INVESTIGATING THE ROLE OF DKK3 IN TEMPOROMANDIBULAR JOINT HOMEOSTASIS IN THE MURINE MODEL

by

Minjie Paark

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Minjie Paark

Approved: _____
Joohyun Lim, Ph.D
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Aimee Jaramillo-Lambert, Ph.D
Committee member from the Department of Biological Sciences

Approved: _____
Carlton Cooper, Ph.D
Committee member from the Board of Senior Thesis Readers

Approved: _____
Michael Chajes, Ph.D
Dean, University Honors Program

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ABSTRACT

The temporomandibular joint (TMJ), also known as the jaw joint, is essential to daily human function. Without proper jaw function, necessary activities like eating, drinking, and speaking can become painful and impaired [1]. In studying the TMJ, both histologically and molecularly, our understanding of how disorders of the jaw joint develop can be furthered.

Controlling the canonical Wnt (cWnt) signaling pathway has been thought to have essential function in the maintenance of the TMJ. Preventing cWnt signaling in putative progenitor cell populations may allow for these cells to differentiate into necessary fibrocartilage for the TMJ articulating surfaces [2]. One likely inhibitor of cWnt, which has been less thoroughly researched *in vivo*, is Dkk3.

To study the role of Dkk3 in TMJ homeostasis *in vivo*, the Dkk3 gene was deleted in transgenic mice and compared to Dkk3 wild type. Then, the TMJs of these mice were studied through H&E histology, fluorescent reporters tagging Wnt signaling activity, and micro-CT.

Our findings suggest that Dkk3 is indeed important *in vivo* to the maintenance of putative stem progenitor populations in the TMJ condyle and fossa, and in the absence of Dkk3 these populations may be depleted and differentiated into bone by excessive Wnt signaling, causing abnormal TMJ features, particularly in the fossa.

INTRODUCTION

1.1 Temporomandibular Joint Disorders

Temporomandibular joint (TMJ) dysfunction is a significant public health issue that affects a multitude of individuals in the human population. A recent meta-analysis and systematic review of over 2500 articles stated that the overall prevalence of temporomandibular joint disorders (TMDs) could be as high as 31% in adults, and 11% in children [3]. Disorders that fall under the category of TMDs include a variety of musculoskeletal and neuromuscular conditions within the jaw joint that can cause jaw locking, clicking, and other painful issues. Moreover, these symptoms impact essential human functions such as talking and eating. These clinical features cause chronic pain and can significantly impact quality of life [1, 4].

Currently, there is no fully effective cure for TMDs. Methods including injections, physical therapy, and surgical bone and joint procedures have been implemented, but remain controversial on their success and efficient symptom relief. Therefore, there is a need for improved and minimally invasive treatments, as well as a deeper understanding of how TMDs develop within this relatively novel field [1].

1.2 TMJ Biology

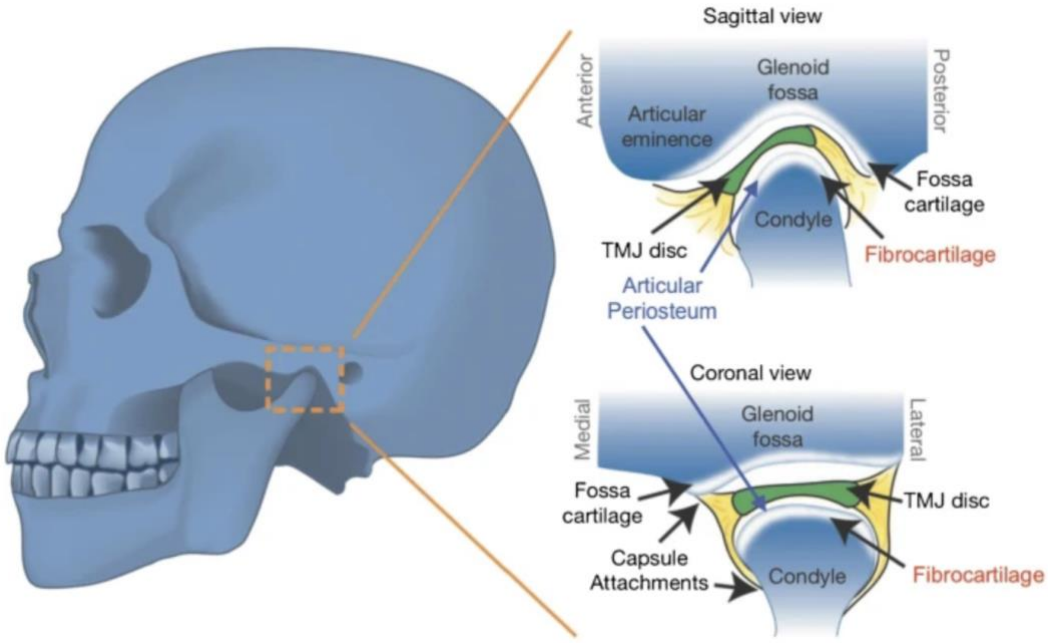

Figure 1 **Temporomandibular joint anatomy.** The temporomandibular joint (TMJ) and basic anatomical features. The region of interest, which includes the articular disc, condyle (below the disc), and glenoid fossa (above the disc), is emphasized [5].

The TMJ is a synovial joint which consists of the glenoid fossa of the temporal bone, the fibrocartilaginous articular disc, and the mandibular condyle. During development, the temporal bone and condyle form first, and the condylar blastema grows towards the temporal blastema while the articular disc forms between them [5].

The TMJ is unique in many ways compared to other synovial joints. It develops through appositional growth, rather than cleavage of continual skeletal condensation [5]. The surface layer of the mandibular condyle consists primarily of type I collagen, rather than type II collagen which most synovial joint surfaces express [6]. The TMJ also consists of fibrocartilage rather than hyaline cartilage, which covers the articular surfaces of most other synovial joints [5].

