{flox/flox}$ conditional knockout mice by studying X rays and microCT of the samples. We determined the differences in size of teeth and bone density of the upper and lower jaws by studying microCT of the samples. We determined the morphological differences in the teeth structure through histological sections and SEM of the teeth. Wnt1-Cre $Lrp4^{flox/flox}$ mice demonstrated presence of supernumerary teeth, fused molars, changes in tooth size, histological morphological differences and syndactyly.

X rays analysis: We observed the presence of premolar-shaped supernumerary teeth in the mandible, as seen in fig.2.

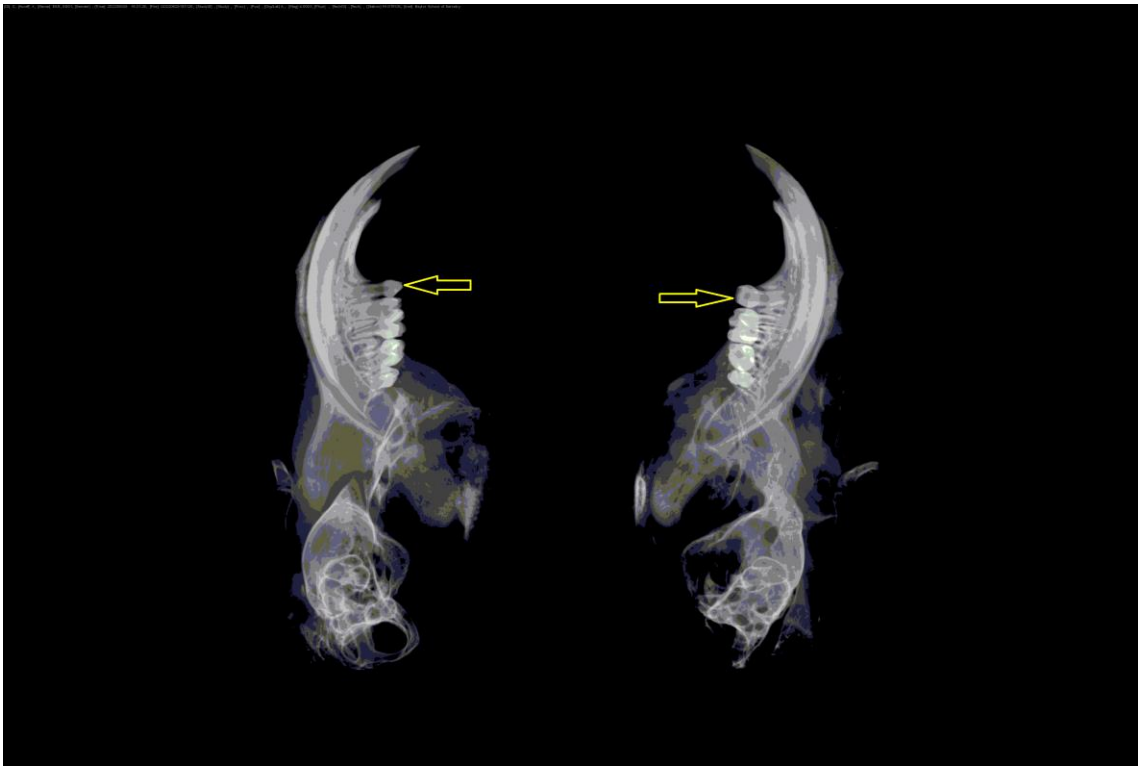


Figure 2: This X Ray shows yellow arrows indicating premolar-shaped teeth in the most mesial position of the molar teeth in Lrp4 knockout mouse. Wild-type mice do not demonstrate any premolar-shaped teeth. These premolar-shaped teeth are seen in Lrp4 conditional knockout mice mandibles.

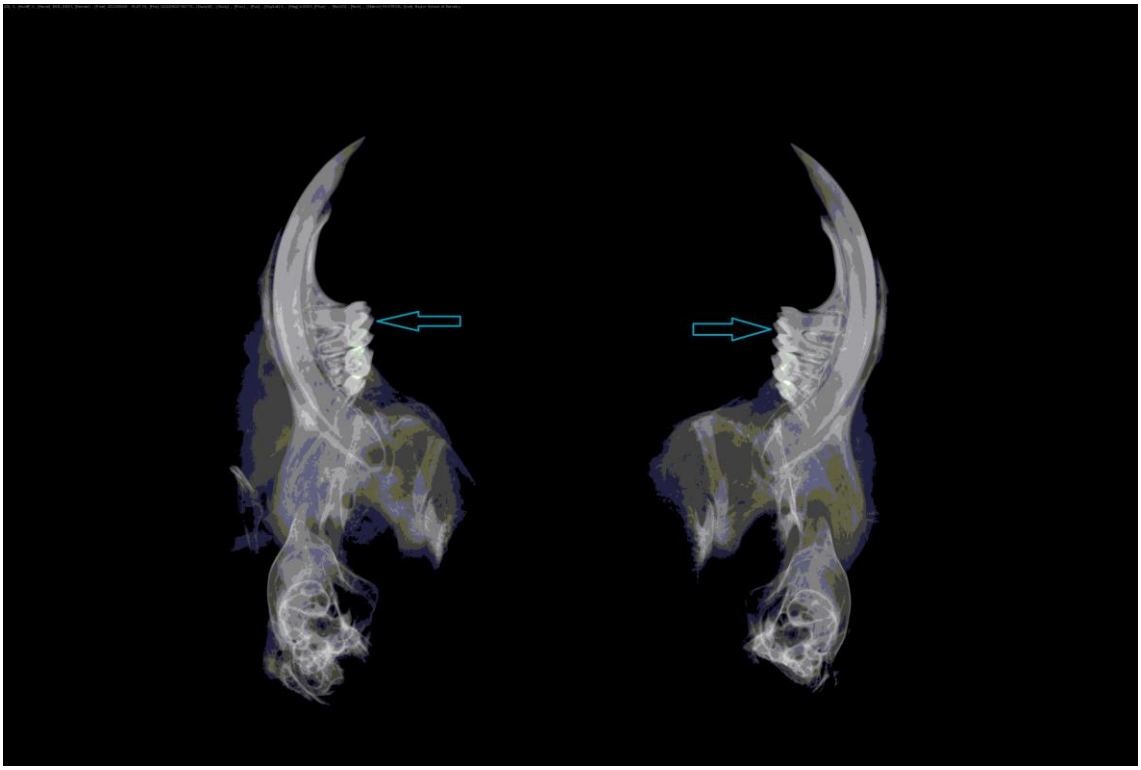


Figure 3: This X Ray shows normally shaped first molars in the mandibles of a wild-type mouse.

MicroCT analysis: We saw the following observations in the microCT images:

Presence of premolar-shaped supernumerary teeth in the mandible of Lrp4 conditional knockout mice. The premolar-shaped tooth usually had a single root. It was in the mesial position of the molar area in the lower jaw.

Presence of fused molar teeth in the mandible and maxilla of Lrp4 neural crest cell conditional knockout mice. Fused molar teeth were more frequently present in the maxilla compared to the mandible. The fused molar teeth were seen to have a continuous pulp chamber throughout as well as fused crown and roots.

Presence of two incisors per quadrant. Usually, mice have one incisor per quadrant. In some of the Lrp4 conditional knockout mice, two incisors per quadrant were observed.

Number of teeth usually present in wild-type mice is 1 incisor and 3 molars per quadrant. In some of the knockout mice, greater or fewer numbers of teeth were observed. This was due to the presence of supernumerary teeth, which increased the number, or the presence of fused teeth, which decreased the number, as we counted each fused tooth as one individual tooth.

The bone density data from microCT analysis revealed that the Lrp4 conditional knockout mice had similar bone density to the wild-type mice.

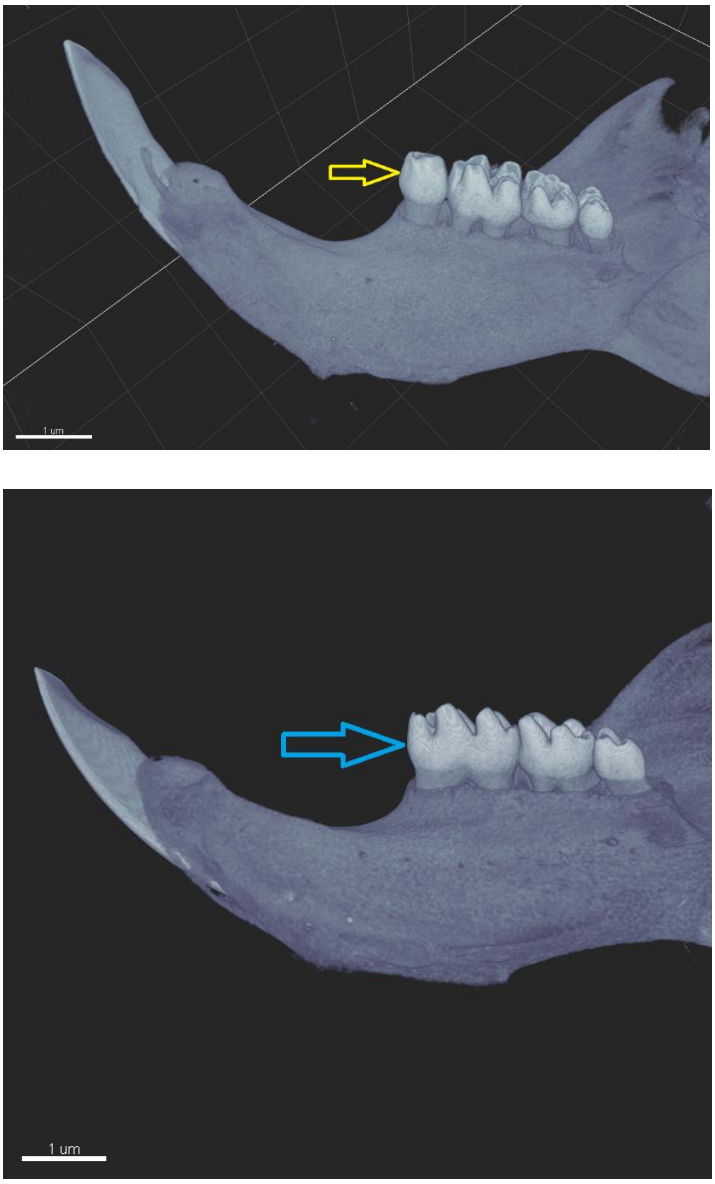


Figure 4: The microCT images show a yellow arrow indicating a premolar-shaped tooth in a Lrp4 neural crest cell conditional knockout mouse mandible. The blue arrow indicates a normal first molar tooth in the wild-type mouse.

