DICKKOPF-1 IN CRANIOFACIAL BONE AND TOOTH DEVELOPMENT

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2014

Major Subject: Biomedical Sciences

ABSTRACT

Dickkopf-1 (Dkk1) is a potent inhibitor of the Wnt/β-catenin signaling pathway in both tooth and bone development. Deletion of the *Dkk1* gene in mice leads to embryonic lethality, whereas transgenic mice over-expressing *Dkk1* in mature osteoblasts result in osteopenia. Targeted expression of *Dkk1* in dental epithelial cells leads to the formation of dysfunctional enamel knots and subsequent tooth defects during embryonic development. However, the direct role of Dkk1 in post-natal dentinogenesis and calvarial osteogenesis is largely unknown. To address this issue, we analyzed 2.3-kb Col1a1 *Dkk1*-transgenic (Tg) mice, which over-express *Dkk1* in immature and mature odontoblasts and osteoblasts.

The *Dkk1* transgene was highly expressed in pulp and odontoblast cells during post-natal developmental stages. A disruption in tooth development was obvious with the 1st mandibular molar displaying short roots, an enlarged pulp/root canal region, and a decreased dentin formation rate. Also within the mandible, the 2nd molar is small and malformed and the 3rd molar is absent. The molars possess an increased number of immature odontoblasts, very few mature odontoblasts, and a sharp reduction in dentinal tubule number accompanying a dramatic change in expression of certain odontoblast markers, such as Osterix and Nestin.

The calvarial bone presents with a significantly pronounced bone defect within *Dkk1*-Tg mice. The defect exhibits a markedly reduced calvarial bone density and multiple regions lacking any bone mineralization, which is indicative of an osteogenic deficiency. A greater than 60% reduction of parietal bone volume as well as an incomplete articulation of the parietal bones abutting the sagittal suture is evident. Irregularly shaped and abnormally small osteocytes are housed within the underdeveloped bone presenting more as osteoblastic-like cells in structure/function. Most obviously, the calvaria rather unusually presents with a considerable increase in osteoclast cell number, maturation, and activity present on the endo-cranial surface. An increase in immature osteoblast markers, as well as a decrease in mature osteoblast

markers combined with an increase in active osteoclast markers was evidence of a disrupted bone homeostasis.

From our studies we propose that within molar development and calvarial bone development, Dkk1 over-expression disrupts post-natal dentin formation and instigates an uncoupling of the osteoblastic/osteoclastic bone remodeling activities in favor of bone resorption with an increase in osteoclast-mediated destruction. Dkk1 is able to exert its effects directly via the inhibition of Wnt signaling or indirectly via its regulation of Osterix expression.

DEDICATION

I dedicate my dissertation work to my husband, Tyler, my parents, John & Denise, and my little brother, Aaron, who have all been a huge support system providing me with unwavering love and encouragement.

ACKNOWLEDGEMENTS

I wish to thank my committee members: Dr. Jerry Feng, Dr. Lynne Opperman, Dr. Paul Dechow, and Dr. Bruno Ruest, who were more than generous with their expertise and precious time. A special thanks is owed to Dr. Jerry Feng, my mentor, for his countless hours of reflecting, reading, encouraging, and most of all patience throughout the entire process. My committee's excitement and willingness to provide feedback made the completion of this research an enjoyable experience.

I would like to acknowledge Texas A&M Baylor College of Dentistry Biomedical Sciences Department for allowing me to conduct my research and providing any assistance requested. This includes a warm thank you to Dr. Kathy Svoboda, our graduate program director, who provided me with guidance and patience throughout the degree process. Another very warm thank you goes to the four ladies that keep everything together within the biomedical sciences department, Nancy Anthony, Jeanne Santa Cruz, Darla Benson, and Marge Palma, as well as Jo Spears within the office of research and graduate studies. These wonderful ladies have never hesitated to help me in times of need.

I would like to extend a very special thank you to Dr. Robert Spears for his co-mentoring on one of my research projects and for his undeniable support and kindness as well as his selfless consideration throughout my Ph.D program.

I would like to thank two lifelong friends that I met during my Ph.D training: Wendy Vu and John Bonds. Their friendships and support brought joy and happiness into my Ph.D. training program.