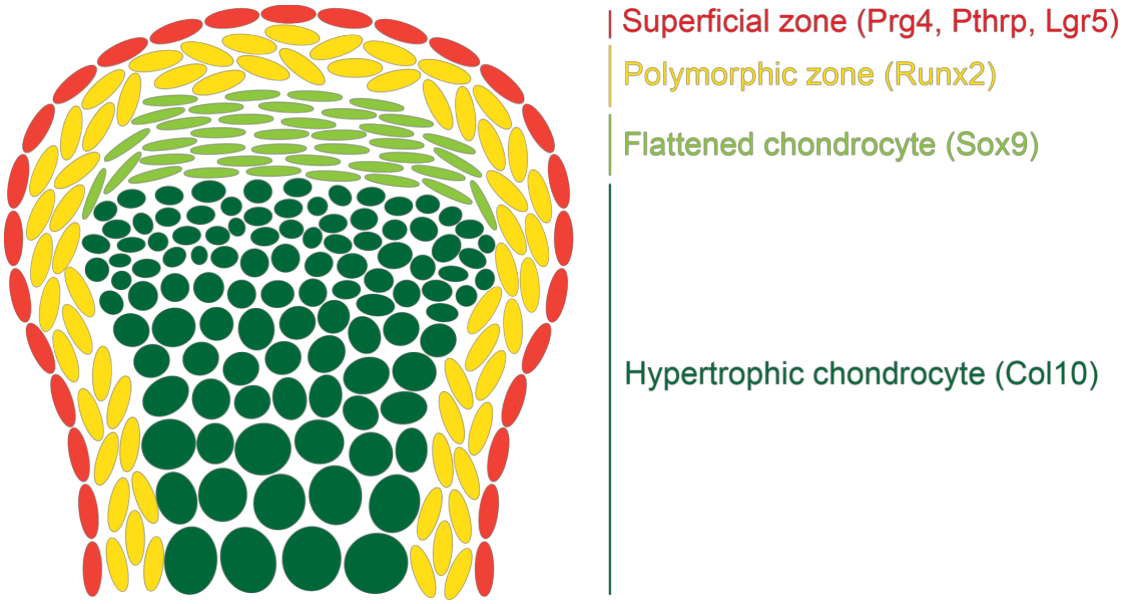

Figure 2 **TMJ histology and zonal compartments.** Distinct compartments of cell types observed on the TMJ condyle [7].

The TMJ also has distinct compartments of cell populations that we can observe in its histology, which possess different gene expression patterns. On the surface of the fossa and condyle, is the superficial layer. Within this superficial zone exists the unique fibrocartilage surface, which consists mainly of type I collagen. This surface layer can be observed through markers like *Prg4*, which tags fibrocartilage cell populations and essential lubricating protein lubricin; *Pthrp*, which marks parathyroid hormone— a crucial regulator of bone formation; and *Lrg5*, which marks fibrocartilage stem progenitor cell populations. [5, 6].

These features can all suggest that there are unique functions and molecular mechanisms at play within the temporomandibular joint— a niche yet to be studied extensively *in vivo*. Thus, observing the TMJ biology, surface structure, and cell and molecular mechanisms could provide insights into how TMDs develop, and what processes and gene expression in TMJ homeostasis are vital for maintaining the jaw joint.

1.3 Current Research

Whereas the articular cartilage and growth plate in the appendicular skeleton have been characterized in detail, our understanding of the TMJ is limited. More recently, several studies have suggested that the superficial and proliferative zones in the mandibular condyle may play a role in harboring stem/progenitor cell populations that give rise to mandibular condyle cartilage and bone. Interestingly, these findings suggest that the organization of the mandibular condyle partly resembles that of the growth plate in which stem/progenitor cells are maintained in the resting zone at the top of the growth plate followed by proliferative and maturing zones of chondrocytes [8, 9].

Skeletal stem/progenitor cells (SSPCs) are a heterogeneous group of cell populations that can derive multiple cell types including those of the chondrogenic and osteoblastic lineage [10]. Recent work in the past decade has revealed that, unlike hematopoietic stem cells (HSCs) which can reconstitute the entire bone marrow following transplantation in irradiated patients, SSPCs have more specialized functions and reside in anatomically distinct locations such as on the surfaces of the bone and blood vessels in the bone marrow cavity, periosteal/periskeletal regions, fibrous cartilage, and other regions in the skeletal microenvironment, i.e., niche [10, 2]. Within the TMJ, putative fibrocartilage stem progenitor cell populations (FSPCs) have the potential to differentiate into chondrocytes and osteoblasts/osteocytes that form, respectively, the mandibular condyle cartilage or bone [11].