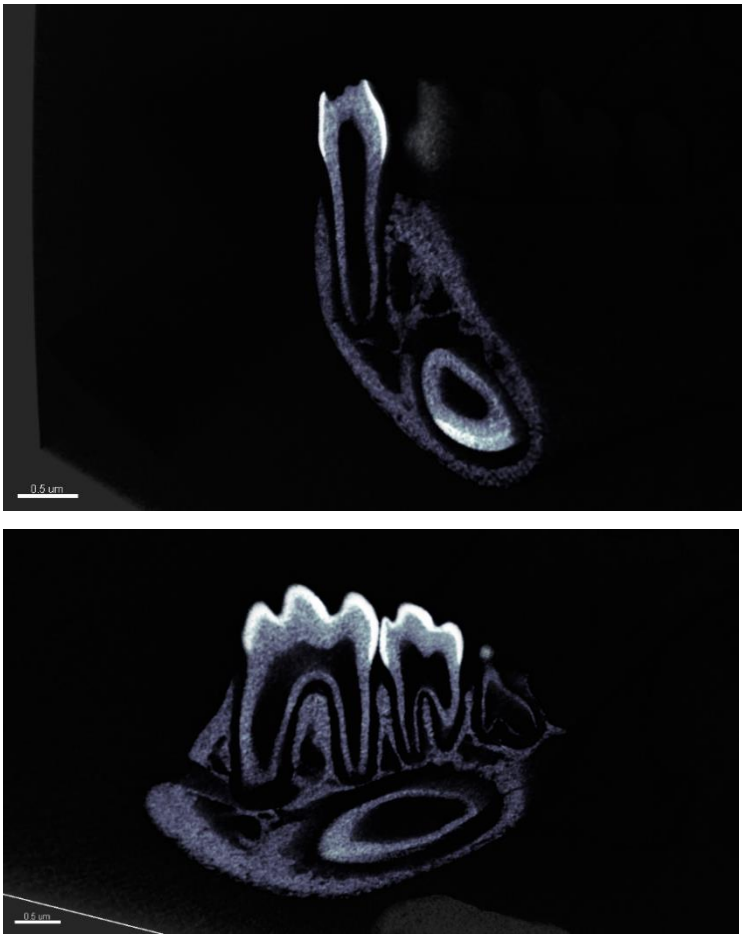


Figure 5: These microCT sectioned images show the section of a premolar-shaped tooth from an Lrp4 conditional knockout mouse mandible on the top, compared to a normal first molar from a wild-type mouse mandible on the bottom. Notice that the premolar-shaped tooth is narrower, has thinner dentin and has only one root.

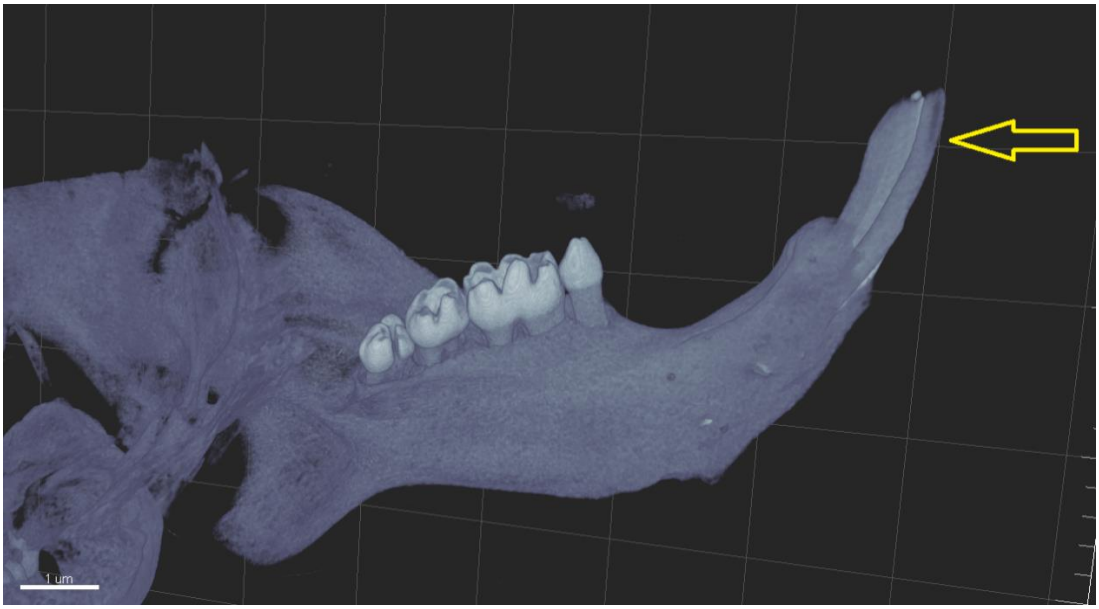


Figure 6: This microCT image shows the presence of two incisor teeth per mandibular quadrant in the Lrp4 knockout mouse.

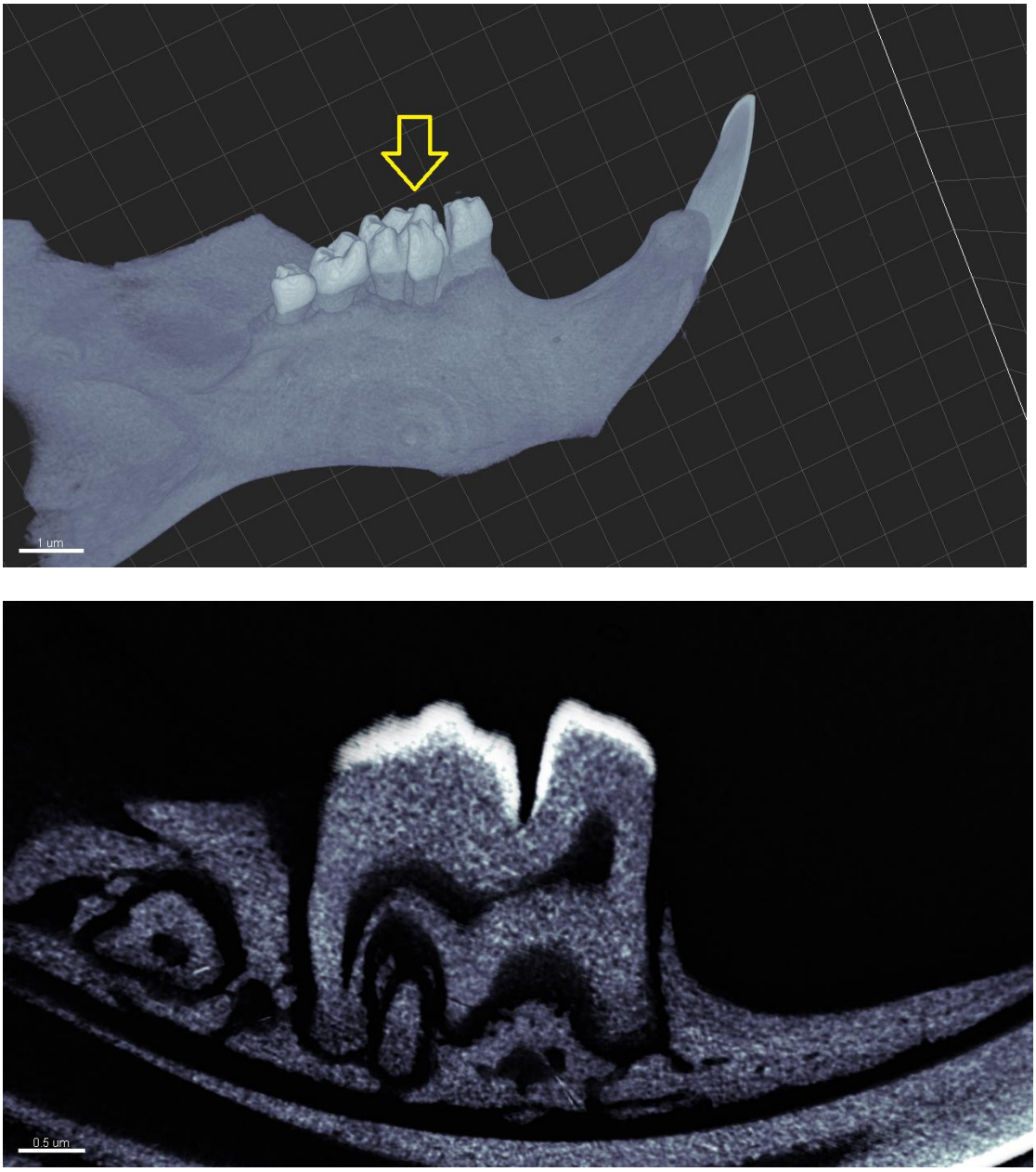


Figure 7: The above microCT image shows a yellow arrow indicating the presence of a fused molar tooth in an Lrp4 neural crest cell conditional knockout mouse mandible. The lower microCT image shows the section of the same tooth. Notice that the roots of the tooth are fused and the pulp chamber is continuous.