Finally, I would like to thank my very special Grandpa Robert, a true scientist, who instigated my love of science at a very young age by showing me my saliva under the microscope. My interest flourished and directed me towards become a scientist, just like him!

NOMENCLATURE

μCT	Microcomputer Tomography
β-Cat	Beta-Catenin
ALP	Alkaline Phosphatase
APC	Adenomatous polyposis coli
AXIN	Axis inhibition protein
BAC	Bacterial Artificial Chromosome
BSP	Bone Sialoprotein
CatK	Cathepsin K
cKO	Conditional Knockout
Cre	Cre “causes recombination” recombinase
DKK1	Dickkopf-1
DMP1	Dentin Matrix Protein 1
ECM	Extracellular Matrix
ER	Estrogen Receptor
FRZD	Frizzled
GOF	Gain of Function
KI	Knock-in
KO	Conventional Knockout
LacZ	β-Galactosidase
LOF	Loss of Function
LoxP	Lox sequence
LRP	Low-density lipoprotein receptor-related protein
MM	Multiple Myeloma
MMP	Matrix metalloproteinase
OB	Osteoblast
OC	Osteoclast

OCN	Osteocalcin
OPG	Osteoprotegerin
OSX	Osterix
PDL	Periodontal Ligament
RANK	Receptor activator of nuclear factor kappa
RANKL	Receptor activator of nuclear factor kappa ligand
SEM	Scanning Electron Microscopy
SOST	Sclerostin
TCF	T cell factor
TG	Transgene/Transgenic
TK	Thymidine Kinase
TRAP	Tartrate-resistant acid phosphatase
Wnt	Wingless
WT	Wild type

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INTRODUCTION AND LITERATURE REVIEW

Introduction

Bone and tooth development have been meticulously investigated in the field of craniofacial research as their formation are instrumental to life. Genes and proteins involved in their formation have been sought after for decades and many have been since discovered and intensely researched to allow for the piecing together of key signaling pathways. It is imperative to continue these studies as therapeutics depends on our discoveries and disclosure of the mechanics of intricate signaling pathways. The Wnt signaling pathway is one signaling pathway that is crucial in all areas of development from early embryogenesis, through organogenesis and beyond. The role of Wnt signaling in bone and tooth formation has been explored but through the generation and utilization of mouse models its role in these developmental processes became less concrete and warranted further analyses for a more thorough understanding.

Craniofacial research has been made possible and has progressed over the years through the creation and implementation of mouse models. Genetically engineered mice are powerful animal models used for studying cutting-edge craniofacial research analyzing developmental and disease states affecting tooth, bone and cartilage. These techniques have been well established over the past few decades and allow for any gene of interest to be genetically modified in a predetermined way based upon basic and clinical research needs. These mouse models are created and analyzed in hope that their use in research will initiate translational approaches for enhanced understanding of human diseases and enhance subsequent patient treatment.

Chapter I provides the basic foundation of knowledge for medical/dental researchers to further understand how genetically engineered mice are generated. It also details on how to select for utilization specific mouse models directed towards specific

research areas, and will elaborate on the advantages and disadvantages of using different mouse models.

Chapters II and III delve in-depth into the specifics of the 2.3-kb Col1a1 *Dkk1*-Tg mouse model. This mouse model over-expresses the *Dickkopf-1* (*Dkk1*) gene under a specific collagen type 1 alpha1 promoter. This promoter is a 2.3-kb fragment of the rat collagen 1 promoter, which is active in immature and mature odontoblasts and osteoblasts. Chapter II focuses on the use of the mouse model to study the role of Dkk1 in tooth morphogenesis, more specifically within dentinogenesis. Chapter III explores the role of Dkk1 in calvarial osteogenesis and how the time and location specific over-expression of the *Dkk1* gene causes major disruptions in bone homeostasis, and hence an altered regulation of bone formation and resorption.