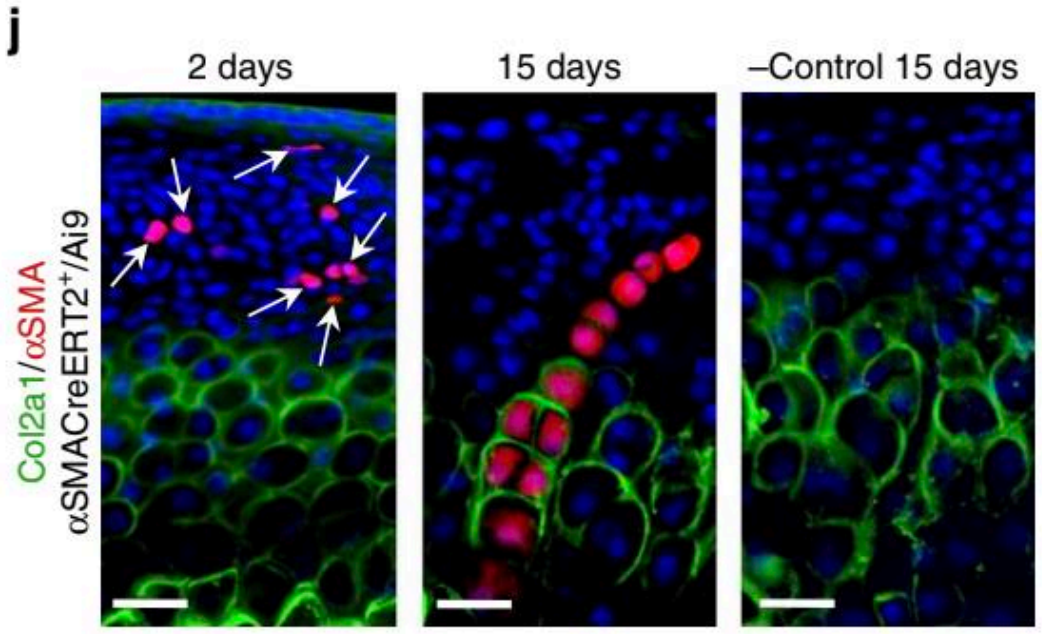

Figure 3 **Dr. Embree et al. FSPC lineage tracing on the TMJ condyle.** Immunohistochemistry for type 2 collagen shown in green, and alpha-smooth muscle actin (αSMA) cells in red using αSMACreERT2/Ai9 mice, treated with tamoxifen at 16 days old to induce Cre recombinase and then quantified at days 2 and 15, or untreated for negative control [11].

As seen in Figure 3, alpha-smooth muscle actin (αSMA), a putative marker of these FSPCs, was utilized along with red fluorescence to visualize the potential fibrocartilage progenitor cell populations differentiating into mature chondrocytes in the deeper layers of the mandibular condyle over time. These findings suggest that these progenitor populations have the potential to differentiate into mature cartilage populations and eventually form the lower layers of mature chondrocytes in the condyle.

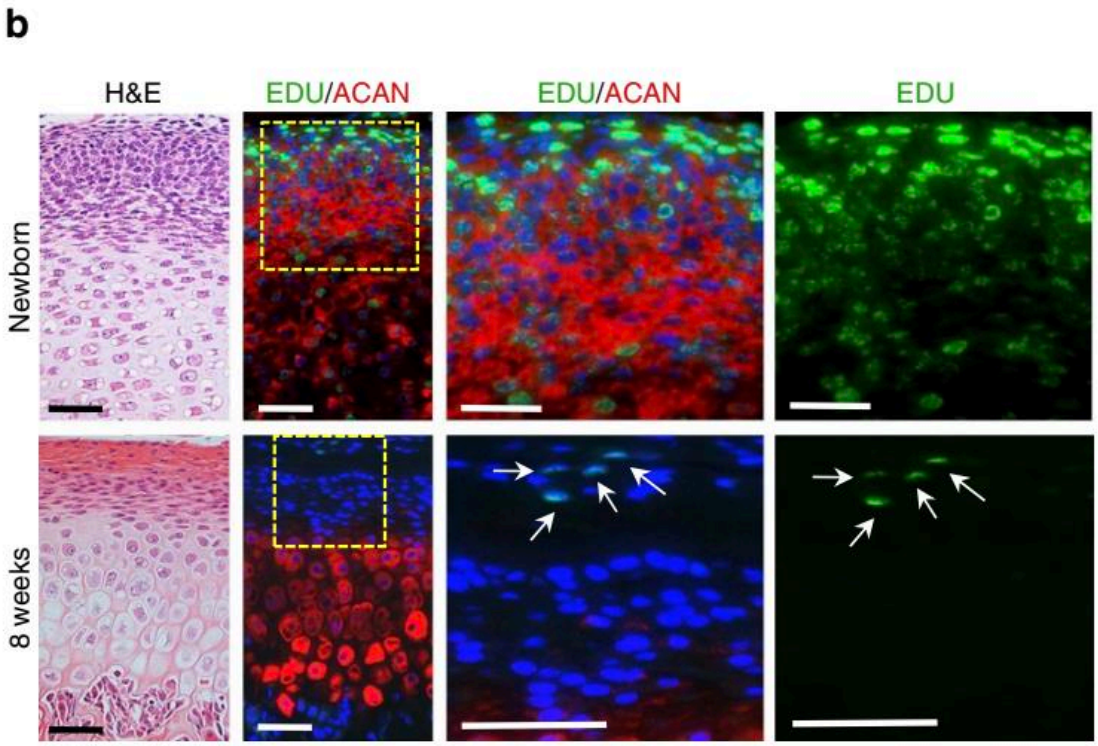

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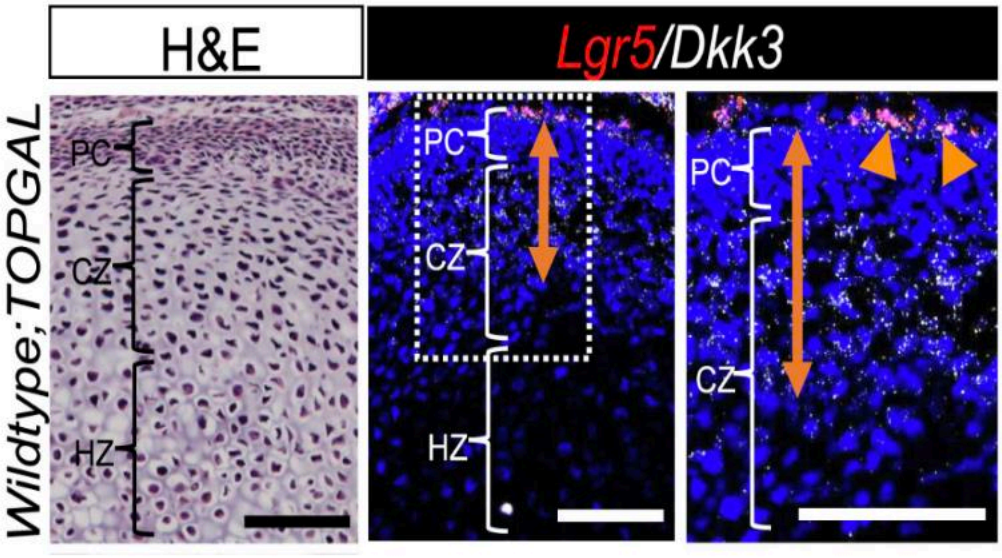
Figure 4 shows the location of these FSPCs within the mandibular condyle utilizing aggrecan, a mature chondrocyte marker, and a thymidine analog (EDU) to mark actively replicating cells. From newborn to 8 weeks, the FSPCs in the mouse TMJ became much more restricted to the surface layer over time [11].