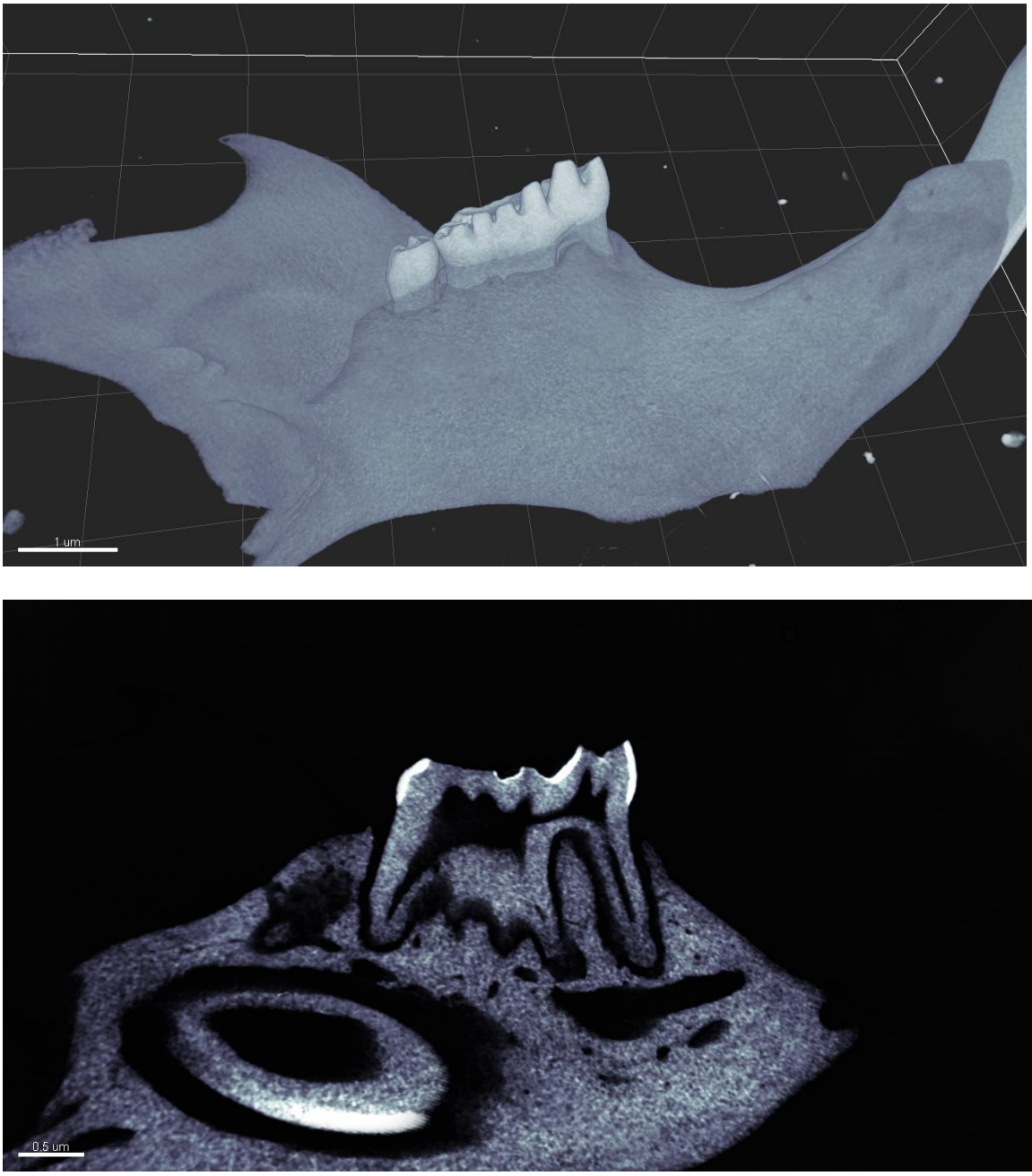
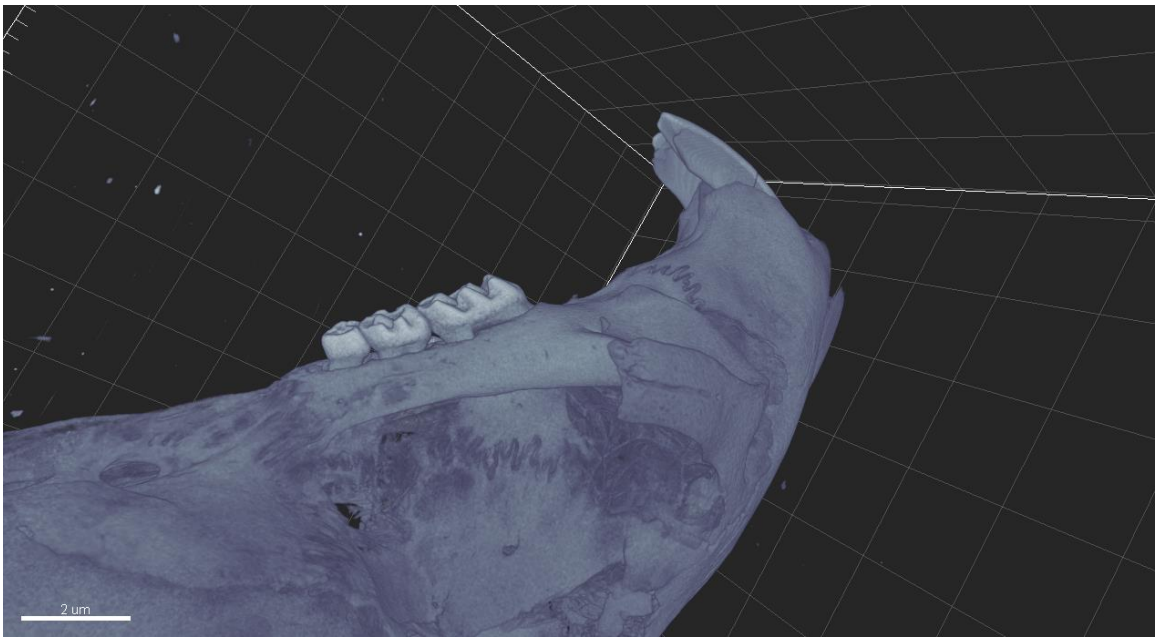


Figure 8: The microCT image above shows a fused molar tooth in a Lrp4 conditional knockout mouse mandible. The microCT image below shows the section of the same tooth. Notice the multiple fused roots and continuous pulp chamber.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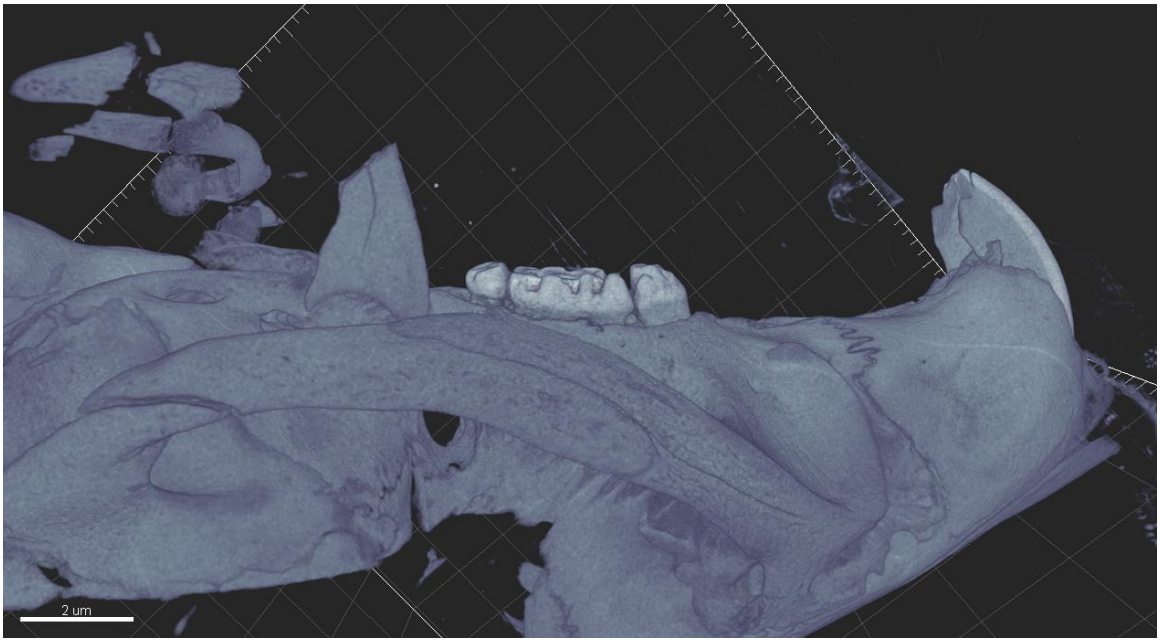
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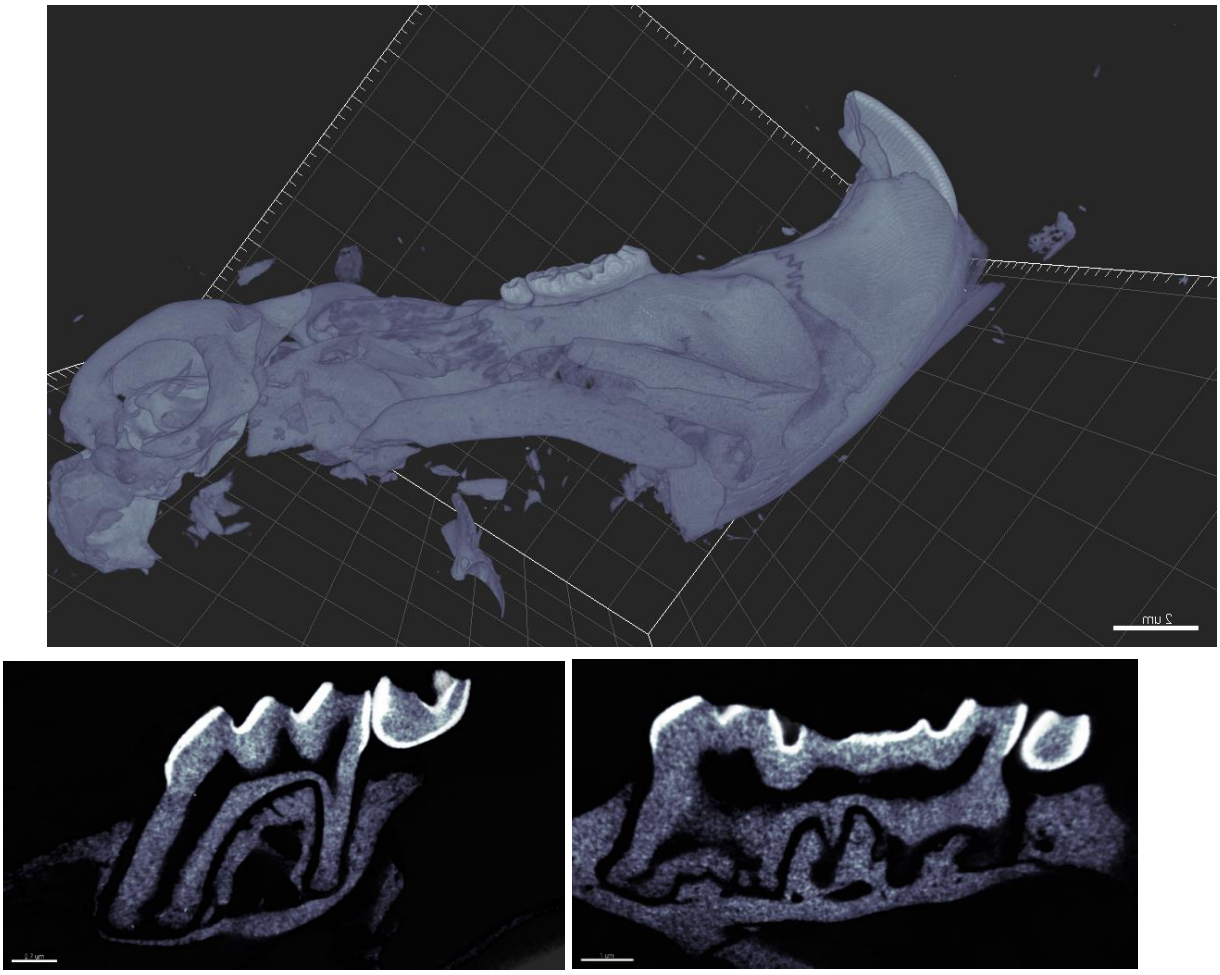