Literature Review

Wnt/β-catenin signaling pathway

The Wnt/β-catenin signaling pathway is inherently a very complex pathway with an indispensible role in many developmental processes including, but not limited to, bone and tooth development (*1-5*). The Wnt proteins form a family of highly conserved secreted glycoprotein signaling molecules that regulate cell-to-cell interactions during embryogenesis. Mutations in the Wnt signaling pathway have known to cause many developmental defects and diseases (*1*). Wnt signaling is greatly involved in controlling cell proliferation, stem cell maintenance and cell fate decisions, as well as organized cell movements and the establishment of tissue polarity (*6*). Key players in the pathway include the classical Wnt receptor and the Frizzled (Frzd) receptor, a seven-pass transmembrane receptor possessing a cysteine-rich domain for the Wnt protein to bind to. Another known family of proteins in the signaling pathway is the low-density Lipoprotein Receptor-related Protein (LRP) family, two of the members, Lrp-5 and Lrp-6, being single-pass transmembrane co-receptors for Frzd. Wnts bind to Fzld family receptors and Lrp-5/6 co-receptors. These co-receptors have a relatively small

intracellular domains and a large extracellular domain containing several potential protein interaction domains.

In the absence of Wnt, β-catenin forms a complex with the APC, Axin and glycogen synthase kinase 3, which facilitates phosphorylation and proteosomal degradation of β-catenin. Stimulation of these receptors by Wnts leads to the intracellular molecule β-catenin to accumulate and translocate into the nucleus, where it interacts with the TCF/Lef1 transcription factor to activate transcription of target genes (*7*). Conditional inactivation of β-catenin in either skeletal progenitor cells or at a later stage of osteoblast development in mouse embryos blocks osteoblast differentiation (*7*). Dkk1 and Sost are antagonists that prevent the Wnt protein from activating the Frzd/ Lrp5/Lrp-6 receptor-signaling pathway leading to a decrease in signaling (*7*). The Wnt pathway in tooth and bone development is very complex as there are many different genes involved that either are Wnt affecters or are Wnt effectors. Studies have been performed *in vivo* and *in vitro* to further understand how Wnt interacts with other key factors and proteins.

Dickkopf-1

Although significant progress has been made studying gene mechanisms in bone and tooth development within the last decade, the role of Dkk1 in their formation during postnatal development is largely unknown. Dkk1, a major inhibitor of the Lrp-5/Lrp-6 co-receptors, is characterized as a soluble, secreted protein that, when bound to the co-receptors blocks their interaction with Wnt proteins resulting in β-catenin degradation and subsequent effects on cell proliferation (*3, 8-10*). Dkk proteins have also been linked to canonical and non-canonical signaling events. Conventional deletion of the *Dkk1* gene in mice led to embryonic lethality with the embryos lacking anterior head structures (*11*) and an elimination of *Dkk1* expression in osteoblasts resulted in an increase in bone formation and mass (*12*). On the contrary, over-expression of *Dkk1* under the 2.3-kb Col1a1 promoter resulted in severe defects in bone formation as well as molar tooth development (*13*).

Dickkopf-1 and tooth development

The mouse has a very reduced dentition compared to humans, but it still possesses molars and incisors and a dental developmental process that is very similar to humans. A major difference is that the mouse has only one dentition instead of a primary and a permanent dentition as seen in humans. Various stages of tooth development occur during embryogenesis and beyond. At E11.5 (embryonic day 11.5) the oral epithelium thickens and cell proliferation increases at the sites of future tooth formation (*14*). The ectoderm-derived epithelium then undergoes an invagination into underlying neural crest-derived mesenchyme, and these processes lead to the formation of a tooth bud. Within the developing tooth, *Dkk1* is expressed in the dental mesenchyme. At E12 the underlying mesenchyme condenses around the tooth bud and takes over the instructive role from the epithelium for future tooth formation. Overexpression of *Dkk1* in the early oral epithelium lead to a cessation of tooth formation at the bud stage (*15*).

Dkk1 has already been established as a major inhibitor of the Wnt signaling pathway and Wnt proteins are thought to play a significant role during the bud stage of tooth development. Within the developing tooth, Dkk1 shows a distinct spatiotemporal pattern of expression. It is not until E13.5, during bud stage, when Dkk1 presents itself for the first time expressed within the molar tooth germ within the epithelial bud.