To continue these findings, further studies focused on the molecular mechanisms which guide the differentiation of these FSPCs. Dysregulated differentiation of these progenitor populations had the potential to be a key cause of TMDs in the jaw joint.

In 2023, the hypothesis rose that the inhibition of canonical Wnt signaling, an essential pathway well known in its role for bone development and homeostasis, might be critical for

maintaining FSPCs, as well as FSPC fate speciation into cartilage rather than skeletal cell populations [2].

Dr. Ruscitto et al. suggested that, at least *in vitro*, Dkk3 may regulate canonical Wnt activity in the TMJ superficial zone. They suggested that the cells of the superficial zone in the mandibular condyle secrete Dkk3 to inhibit canonical Wnt signaling.



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In Figure 5, we can observe that Dkk3 expression is robust in the TMJ condyle, with particularly high expression within the superficial and chondrocyte zones. This provides promising support that Dkk3 has a role in FSPC maintenance within the temporomandibular joint [2]. However, because their study used *in vitro* culture, which is an isolated environment,

there exists a research niche to pursue this hypothesis *in vivo* through the generation of cell-specific Dkk3 knockout in live model organisms.

1.4 Significance of Wnt Signaling

As the role of stem progenitor cell populations became an interest in TMJ research, the canonical Wnt (cWnt) signaling pathway rose in focus due to its widely suggested roles in development.

Wnt signaling has been extensively studied in its role as a regulator of various developmental and adult homeostasis processes [12]. There are two pathways utilized in Wnt signaling: β-catenin dependent, or canonical Wnt signaling, and β-catenin independent, also known as noncanonical Wnt signaling.

The cWnt signaling pathway is thought to provide an essential role in bone development and homeostasis, especially in the proliferation and differentiation of mesenchymal stem cells and stem progenitor cell populations into bone [13]. This causes it to be an ideal pathway to study in relation to TMJ homeostasis, as TMDs often display dysregulated and abnormal bone and cartilage populations [1].