Figure 9: The first microCT image shows a wild-type maxilla with normal teeth. The second and third microCT images show Lrp4 neural crest cell conditional knockout mice maxilla with the presence of fused teeth. The last two microCT images show sections, the one on the left is a section of a wild-type first molar tooth in the maxilla. The one on the right shows the section of a fused tooth. Notice the fused roots, fused crowns and continuous pulp chamber.

Histological analyses: We did H&E staining to observe the tissues. We found that while there were morphological differences between Lrp4 neural crest cell conditional knockout and wild-type such as the presence of supernumerary premolar-shaped teeth, fused teeth with multiple roots and connected pulp chambers and differences in dentin thickness, the appearance of the tissues was normal in Lrp4 mutants similar to the wild-type. This leads us to state that while there are morphological differences between Lrp4 knockout and wild-type, the tissues themselves are normal in the Lrp4 mutants.

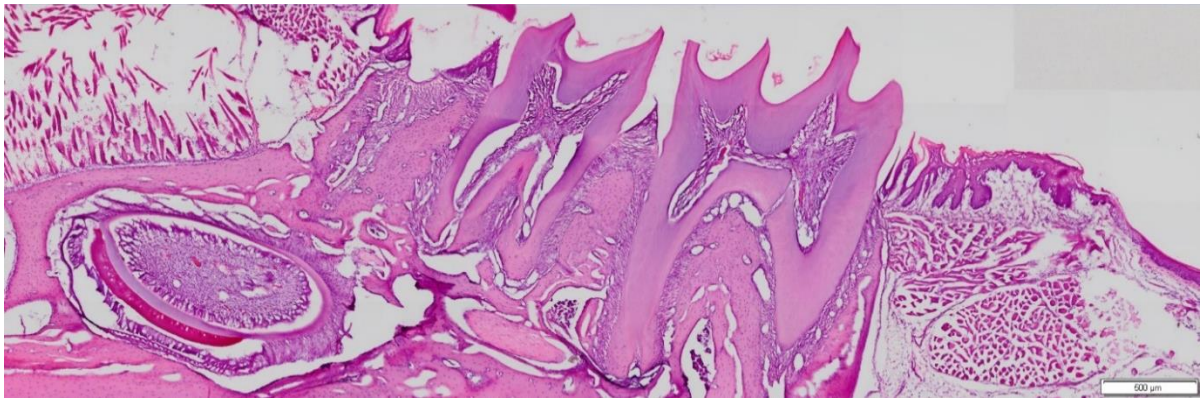

Figure 10: Wild-type H&E stained section showing molar teeth and surrounding tissues.

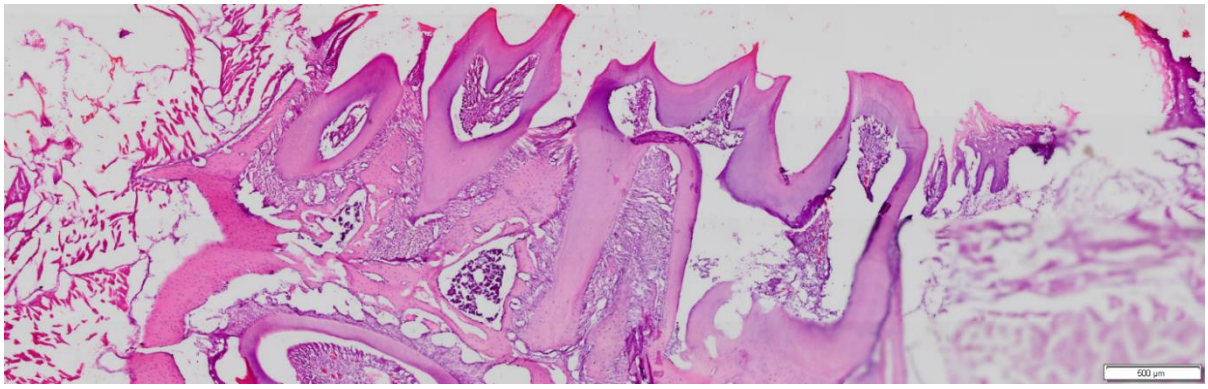

Figure 11: Lrp4 knockout H&E stained section showing fused molar tooth with fused crown and root, with a continuous pulp chamber.

SEM: There were no differences in microstructure observable between Lrp4 conditional knockout and wild-type on SEM. Therefore, we can conclude that the microstructure of the Lrp4 mutant tissues is normal, similar to the wild-type.

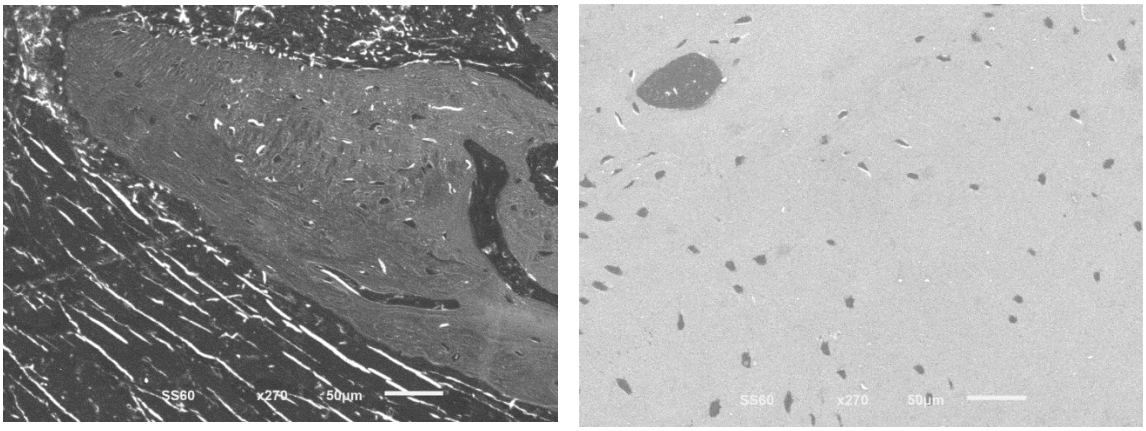

Figure 12: SEM image at 50 µm showing alveolar bone in wild-type (left) and mutant (right).

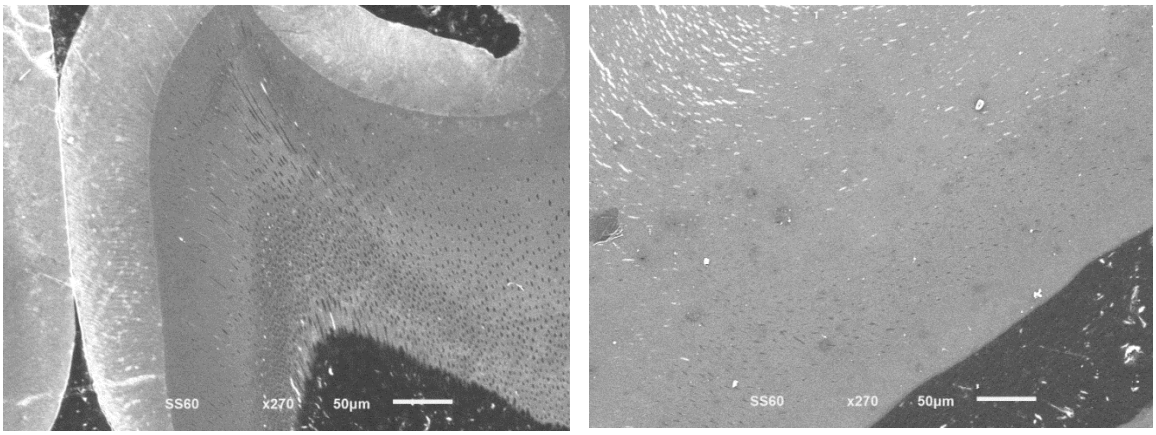

Figure 13: SEM image at 50 µm showing dentin in wild-type molar tooth (left) and mutant (right).