A specific structure, known as the enamel knot, then forms at the tip of the bud driven by mesenchymal signaling to the overlying epithelium. The enamel knot has been heavily studied and expresses a plethora of tooth signaling molecules, therefore this structure became known as a very important signaling center for tooth morphogenesis. Although the cells of the enamel knot do not proliferate, the structure itself has been associated with cusp formation. Disruption in knot formation leads to cuspal defects. Secondary enamel knots then form and the inner enamel epithelium then folds. This initiates the bell stage, which occurs around E16.5. Secondary knots dictate the formation of multiple cusps on a mouse molar tooth. At E14 during early cap stage through E14.5 late cap stage and through to E16 early bell stage, Dkk1 expression is seen at the cervical region of the mesenchymal dental papilla (*16*). After the loss of the

secondary enamel knots, cells within the tooth organ start their differentiation into their final forms: inner enamel epithelial cells differentiate into ameloblasts and dental pulp mesenchymal cells differentiate into odontoblasts. At around E18 (right before birth) Dkk1 expression shifts to the cuspal region within the dental papilla, more specifically to the pre-odontoblasts, which are located under the inner dental epithelium at the tips of the future cusps.

Differentiated ameloblasts form a mineralized enamel matrix and differentiated odontoblasts form dentin. Pre-odontoblasts form first and then differentiate into polarizing odontoblasts, then into secretory/functioning odontoblasts and lastly into mature terminally differentiated odontoblasts (*17*). At PN2 (post-natal day 2), Dkk1 expression is still very intense in the pre-odontoblasts. The odontoblasts and dental lamina expresses much weaker Dkk1 signal at this time period but the signal is sustained into the secretory odontoblast stage (*16*). The overall signaling pattern of Dkk1 throughout tooth development shows a shift from epithelium to mesenchyme but is primarily expressed in postnatal mesenchymal-derived cells.

Wnt/β-catenin signaling in the dental epithelium is critical for dental patterning during multiple stages of early tooth development. Supportive evidence includes constitutive activation of β-catenin in the epithelium, which causes the formation of large malformed tooth buds and ectopic teeth (*18*). Alternatively, blocking Wnt/β-catenin signaling through the targeted expression of *Dkk1* in epithelial and underlying mesenchymal cells led to the formation of blunted molar cusps (*i.e.*, blocking the secondary enamel knots) (*18*). The role of Wnt signaling in the dental mesenchyme during tooth patterning is controversial. Chen *et al*. showed that the inactivation of β-catenin resulted in tooth arrest at the bud stage in both molars and incisors (*19*). Recently, Lohi *et al*. showed that Axin2 lacZ signal, which reflects the canonical Wnt signaling pathway, is expressed in dental pulp and developing odontoblast cells, but not in ameloblast cells postnatally (*20*), suggesting a potential role for canonical Wnt signaling in post-natal tooth formation. Although *Dkk1* mRNA is expressed in the dental

papilla, pre-odontoblasts, and odontoblasts (*16*), its function in post-natal dentin formation is largely unknown.

Cranial vault development

The entire process of cranial vault development occurs first by the formation, migration and commitment of skeletogenic mesenchymal precursor cells followed by subsequent differentiation and proliferation of osteogenic bone cells. In contrast to long bones, cranial vault bones are derived from two tissues with separate origins: paraxial mesoderm and neural crest. The vault bones are formed through intramembranous bone formation during which mesenchymal cells condense and differentiate into osteoprogenitor cells then to osteoblast cells, a process which is different endochondral bone formation (*21, 22*). The cranial vault is composed of the paired frontal and parietal bones with a lesser contribution from the post-parietal (inter-parietal) bone. Growth of the mammalian cranial vault takes place mainly in the fibrous joints (sutures) between these bones, all of which differentiate directly within the mesenchyme that lies between the brain and the surface ectoderm. The overall growth of the cranial vault, made up of multiple different bones, including the paired frontal and paired parietal bones, allows for brain growth and suture formation within the boundaries of the vault bones (*23*). The osteogenic fronts, or leading edges of the bones, grow towards each other and when they come together there is either fusion between the two to create a single bone (only the posterior aspect of the frontal bones in mice fuses) or the bones abut one another and sutural tissue remains. A suture is composed of the two osteogenic fronts and the interposing mesenchymal tissue (*9*). After migration and commitment of mesenchymal cells, osteogenic precursors differentiate into osteoblasts and secrete a mineralized matrix to form the cranial vault bones. The specific process of cranial vault bone formation occurs by intramembranous ossification involving a direct differentiation of condensed mesenchymal cells rather than the process of endochondral ossification involving mesenchymal condensation to form a cartilaginous intermediate (*24*). Bone formation occurs in stages during mouse development and a complex variety of genes