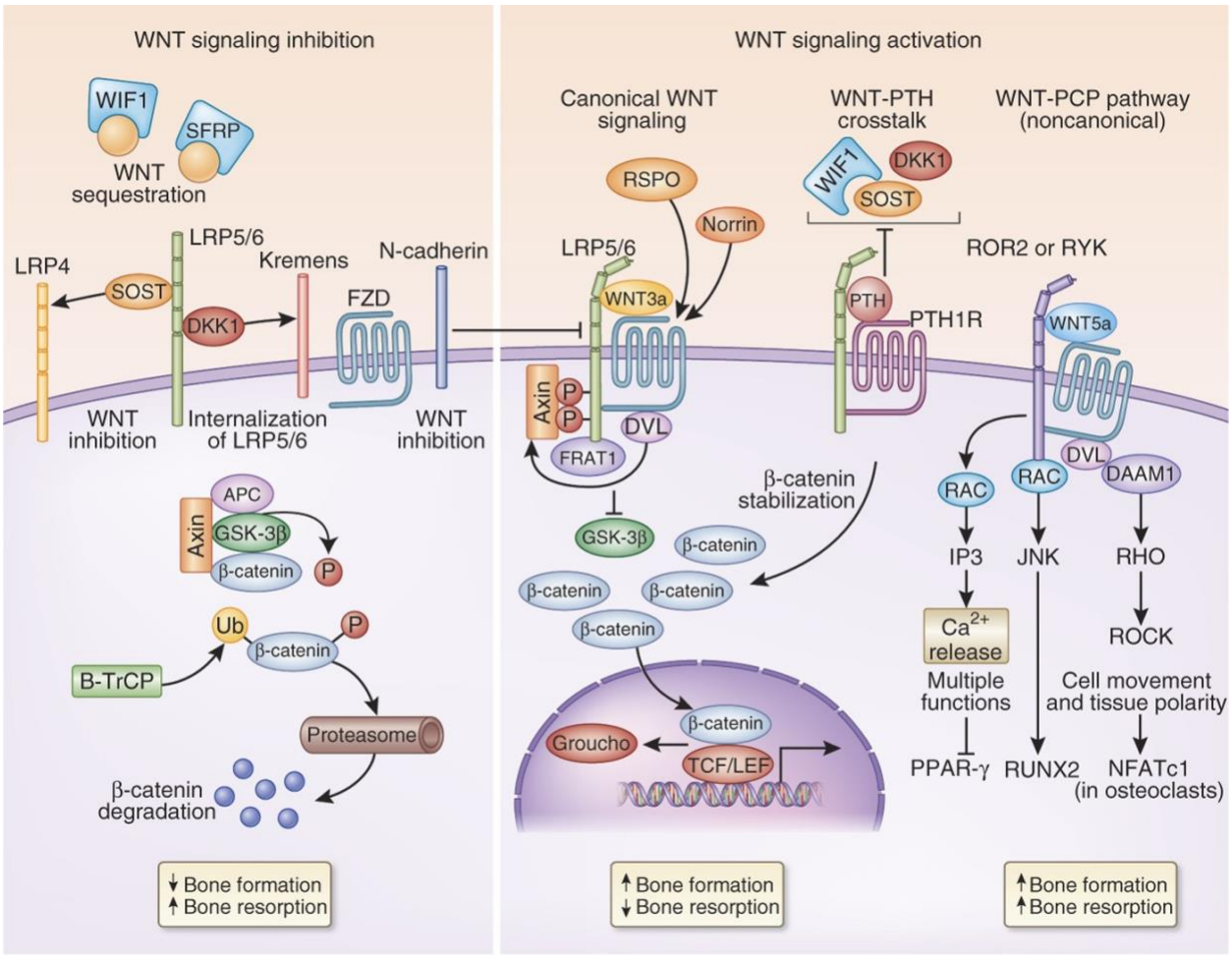

Figure 6 **Simplified overview of Wnt signaling pathways.** On the left marking cWnt signaling inhibition, note the Dkk3 competitive inhibition of Wnt on the LRP5/6 receptor, and β-catenin phosphorylating destruction complex. On the right is a depiction of cWnt signaling activation, including the disassociation of the protein complex and mechanisms of β-catenin stabilization. In addition, a short review of noncanonical Wnt signaling is shown which does not utilize β-catenin [12].

Shown on the left of Figure 6, in the absence of Wnt. a protein complex in the cytoplasm phosphorylates β-catenin, which ultimately marks it for destruction and prevents it from entering the nucleus. Furthermore, in the nucleus, transcription factor T-cell factor (TCF) and Groucho generate a constitutive repressor complex, inhibiting target gene transcription [12].

On the right of Figure 6, it is shown that in the presence of the Wnt ligand, signaling from the coupling of the Frizzled receptor and LRP5/6 coreceptor causes dishevelled (DVL or DSH) to be

recruited to the plasma membrane. Dishevelled then acts as a site which allows for the phosphorylation of LRP6 and recruits Axin, which was previously a part of the protein destruction complex. This allows for β-catenin to accumulate within the cell and eventually enter the nucleus and interact with transcription factors, turning on target genes [12].

1.5 Significance of Dkk3

Canonical Wnt signaling, as an essential and ubiquitous pathway, has a variety of antagonists that inhibit its activation. One of the most extensively researched is the Dickkopf (Dkk) family of proteins.

There are four proteins in the Dkk family, labelled as Dkk1 through 4. Dkk1 is the most well studied and is widely believed to be a competitive inhibitor which prevents Wnt binding to the receptor LRP5/6. Dkk1, 2, and 4 all possess similar structure, including the LRP5/6 binding domain. However, the Dkk3 structure is significantly different from Dkk1, suggesting unique function [14].

Especially with the findings of Dr. Ruscitto et al., which suggested that Dkk3 expression was robust within the FSPCs of the TMJ and provided these populations protection from cWnt signaling, studying the effects of Dkk3 knockout *in vivo* may provide realistic insight into the function of Dkk3 in cWnt signaling, particularly for maintaining TMJ homeostasis.

1.6 Hypothesis and Proposed Aims

Our goal is to investigate the role of Dkk3 in TMJ homeostasis by observing the consequence of Dkk3 deletion in the mouse model. Based on the findings of current research, our hypothesis is that Dkk3 provides negative regulation of cWnt signaling in the TMJ *in vivo*.

To observe the effects of Dkk3 deletion on TMJ homeostasis, we have proposed two aims as following:

- **Aim 1. To examine if Dkk3 deficiency affects chondrogenesis and osteogenesis in TMJ:**
    - Visualization of bone volume and mandibular geometry by micro-computed tomography analysis
    - Histological analysis of TMJ to examine cartilage and bone histomorphometry
- **Aim 2. To test if Dkk3 deletion alters cWnt signaling**:
    - Fluorescence imaging of Dkk3 knockout mice with TCF/Lef:H2B-GFP
    - Immunofluorescence (IF) analysis of Dkk3 knockout mice to examine cell-specific markers

With these aims, we seek to provide insight into Dkk3's relationship to cWnt signaling *in vivo* and how issues within the TMJ develop. Understanding the molecular and genetic mechanisms of the TMJ may create new opportunities for therapies of TMJ dysfunction.

Chapter 2

METHODS

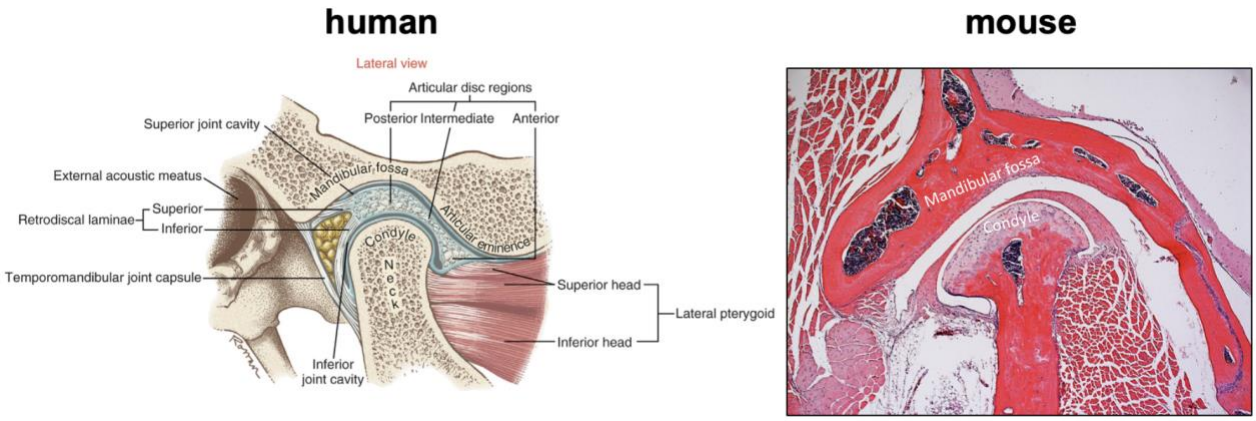

Figure 7 **Conserved features in human and mouse TMJ anatomy.** The mouse model provides comparable features in relation to the human TMJ, including a similar mandibular fossa, articular disc, and condyle [15, 7].