Statistical analyses: We measured different parameters on microCT images and compared the Lrp4 neural crest cell conditional knockout to the wild-type to see the differences between them using the Student t-test.

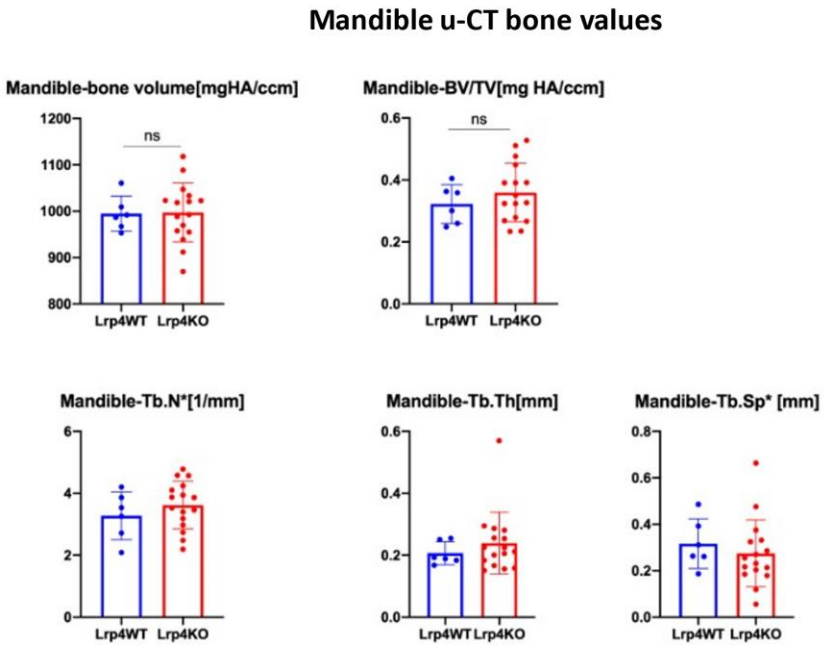

Fig 14: Mandibular bone density values on microCT.

We compared the values obtained through microCT of mandibular bone volume, mandibular bone volume/total volume, mandibular trabecular number, mandibular trabecular thickness and mandibular trabecular separation between Lrp4 conditional knockout and wild-type. There were no statistically significant differences between them.

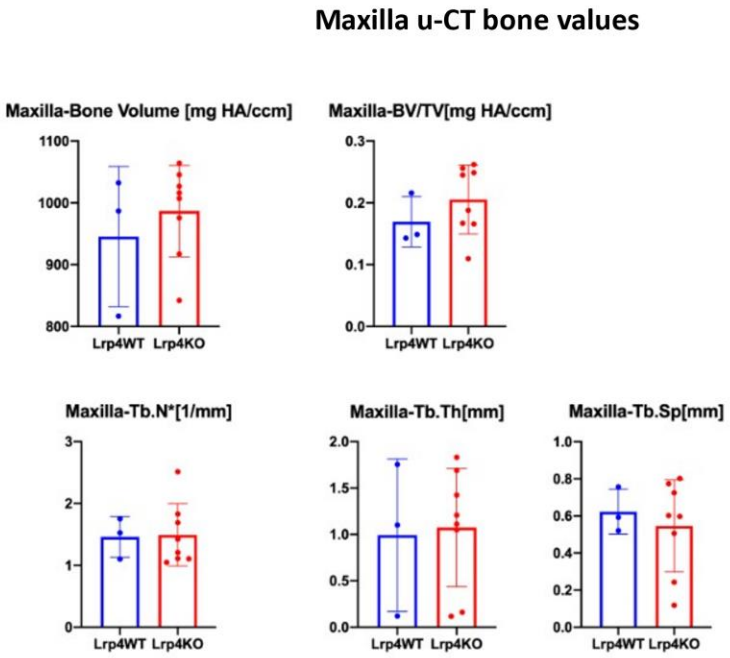

Figure 15: Maxillary bone density values on microCT.

We compared the values obtained through microCT of maxillary bone volume, maxillary bone volume/total volume, maxillary trabecular number, maxillary trabecular thickness and maxillary trabecular separation between Lrp4 mutant and wild-type mice. There were no statistically significant differences between them.

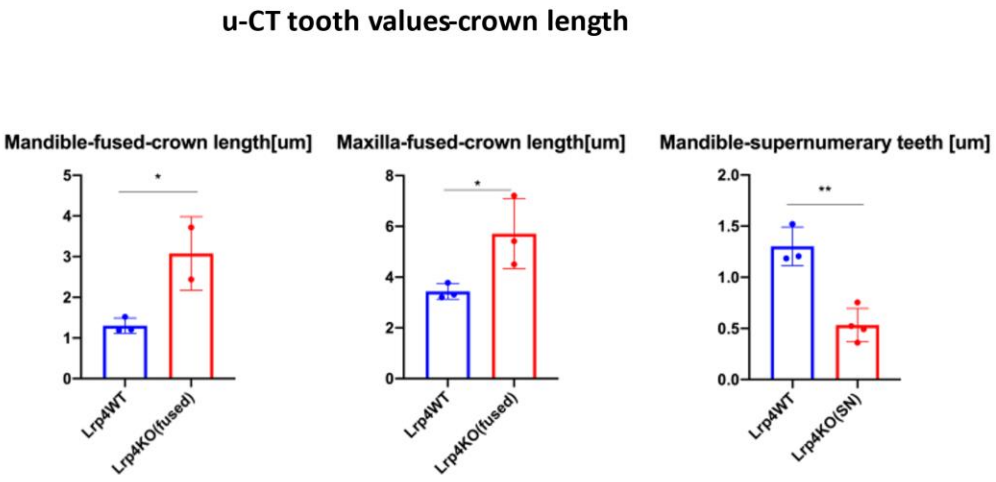

Figure 16: Length of first molar teeth on microCT images in mandible and maxilla.

We compared the mesiodistal crown lengths of Lrp4 conditional knockout fused molar teeth to wild-type first molar teeth on microCT images in mandible and maxilla. We compared mandibular supernumerary premolar-shaped teeth to wild-type first molar. We found that the mandibular fused molar teeth were longer than the wild-type first molar, the maxillary fused molar teeth were longer than the wild-type first molar, and the mandibular supernumerary premolar-shaped teeth were shorter than the wild-type first molar. These differences were statistically significant at $p<0.05$.

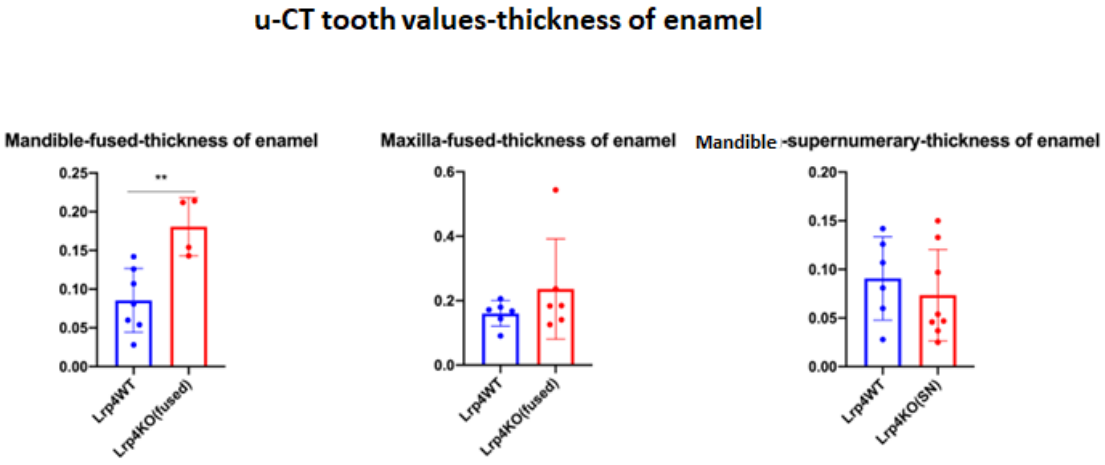

Figure 17: Thickness of enamel on microCT images.

We compared the thickness of enamel on microCT images between mandibular fused molar teeth and wild-type first molar, maxillary fused molar teeth and wild-type first molar, and mandibular supernumerary premolar-shaped teeth and mandibular first molar teeth. We found that the thickness of enamel was greater in Lrp4 knockout mandibular fused molar teeth compared to wild-type first molar teeth; this difference was statistically significant. We also found that the Lrp4 mutant maxillary fused molars had greater enamel thickness than wild-type first molar teeth and that the Lrp4 conditional knockout supernumerary premolar-shaped teeth had thinner enamel than the wild-type first molar teeth; however, these differences were not statistically significant.