are involved. By E12.5 (embryonic day 12.5) during mouse development mesenchymal condensations on the lateral sides of the head start to form the frontal and parietal bone rudiments. These condensations, bearing osteogenic activity, spread dorsally. These bones are intramembranous and thus develop without a preceding cartilaginous primordium (*25*) with apposition of these bones occurring mostly on the outer bone surfaces (*25*). At E14.5, the immature osteoblasts residing at the osteogenic fronts of the developing bones differentiate to produce a bone matrix. Ossification of the calvarial bones starts within centers of condensing mesenchymal cells, in which the osteoblasts subsequently differentiate. The sutures that form between the two bone fronts are the primary sites of osteoblasts differentiation and bone formation (*26*). By E15, the osteogenic fronts of the parietal bone are approximating each other, but are still widely separated by a region of mesenchymal tissue within the sagittal sutural region (*10*). By E15.5, a primary sagittal suture is formed in the midline where the ends of the cellular masses meet. From this point until birth (around E16.5), a relative amount of mesenchyme is reduced as the osteogenic fronts move together in an end-to-end fashion.

It is important to note that mid-sutural mesenchymal cell populations are not likely to contribute to calvarial bone formation but rather remain undifferentiated. Conversely, the small population of mesenchymal cells lining the parietal bone osteogenic fronts are able to differentiate into the osteoblastic lineage, displaying a relationship between mesenchymal cell position and its contribution to osteogenic activity within the suture. Intramembranous bone growth is therefore shown to occur through proliferating osteoprogenitor cells at the osteogenic bone fronts as well as a contribution from the sutural mesenchyme (*9*).

Genetic regulation of bone

Bone extracellular matrix (ECM) contains two types of proteins: the collagens, mostly type I collagen, which accounts for 90% of the bone matrix proteins, and the non-collagenous proteins, including Osteocalcin, Osteopontin, and Bone sialo-protein (*27*). In 1999, Runx2 was the only osteoblast-specific transcription factor that had been

identified, and it was seen to act as an activator of osteoblast differentiation during embryonic development (*27*). Runx2 has an indispensable role in ECM deposition, and inhibition of this auto-regulatory cascade results in an osteopenic phenotype caused by the near abolition of expression of ECM-related genes, including type I collagen-encoding genes, without any overt effect on osteoblast differentiation (*27*). No membranous or endochondral bones are formed in *Runx2*-null mice (*7*) and therefore it is certain that Runx2 is needed for mesenchymal cell differentiation into pre-osteoblastic cells (*7*). Runx2 regulates osteoblast-specific expression of *Osteocalcin* (*Ocn*) and *Osteopontin* (*Opn*) (*27*). Runx2 has also been documented to regulate the expression of Osterix (Osx), a zinc finger transcription factor involved in osteoblast differentiation. Inactivation of *Runx2* in mice has shown that this gene is an indispensable regulator of osteoblast differentiation as the mice have a total lack of osteoblasts in homozygous mutant animals and die prenatally (*27*). *Osteocalcin* (*Ocn*) is a gene that is not expressed prenatally and has been dubbed as a hallmark gene for a differentiated osteoblast (*27*). The expression of this gene is virtually absent before birth and its restricted to differentiated osteoblasts able to produce a bone ECM, and is absent in osteoblast progenitors (*27*).

In the presence of a normal number of osteoblasts in the bone matrix, an osteopenic phenotype could develop only if each osteoblast produces less of the various proteins required to form the bone ECM (*27*). The differentiation of the osteoblasts forming the bone matrix in the process of intramembranous ossification is regulated by a number of transcription factors and signaling factors including Osx, Runx2 and the Wnt signaling pathway (*28*).

Bone formation is regulated by many different bone markers expressed through a few different pathways. Osx is a key player in bone formation whose expression has been previously documented to be under the control of Runx2. *Runx2* knock-out (KO) mice lack bone formation completely and *Osx* KO mice are unable to form bone due to an arrest of osteoblast differentiation. Interestingly, an overexpression of both *Runx2* and *Osx* under the expression of the 2.3-kb Col1a1 promoter leads to an osteopenic

phenotype in the long bones similar to that which is seen in 2.3-kb Col1a1 *Dkk1*-Tg mice. Osx is known to be pivotal in osteoblastic differentiation during the early stage of osteoblast differentiation, but during the later stages it has become evident that Osx has an inhibitory function leading to an accumulation of immature osteoblasts. Runx2 regulation of *Osx* expression has been previously documented as well as *Osx* expression being regulated by the Bone morphogenetic protein (Bmp) pathway. A potential interaction between the Wnt signaling pathway and the Bmp pathway has been identified that could lead to the regulation of *Osx* expression independent of Runx2.