In our studies, we utilized the mouse model. In Figure 7, one can observe the similarities and conserved anatomical features between the human and murine jaw joint. The consistency of these features allows us to use the mouse as an ideal model for the human TMJ.

2.1 Mouse Mating Strategy

Dkk3 knockout mice (B6.Cg-Dkk3<tm1.1>, RIKEN) and TCF/Lef:H2B-GFP transgenic mice (Jackson Laboratory, Stock #000664) were kindly provided by Melinda Duncan. All animal work was approved by the University of Delaware Institutional Animal Care and Use Committee.

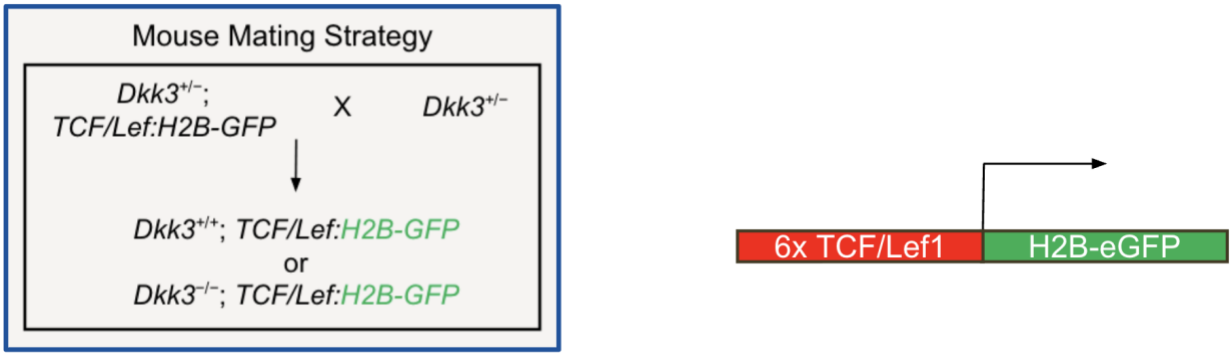

Figure 8 **Fluorescence marker mouse mating strategy.** Utilizing a mouse cross between two heterozygous mice, one with Wnt fluorescent reporter, allowed us to obtain our ideal Dkk3 KO and WT mice. TCF/Lef1, T cell factor and lymphoid enhancer-binding factor, as shown in Figure 6, are the downstream effectors of β-catenin dependent Wnt signaling. By overexpressing these, we would obtain stronger signal to visualize the presence of β-catenin activity in the TMJ.

2.2 Mouse Processing

Mice were euthanized with carbon dioxide, and mouse heads were collected in 10% neutral buffered formalin, and then incubated with shaking at 75 rpm at 4ºC for 16-24 hours. Subsequently, collected heads were rinsed in cold PBS, and decalcified in 14% EDTA for 2 to 3 weeks, incubated with shaking at 75 rpm at 4ºC. Between weeks 1-2 of decalcification, the decalcification reagent was discarded, and heads were dissected from the frontonasal suture to the coronal suture, taking care not to disrupt the TMJ. 14% EDTA was then replenished and decalcification continued with 2-3 reagent changes per week.

For fixed frozen sectioning, mouse head sections were further dissected to contain only one TMJ, incubated in 30% sucrose/PBS at 4ºC with shaking at 75 rpm overnight, and embedded with OCT (optimal cutting temperature) mounting medium. Frozen blocks and sections were stored at -20ºC, and sectioning was performed with a Leica cryostat at 7µm.

Paraffin sectioning was done using a Leica tissue processor on similarly further dissected mouse TMJs embedded in paraffin. Sectioning was done using a Leica microtome at 7µm and stored at room temperature. Hematoxylin and Eosin staining was performed according to manufacturer's protocols.

Sectioned slides were imaged using Zeiss AxioImager equipped with Apotome 3 for deconvolution microscopy.

2.3 Micro-computed tomography

Mouse heads were scanned utilizing a Brüker Skyscan 1276 uCT. Scans were obtained at X-ray source intensity of 85kV and 47mA, and resolution of 10.6µm. Analysis and reconstruction were done using NRecon, Data Viewer, and CTan. Threshold minimum and maximum of reconstruction was kept constant between specimen at 0 and .035918 respectively, to obtain comparable results.