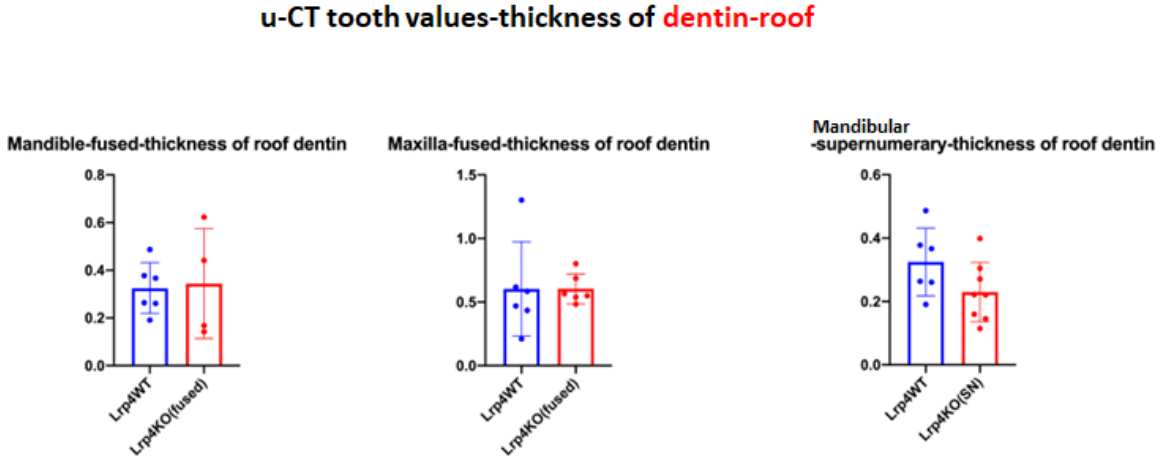

Figure 18: Thickness of the dentin at the roof of the pulp chamber in microCT images.

We compared the thickness of the dentin at the roof of the pulp chamber in microCT images by comparing Lrp4 conditional knockout mandibular fused molar teeth, maxillary fused molar teeth and supernumerary premolar-shaped teeth with wild-type first molar teeth. We found not much difference in the Lrp4 mutant mandibular and maxillary fused molar teeth. The Lrp4 conditional knockout mandibular supernumerary premolar-shaped teeth had thinner roof dentin than the wild-type first molar; however, this was not statistically significant.

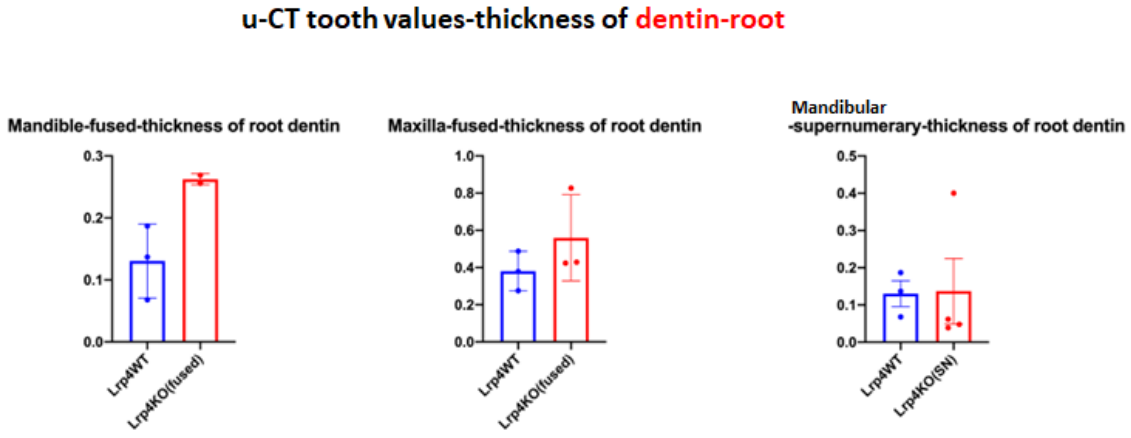

Figure 19: Thickness of dentin in the roots of molar teeth in microCT images.

We measured the thickness of dentin in the roots of molar teeth in microCT images. We compared the Lrp4 conditional knockout mandibular fused molar teeth, maxillary fused molar teeth and mandibular supernumerary premolar-shaped teeth to the respective wild-type first molar teeth. We found that the dentin thickness in Lrp4 conditional knockout mandibular and maxillary fused molars was greater than wild-type first molar teeth and that there were no differences between Lrp4 conditional knockout mandibular supernumerary premolar-shaped teeth and wild-type mandibular first molars. The differences were not statistically significant.

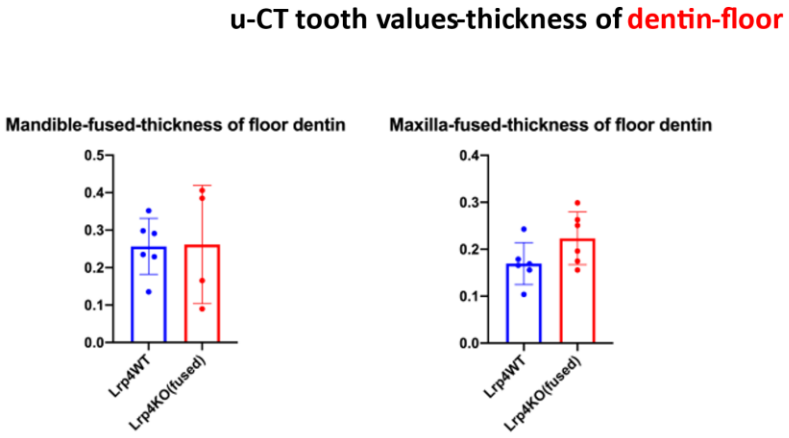

Figure 20: Thickness of the dentin in the floor of the pulp chamber in microCT images.

We compared the Lrp4 neural crest cell conditional knockout maxillary and mandibular fused teeth to their respective wild-type first molar teeth in terms of thickness of the dentin in the floor of the pulp chamber in microCT images. There was not much difference.

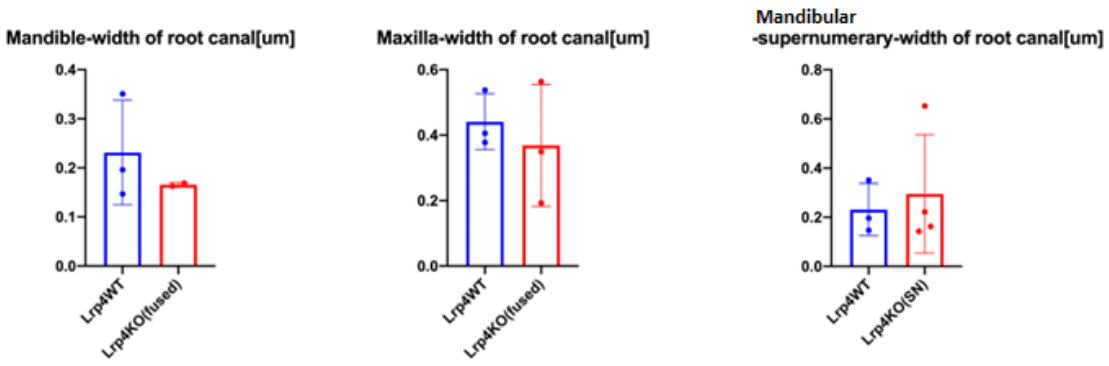

Figure 21: Width of the root canal in microCT images.

We compared the width of the root canal in microCT images in Lrp4 conditional knockout mandibular fused molars, maxillary fused molars and mandibular supernumerary premolar-shaped teeth to their respective wild-type first molar teeth. We found that the Lrp4 conditional knockout mandibular fused teeth had narrower root canals than wild-type mandibular first molars. The mutant maxillary fused teeth had narrower root canals than the wild-type maxillary first molar teeth and mandibular supernumerary premolar-shaped teeth had wider root canals than the wild-type mandibular first molars. None of these findings were statistically significant.

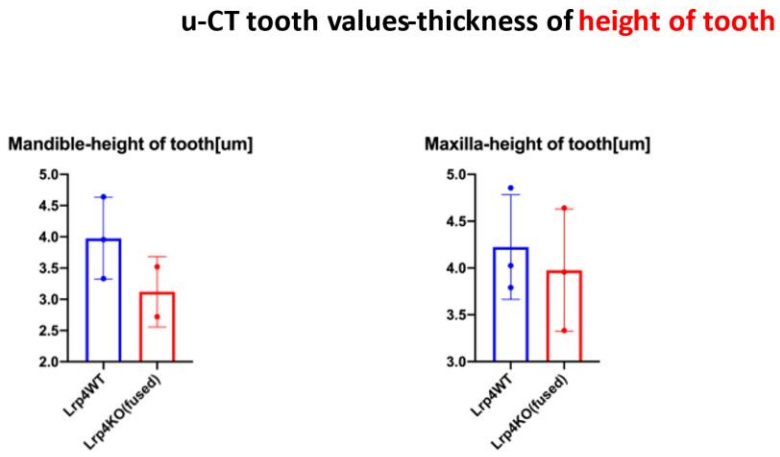

Figure 22: Height of teeth on microCT images.

We compared the height of teeth on microCT images. We compared the height of teeth of Lrp4 conditional knockout maxillary and mandibular teeth to their respective wild-type teeth. We found that the teeth from mutant mice were shorter than their wild-type counterparts but the differences were not statistically significant.

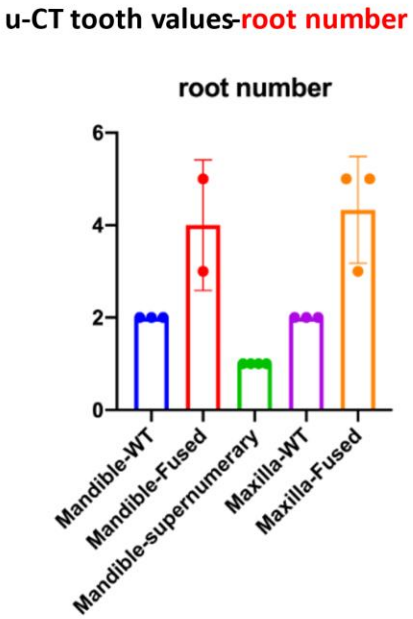

Figure 23: Number of roots on microCT images.

We compared the number of roots on microCT images. We looked at maxillary and mandibular wild-type molar teeth, which had normally 2 roots. We looked at Lrp4 conditional knockout maxillary and mandibular fused molar teeth, which had 4 roots. The mutant mandibular supernumerary premolar-shaped teeth were single-rooted.