Bone homeostasis: osteoblasts and osteoclasts

Pivotal cells in bone formation, regulation and turnover include the osteoblasts and the osteoclasts. The osteoblasts are the cells responsible for bone formation whereas the osteoclasts are terminally differentiated, multinucleated cells responsible for bone resorption. These two cell types work in collaboration with one another to maintain bone homeostasis (*29*). To promote osteoclast differentiation osteoblasts secrete Receptor activator of nuclear factor kappa-B ligand (Rankl), which is then free to bind to Rank, a protein expressed by osteoclasts (*30*). Osteoblasts also secrete macrophage-colony stimulating factor (M-csf), which has shown an effect in osteoclast differentiation, but is not needed for the resorbing activity of osteoclasts (*31*). The counter-response to Rankl-Rank binding and osteoclast differentiation into a multinucleated osteoclast is modulated by the osteoblasts' expression of Osteoprotegerin (Opg), a decoy receptor. Opg binds to Rankl, preventing Rankl from binding Rank, thereby preventing osteoclastogenesis (*32*). Determination of the extent of normal bone resorption depends heavily on the ratio of Rankl:Opg. Once activated, osteoclasts stimulate resorption and degradation of the boney matrix, by expressing enzymes, such as tartrate-resistant acid phosphatase (Trap), a glycosylated monomeric metalloenzyme, Cathepsin K, a lysosomal cysteine protease, and matrix metalloproteinase-9 (Mmp-9), an extracellular matrix proteinase. These proteins are secreted into the osteoclasts' lacunae, the space between the cell and their attachment site to the extracellular mineralized matrix, and degrade the matrix (*33*).

Dickkopf-1 and multiple myeloma disease

Osteoclastic bone resorption has been indicated in patients with osteolytic bone diseases such as multiple myeloma (*34*). Speculation as to the driving force behind multiple myeloma lytic bone disease include both osteoblastic inhibition (such as with an over-expression of Dkk1) (*35*), and osteoclastic stimulation leading to an uncoupling of bone homeostasis. Dkk1 negatively regulates Wnt/β-catenin signaling and in turn functions as a critical regulator of bone physiology. Specific aspects of the Wnt/β-catenin signaling pathway have been studied within osteoblasts but a relatively scant amount of research has focused on the crosstalk of Wnt/β-catenin signaling and Bmp signaling in osteoblasts. Studying the effects of Dkk1 on bone in the very serious and devastating lytic bone disease in humans (*36, 37*). Research has shown that there is a definitive link between patients with multiple myeloma and an over-secretion of Dkk1 from the patients' plasma cells (*35, 38, 39*). Elevated levels of Dkk1, present in the plasma and peripheral blood, were then subsequently correlated with the formation of lytic bone lesions and the "punched out" bone lesions in the calvarium. Bone marrow biopsy specimens stained for Dkk1 exhibited very elevated levels, and also presented with an increased Dkk1 antibody staining within plasma cells adjacent to the bone (*36, 40*). Furthermore, Dkk1's involvement in multiple myeloma links the over-expression of the Dkk1 protein to a disruption in the balance between osteoblastic and osteoclastic activity. Patients with multiple myeloma have presented with markedly increased osteoclastic activity in contrast to a significantly diminished osteoblastic activity. The osteolytic lesions formed in a significant amount of patients with multiple myeloma seem to occur due to osteoclastic hyper-activation combined with osteoblastic inhibition.

Increased Dkk1 expression has been found to be associated with lytic bone lesions, which form in patients with multiple myeloma, a devastating disease. This suggests that Dkk1 might therefore inhibit osteoblast differentiation and/or function (*36, 41*). This finding has been reinforced by data disclosing Dkk1 as a powerful negative regulator of osteoblast function *in vitro* and *in vivo*. It has also been shown that a decrease in Dkk1 expression is sufficient to induce a potent anabolic response in bone

(*12, 13, 42*). Studies have also shown that there is an association between serum Dkk1 levels and BMD (bone mineral density) (*41*).