Chapter 3

RESULTS & DISCUSSION

3.1 Results

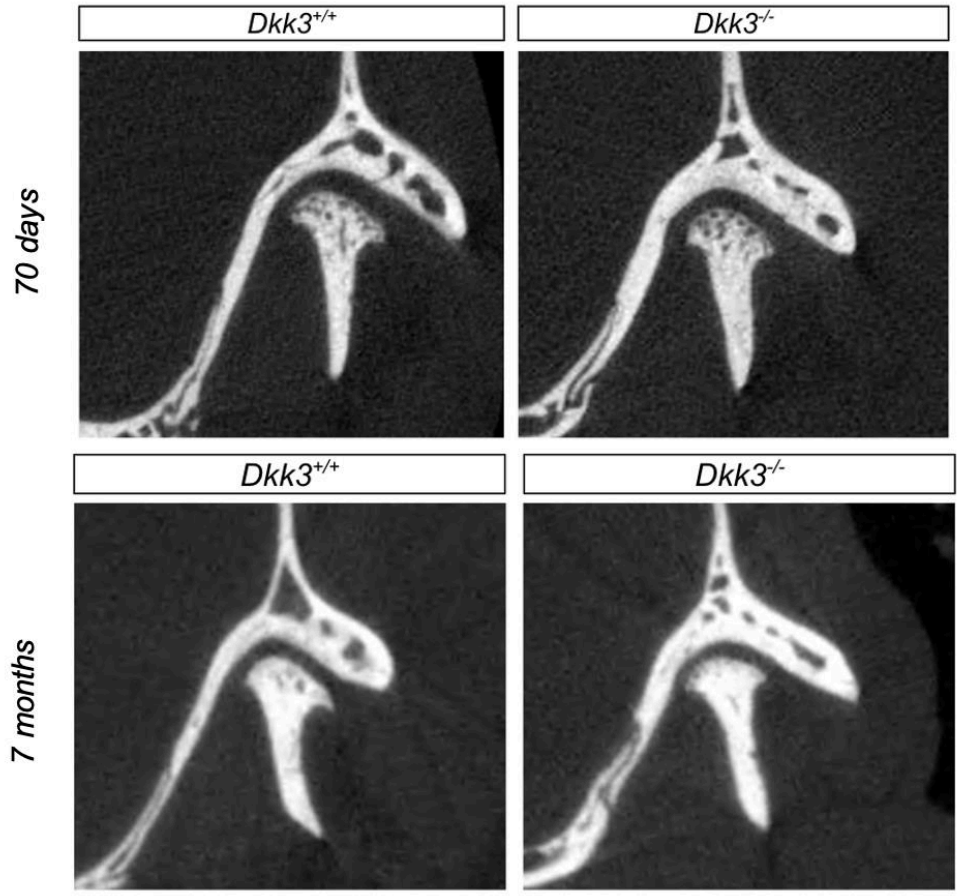

Figure 9 **Micro-CT shows higher bone signal and fossa elongation in Dkk3 KO mice at 7 months.** 70 days and 7 months high resolution CT scan comparison between Dkk3 KO and WT. Threshold is constant across all scans.

At 70 days of age, the removal of Dkk3 does not appear to have a phenotypic impact to the condyle or fossa, but at 7 months of age the previously observed Dkk3 knockout (KO) fossa elongation is shown through μCT. Moreover, there may be higher signal intensity in the 7-

month knockout specimen TMJ, which would suggest higher bone volume and align with increased Wnt signaling.

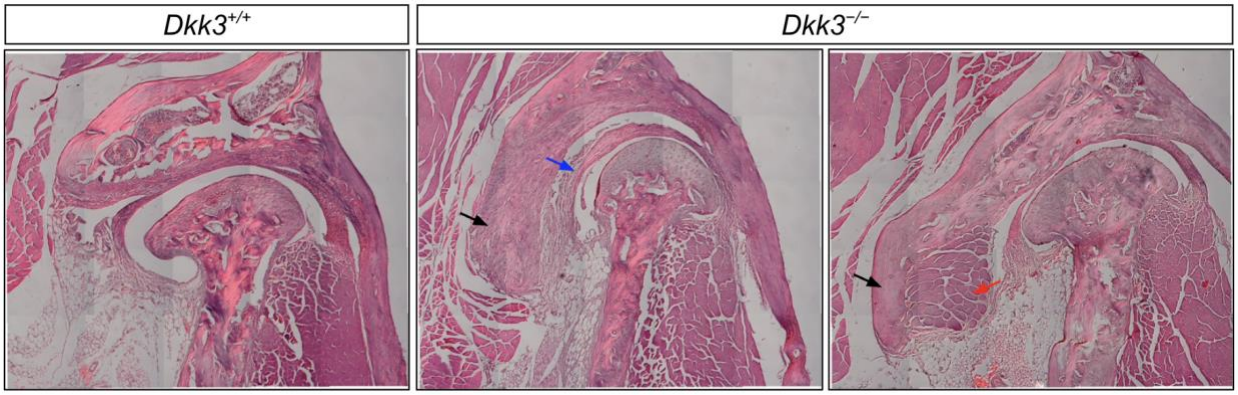

Figure 10 **Loss of Dkk3 causes ectopic muscle, bone, and fibrocartilage growth in the mandibular fossa.** H&E Histology of Dkk3 KO and WT mice. Black arrow shows elongation of the mandibular fossa, including ectopic bone (rightmost) or fibrotic tissue (center). Blue arrow shows disc degeneration, and red arrow signifies ectopic muscle. Stronger pink intensity signifies bone formation, while lighter represents cartilage.

Abnormal fossa elongation was further visualized through our histological study, and gave further insight into the Dkk3 KO phenotype. In the Dkk3 knockout mice, there was observable ectopic bone, muscle, or fibrous tissue growth on the glenoid fossa. It is also notable that the abnormal growth is variable, presenting as a variety of tissue types in different samples. There was also evidence of disc degeneration, which also suggests that Dkk3 is important for TMJ homeostasis. Loss of Dkk3 may also increase bone volume in the glenoid fossa at the expense of bone marrow space. In the wild type, there is a considerable lack of staining within the bone where the marrow typically presents, but in the KO specimens there is much less space for bone marrow in the mandibular fossa.