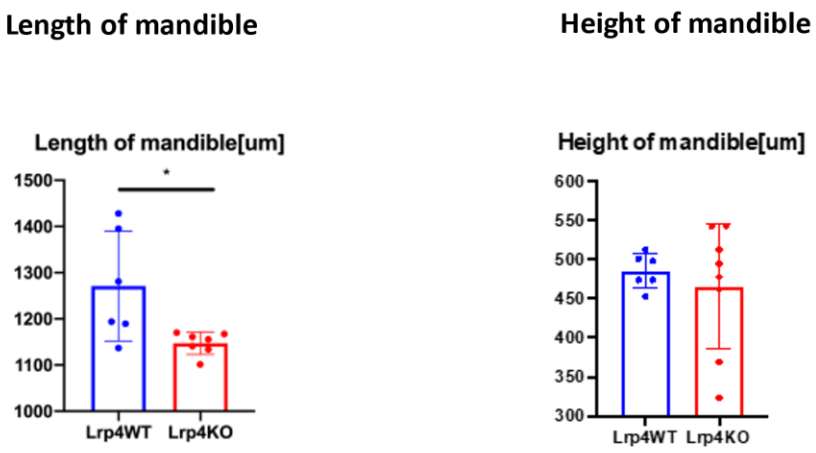

Figure 24: Length and height of mice mandibles.

We compared the length and height of the Lrp4 knockout mice mandibles to the wild-type mandibles on X Ray images. The mutant mandibles were shorter in length than the wild-type mandibles; this difference was statistically significant ($p<0.05$). There was not much difference between the height of Lrp4 knockout mice mandibles and wild-type mice mandibles.

Figure 25: Number of incisors per mandibular quadrant.

We compared the number of incisors per quadrant between Lrp4 conditional knockout mice mandibles and wild-type mice mandibles. The wild-type mice mandibles had 1 incisor per quadrant. 18.75% of the total were Lrp4 mutant mice had 2 incisors per quadrant.

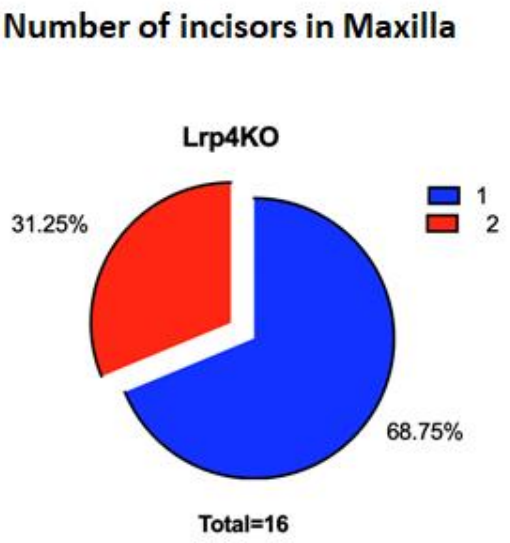

Figure 26: Number of incisors per maxillary quadrant.

We compared the number of incisors per quadrant between Lrp4 conditional knockout mice maxilla and wild-type mice maxilla. The wild-type mice maxilla had 1 incisor per quadrant. 31.25% of the mutant mice had 2 maxillary incisors per quadrant.

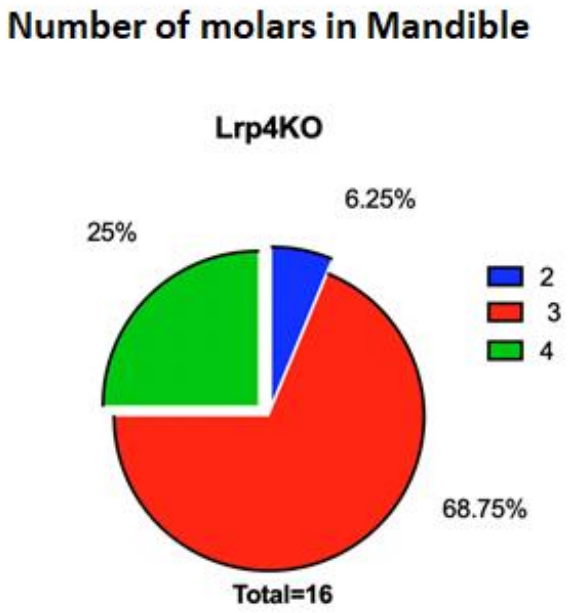

Figure 27: Number of molars per mandibular quadrant.

We compared the number of molars per quadrant in Lrp4 conditional mutant mice mandible to wild-type mice mandible. All the wild-type mice mandibles had 3 molars per quadrant. Out of the Lrp4 mice, 68.75% had 3 mandibular molars, 25% had 4 molars due to the presence of a supernumerary tooth, and 6.25% had 2 molars due to the presence of fused molar teeth.

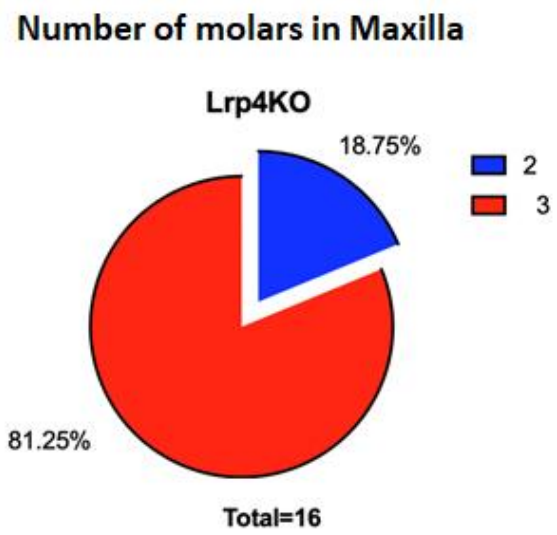

Figure 28: Number of molars per maxillary quadrant.

We compared the number of maxillary molars per quadrant in Lrp4 conditional knockout mice maxilla to wild-type mice. All wild-type mice maxilla had 3 molars per quadrant. Out of the Lrp4 conditional knockout mice maxillae, 81.25% had 3 molars, 18.75% had 2 molars due to the presence of fused molar teeth.

5. SUMMARY AND CONCLUSIONS

In this study, we demonstrated the presence of supernumerary teeth and fused molars in Wnt1-Cre;Lrp4$^{flox/flox}$ conditional knockout mice. This demonstrates the function of Lrp4 in normal tooth development. Since Lrp4 is involved in normal tooth patterning, the Lrp4 conditional knockouts show abnormal tooth morphology such as supernumerary premolar-shaped teeth when wild-type mice only have incisors and molars and do not have any premolar-shaped teeth. The mutants also show the presence of supernumerary incisor teeth and fused molar teeth. Since the Lrp4 conditional knockout mice showing fused teeth have a reduced number of molars per quadrant, it is ascertained that these fused molar teeth are the result of nearby molar teeth getting fused to each other during the developmental stage (Ohazama et al, 2008). As a result, the fused teeth show fused crowns and roots, with an abnormal number of roots per tooth, as well as the fused tooth having an abnormally large mesiodistal length compared to the wild-type molar tooth. The supernumerary premolar-shaped teeth are only present in the Lrp4 mutant mice mandible. The supernumerary incisors are present in both Lrp4 knockout mice maxilla and mandible. The fused molar teeth are present in both mutant maxilla and mandible; however, they occur more frequently in the maxilla. This is consistent with the findings of Ohazama et al, 2008 and Murashima-Suginami A et al., 2007.

Lrp4 function in tooth development resides in the dental mesenchyme, derived from the neural crest cells. Due to the early nature of Wnt signaling, it could be that the transition from the dental lamina to the well-restricted bud stage was affected. This must have affected the number and shape pattern of the teeth. As the number of buds develop from the dental lamina, the number of buds could have been affected, leading to supernumerary teeth. As the shape of the tooth is

established during tooth development, the fusion of multiple tooth buds must have led to the formation of fused molar teeth. Normal tooth development progresses along a pathway where the dental lamina and surrounding mesenchyme differentiate into tissues that eventually deposit hard tissue matrix in the shape of the tooth, leading to the formation of the crown and root of the tooth. When this process is disrupted, it leads to the formation of supernumerary teeth and malformed fused teeth. The overexpression of Wnt in mutant mice seems to lead to these dental abnormalities.

There were also significant size differences in the length of the crown of the molar teeth of the mutants compared to the wild-type. This indicates that the fused molar teeth were longer than the wild-type molars. As the fused molar teeth were probably made as a result of a supernumerary tooth germ fusing to one or more molar tooth germs during tooth development, this led to the fused molar tooth being longer than one wild-type molar tooth. Hence, there was a difference of length between wild-type and mutants. However, there was no difference between the height of wild-type and mutant teeth. This means that the height of the mutant teeth was not affected by the abnormalities present during tooth development.

Another difference in crown length was between the supernumerary premolar-shaped mutant teeth and the wild-type molar. The supernumerary premolar-shaped mutant tooth was shorter in length than the wild-type molar. The wild-type molar was longer in length and had two roots per tooth. The supernumerary mandibular mutant tooth was shaped like a premolar; hence it was shorter in length and only single-rooted. This explains the difference in length between the mutant premolar-shaped tooth and the wild-type molar tooth.

One significant difference in the thickness of enamel was found in mandibular mutant molar teeth. The mandibular mutant molar teeth had thicker enamel layer compared to the wild-

type molar teeth. Disrupted tooth development during deposition of hard tissue matrix must have led to increased deposition of enamel in the mandibular mutant molar teeth.

The mutant mandibles were significantly longer than the wild-type mandibles on X ray. However, the height of the mutant mandibles were not significantly different than the wild-type mandibles. This means that overexpression of Wnt led to the increase in length of the mandible during bone formation in the mandible.