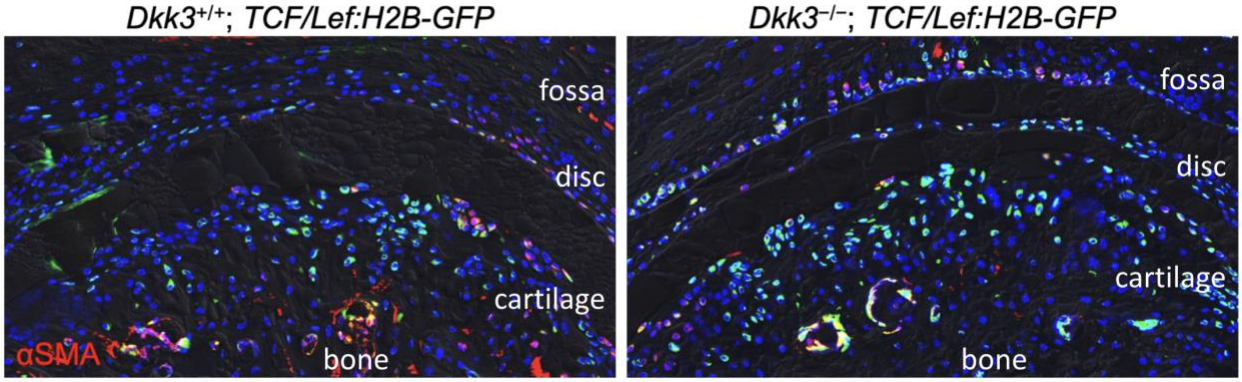

Figure 11 **Loss of Dkk3 increases Wnt signaling activity *in vivo*.** Fluorescent Wnt reporter activity in Dkk3 KO and WT mice. Wnt reporter activity is shown in green most notably on the condyle and fossa surfaces. Alpha smooth muscle actin (αSMA, red) immunofluorescence also shows putative SPCs across the superficial and chondrocyte zones. Colocalization of these green and red markers on the fossa and condyle surface is shown as yellow.

In preliminary data, we observed localized up-regulation of TCF/Lef:H2B-eGFP reporter activity in the articular surface of the glenoid fossa, disc, and surface layers of the mandibular condyle cartilage in Dkk3 knockout mice compared to WT littermate control. In addition, αSMA-positive cell populations appeared to decrease with the removal of Dkk3. These populations were colocalized with Wnt activity in some regions on the condyle and disc surface. These results suggest that Dkk3 may inhibit canonical WNT signaling in the articular surface of the mandibular condyle and glenoid fossa, as well as the mandibular disc and loss of Dkk3 may promote abnormal osteogenic differentiation of putative SPCs in TMJ.

3.2 Discussion

TMJ dysfunction can cause chronic pain and impair necessary human functions including consumption and communication [1]. By studying the function of Dkk3 in the TMJ *in vivo*, our aim was to observe if it has a role in the protection of putative FSPCs from cWnt signaling as

research *in vitro* suggests [2]. To address this goal, we utilized histological, morphological, and molecular analysis to compare Dkk3 deletion mice from wild type.

Our findings support our hypothesis that Dkk3 provides a cWnt inhibitory niche for the chondrocyte populations *in vivo*. Surprisingly, Dkk3 knockout mice appeared to have significant cWnt signaling presence on the glenoid fossa surface, based on fluorescent Wnt reporter activity. The majority of TMJ research focused extensively on the mandibular condyle over the fossa of the jaw joint, but in our Dkk3 experiments the most significant changes appear to have occurred in the fossa, including elongation and ectopic growth.

In our histological studies, we can observe that in the Dkk3 knockout compared to the wild type there appears to be a significant lengthening of the glenoid fossa, and possibly higher bone volume indicated by a greater amount of strong pink intensity from the H&E staining. This increased bone volume in the absence of Dkk3 may also be supported by micro-CT reconstruction, in which the bone signal appeared brighter in the knockout. Further micro-CT analysis and quantification would need to be done to determine the significance of this signal.

Our micro-CT scans suggest that the alterations in the TMJ occur post-development sometime after 70 days, presenting questions as to why this phenotype appears so late into the life cycle. In further research, testing ages between our two time points may provide useful in determining when this phenotype presents, and what might induce this conformational change.

We also observed, through fluorescence marker alpha smooth muscle actin (αSMA), that the stem progenitor cell populations appeared to be being depleted at a higher rate in the Dkk3 knockout specimen, suggesting that the uninhibited cWnt signaling in Dkk3 absence did indeed cause the putative SPCs to differentiate rapidly into bone, causing deformation of the TMJ.

Our findings are consistent with those of previous studies which have suggested the existence of FSPCs and a Dkk3 cWnt inhibitory niche in the TMJ condyle surface [2, 11]. However, we also provide new insights into the role of Dkk3 and FSPCs in the mandibular fossa, which has not been as emphasized in TMJ research as the condyle.

In our study, Dkk3 knockout mice were viable and presented with no gross abnormalities that would impact craniofacial development. However, there are limitations including that we were unable to obtain statistical analysis of certain findings, such as quantification of fluorescent signal and micro-CT bone signal. Individual specimens were presented for our comparisons, and a small sample of mice was used in our research. In future experiments, a higher number of data points and statistical analysis may be utilized to determine the statistical significance of Dkk3 knockout findings. Further study could also determine the effects of other well-known cWnt signaling inhibitors, such as Dkk1, at the consequence of Dkk3 deletion. These findings on the role of Dkk3 in TMJ homeostasis could present use in determining the causes of TMDs and how dysregulation in TMJ development and homeostasis occurs.

Chapter 4

CONCLUSION

TMDs continue to be a significant healthcare burden impacting patients across the world [1, 3]. To understand how dysfunction in the TMJ occurs, our aim was to understand the morphological, histological, and molecular mechanisms of the TMJ when its homeostasis may be disrupted by the absence of Dkk3, a suggested regulator of the β-catenin dependent Wnt signaling pathway [2, 14]. To achieve these aims, micro-computed tomography, H&E staining, and fluorescent reporters were utilized.

In our findings, we noted an elongation of the fossa and possibly higher bone signal intensity at a timepoint between 70 days and 7 months of age from our micro-CT analysis. Moreover, in our sectioned specimen, we determined that ectopic growth of variable cell populations occurred on the fossa causing this elongation. Lastly, in our fluorescent Wnt reporter study, we observed increased Wnt signaling activity on the surface, as well as a decrease in putative SPC populations in our preliminary data.

The results of our studies, though limited by a need for statistical analysis, suggest a novel TMJ phenotype for Dkk3 loss *in vivo* in the mouse model and encourage fossa study in relation to TMDs. Our findings support that Dkk3 provides a role in the surface layers of the TMJ as a cWnt inhibitor for FSPC populations *in vivo*. By researching the role of Dkk3 in TMJ homeostasis, our work may provide use in finding effective therapies for patients suffering from TMDs.

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