According to Glass II and Karsenty, 2006 we should have expected differences in bone density between Lrp4 conditional knockout mice and wild-type mice, however we did not observe any difference in bone density on microCT data comparing Lrp4 knockout to wild-type. Glass II and Karsenty observed the differences in long bone (endochondral bone), whereas in our study we observed maxilla and mandible (intramembranous bones). Intramembranous bones form differently than endochondral bone. Endochondral bone forms from cartilaginous growth plates. First cartilage forms, which then turns into bone. Intramembranous bone, on the other hand, forms directly, without the involvement of cartilage.In conclusion, the findings of supernumerary teeth and fused teeth were consistent with the literature, although we did not find differences in bone density as stated in the literature.

REFERENCES

Ahn Y, Sanderson BW, Klein OD, Krumlauf R. Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning. Development. 2010;137(19):3221-3231. doi:10.1242/dev.054668

Ahn Y, Sims C, Murray MJ, et al. Multiple modes of Lrp4 function in modulation of Wnt/β-catenin signaling during tooth development. Development. 2017;144(15):2824-2836. doi:10.1242/dev.150680

Ahn Y. Signaling in tooth, hair, and mammary placodes. Curr Top Dev Biol. 2015;111:421-459. doi:10.1016/bs.ctdb.2014.11.013

Bonewald LF, Johnson ML. Osteocytes, mechanosensing and Wnt signaling. Bone. 2008;42(4):606-615. doi:10.1016/j.bone.2007.12.224

Chang MK, Kramer I, Huber T, et al. Disruption of Lrp4 function by genetic deletion or pharmacological blockade increases bone mass and serum sclerostin levels. Proc Natl Acad Sci U S A. 2014;111(48):E5187-E5195. doi:10.1073/pnas.1413828111

Chen G, Ishan M, Yang J, et al. Specific and spatial labeling of P0-Cre versus Wnt1-Cre in cranial neural crest in early mouse embryos. Genesis. 2017;55(6):10.1002/dvg.23034. doi:10.1002/dvg.23034

Collette NM, Genetos DC, Economides AN, et al. Targeted deletion of Sost distal enhancer increases bone formation and bone mass. Proc Natl Acad Sci U S A. 2012;109(35):14092-14097. doi:10.1073/pnas.1207188109

Collette NM, Yee CS, Murugesh D, et al. Sost and its paralog Sostdc1 coordinate digit number in a Gli3-dependent manner. Dev Biol. 2013;383(1):90-105. doi:10.1016/j.ydbio.2013.08.015

Cruciat CM, Niehrs C. Secreted and transmembrane wnt inhibitors and activators. Cold Spring Harb Perspect Biol. 2013;5(3):a015081. Published 2013 Mar 1. doi:10.1101/cshperspect.a015081

Dassule HR, Lewis P, Bei M, Maas R, McMahon AP. Sonic hedgehog regulates growth and morphogenesis of the tooth. Development. 2000;127(22):4775-4785. doi:10.1242/dev.127.22.4775

Davidson G. LRPs in WNT Signalling. Handb Exp Pharmacol. 2021;269:45-73. doi: 10.1007/164_2021_526. PMID: 34490514.

Duan P, Bonewald LF. The role of the wnt/β-catenin signaling pathway in formation and maintenance of bone and teeth. Int J Biochem Cell Biol. 2016;77(Pt A):23-29. doi:10.1016/j.biocel.2016.05.015

Ellies DL, Krumlauf R. Bone formation: The nuclear matrix reloaded. Cell. 2006;125(5):840-842. doi:10.1016/j.cell.2006.05.022

Glass DA 2nd, Karsenty G. Molecular bases of the regulation of bone remodeling by the canonical Wnt signaling pathway. Curr Top Dev Biol. 2006;73:43-84. doi:10.1016/S0070-2153(05)73002-7

Gritli-Linde A, Bei M, Maas R, Zhang XM, Linde A, McMahon AP. Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. Development. 2002;129(23):5323-5337. doi:10.1242/dev.00100

He X, Semenov M, Tamai K, Zeng X. LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development. 2004;131(8):1663-1677. doi:10.1242/dev.01117

Herz J, Bock HH. Lipoprotein receptors in the nervous system. Annu Rev Biochem. 2002;71:405-434. doi:10.1146/annurev.biochem.71.110601.135342

Hsu YH, Kiel DP. Clinical review: Genome-wide association studies of skeletal phenotypes: what we have learned and where we are headed. J Clin Endocrinol Metab. 2012;97(10):E1958-E1977. doi:10.1210/jc.2012-1890

Itasaki N, Jones CM, Mercurio S, et al. Wise, a context-dependent activator and inhibitor of Wnt signalling. Development. 2003;130(18):4295-4305. doi:10.1242/dev.00674

Johnson EB, Hammer RE, Herz J. Abnormal development of the apical ectodermal ridge and polysyndactyly in Megf7-deficient mice. Hum Mol Genet. 2005;14(22):3523-3538. doi:10.1093/hmg/ddi381

Kassai Y, Munne P, Hotta Y, et al. Regulation of mammalian tooth cusp patterning by ectodin. Science. 2005;309(5743):2067-2070. doi:10.1126/science.1116848

Klein OD, Minowada G, Peterkova R, et al. Sprouty genes control diastema tooth development via bidirectional antagonism of epithelial-mesenchymal FGF signaling. Dev Cell. 2006;11(2):181-190. doi:10.1016/j.devcel.2006.05.014

Leupin O, Piters E, Halleux C, et al. Bone overgrowth-associated mutations in the LRP4 gene impair sclerostin facilitator function. J Biol Chem. 2011;286(22):19489-19500. doi:10.1074/jbc.M110.190330

Li X, Ominsky MS, Niu QT, et al. Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. J Bone Miner Res. 2008;23(6):860-869. doi:10.1359/jbmr.080216

Li Y, Pawlik B, Elcioglu N, et al. LRP4 mutations alter Wnt/beta-catenin signaling and cause limb and kidney malformations in Cenani-Lenz syndrome. Am J Hum Genet. 2010;86(5):696-706. doi:10.1016/j.ajhg.2010.03.004

Liu J, Xiao Q, Xiao J, et al. Wnt/β-catenin signalling: function, biological mechanisms, and therapeutic opportunities. Signal Transduct Target Ther. 2022;7(1):3. Published 2022 Jan 3. doi:10.1038/s41392-021-00762-6

MacDonald BT, He X. Frizzled and LRP5/6 receptors for Wnt/β-catenin signaling. Cold Spring Harb Perspect Biol. 2012;4(12):a007880. Published 2012 Dec 1. doi:10.1101/cshperspect.a007880

MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell. 2009;17(1):9-26. doi:10.1016/j.devcel.2009.06.016

Michigami T. [Wnt Signaling and Skeletal Dysplasias.]. Clin Calcium. 2019;29(3):323-328. Japanese. doi: 10.20837/4201903323. PMID: 30814377.

Moon RT, Bowerman B, Boutros M, Perrimon N. The promise and perils of Wnt signaling through beta-catenin. Science. 2002;296(5573):1644-1646. doi:10.1126/science.1071549

Murashima-Suginami A, Takahashi K, Kawabata T, et al. Rudiment incisors survive and erupt as supernumerary teeth as a result of USAG-1 abrogation. Biochem Biophys Res Commun. 2007;359(3):549-555. doi:10.1016/j.bbrc.2007.05.148

Mustonen T, Pispa J, Mikkola ML, et al. Stimulation of ectodermal organ development by Ectodysplasin-A1. Dev Biol. 2003;259(1):123-136. doi:10.1016/s0012-1606(03)00157-x

Ohazama A, Johnson EB, Ota MS, et al. Lrp4 modulates extracellular integration of cell signaling pathways in development [published correction appears in PLoS ONE. 2009;4(1). doi: 10.1371/annotation/f19bff3b-227a-4159-b166-7dc3c19bec43. Choi, Hong J [corrected to Choi, Hong Y]]. PLoS One. 2008;3(12):e4092. doi:10.1371/journal.pone.0004092

Thomas PA, Wilson-Delfosse AL, Mehta N, Papp KK, Bierer SB, Isaacson JH. Case Western Reserve University School of Medicine, Including the Cleveland Clinic Lerner College of Medicine. Acad Med. 2020;95(9S A Snapshot of Medical Student Education in the United States and Canada: Reports From 145 Schools):S396-S401. doi:10.1097/ACM.0000000000003411

Visweswaran M, Pohl S, Arfuso F, et al. Multi-lineage differentiation of mesenchymal stem cells - To Wnt, or not Wnt. Int J Biochem Cell Biol. 2015;68:139-147. doi:10.1016/j.biocel.2015.09.008

Wang K, Ren Y, Lin S, et al. Osteocytes but not osteoblasts directly build mineralized bone structures. Int J Biol Sci. 2021;17(10):2430-2448. Published 2021 Jun 11. doi:10.7150/ijbs.61012

Wang Z, Ma C, Chen D, et al. Tendon Cells Root Into (Instead of Attach to) Humeral Bone Head via Fibrocartilage-Enthesis. Int J Biol Sci. 2023;19(1):183-203. Published 2023 Jan 1. doi:10.7150/ijbs.79007

Weatherbee SD, Anderson KV, Niswander LA. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. Development. 2006;133(24):4993-5000. doi:10.1242/dev.02696

Willnow TE, Christ A, Hammes A. Endocytic receptor-mediated control of morphogen signaling. Development. 2012;139(23):4311-4319. doi:10.1242/dev.084467

Yu T, Klein OD. Molecular and cellular mechanisms of tooth development, homeostasis and repair. Development. 2020;147(2):dev184754. Published 2020 Jan 24. doi:10.1242/dev.184754

Yuan G, Yang G, Zheng Y, et al. The non-canonical BMP and Wnt/β-catenin signaling pathways orchestrate early tooth development. Development. 2015;142(1):128-139. doi:10.1242/dev.117887

Zheng J, Chen S, Albiero ML, et al. Diabetes Activates Periodontal Ligament Fibroblasts via NF-κB In Vivo. J Dent Res. 2018;97(5):580-588. doi:10.1177/0022034518755697