Dickkopf-1: further evidence unveiling crosslinking of Wnt and BMP pathways

In this study we aimed to determine the effects of over-expressing *Dkk1* in mature osteoblasts to further study the effect on osteoclastic activity and bone homeostasis. The *Dkk1*-Tg mouse model allows for concentration on determining the relationship between Dkk1 expression and osteoblastic/osteoclastic activity as it is still unclear how the over-expression of *Dkk1* in osteoblasts affects osteoclastic activity. Here we present a hypothesis by which Dkk1 negatively regulates the Wnt signaling pathway, which then has the potential to release the Wnt inhibitory effect on the BMP pathway which allows for the stimulation of Osterix expression. Osterix expression at this later time point in osteoblast differentiation acts as a blocker of osteoblast differentiation from immature osteoblasts into mature osteoblasts. Osterix expression also regulates *RANKL* and *M-CSF* expression increasing osteoclastogenesis (*43, 44*).

CHAPTER II

APPLICATION OF GENETICALLY ENGINEERED MOUSE MODELS IN CRANIOFACIAL BIOLOGY RESEARCH

Overview

Genetically engineered mice are powerful animal models used for studying cutting-edge craniofacial research analyzing developmental and disease states affecting tooth, bone and cartilage. These techniques have been well established over the past few decades and allow for any gene of interest to be genetically modified in a predetermined way based upon basic and clinical research needs. Up to the present, thousands of genes have been modified in mice and these mouse models are available for utilization in research. Importantly, many of them develop craniofacial phenotypes. These mouse models are created and analyzed in hope that this research will initiate translational approaches for enhanced understanding of human diseases and enhance subsequent patient treatment. This chapter will provide the basic foundation of knowledge for medical/dental researchers to further understand how these genetically engineered mice are generated, how to select specific mouse models directed towards their research needs, and will elaborate on the advantages and disadvantages of using different mouse models.

Introduction

The question still asked is: Why the “Mouse” as a Research Model Organism? The most direct answer to this question reasons that biomedical research is dependent upon “biological mouse models” to emphasize the genetic and physiological similarities between mice and humans. These mouse models have propelled craniofacial research, which is directed more specifically towards areas of developmental and genetic diseases encompassing but not limited to skull/cranial vault development and tooth/bone/cartilage

formation. It is the creation and characterization of these mice that has led to the understanding of molecular mechanisms and cellular pathways that underlie craniofacial development and emerging disease states (*45*).

Major benefits of using mouse models include

a) The most important benefit is that the mouse, like humans, is a mammal and has similar biochemical pathways to humans.
b) The ease with which the mouse genome can be manipulated and analyzed.
c) Complete genome sequencing allows for full gene studies.
d) Being mammals, mice have immune, endocrine, nervous, cardiovascular, skeletal and other complex physiological systems that are similar to humans and therefore can be used in comparison to humans.
e) Mice are small in size, easy to maintain, have a relatively low cost of maintenance and can multiply quickly – reproducing as often as every month (with 21 day gestation periods)
f) Mice can be maintained well in laboratory situations and therefore can be controlled for environmentally (*46*).
g) Inbred strains of mice are available allowing for rigorous genetic control within the strain but between strains genetic variation can be introduced (*46*).
h) Mouse embryo developmental stages have been extensively studied and documented and therefore embryonic research is possible.
i) Depending upon which mouse strain is used, mice can produce 6-15 offspring per litter.
j) This model organism allows for transgenic mice to be made in which any foreign gene of interest can be inserted into the genome. (Model organisms: The mouse. <http://genome.wellcome.ac.uk/doc_WTD020804.html>)
k) Complex human diseases often result from the cumulative effects of many genes and these effects can be replicated well in mice using genetic modification techniques, which are already very well tested and utilized. For

example, double knockouts or triple knockouts of specified genes can be generated to study a certain genetic phenotype in one animal.

l) Mice are genetically very similar to humans, and approximately 90% of human genes are found in mice.

m) The rate of homologous recombination in mouse ES cells is much higher than that of most other cells regarding generation of genetic engineered animals. Currently, the reasons are not yet fully understood.

n) There are already established mouse lines created for studying craniofacial development/malformation and disease.

Terminology

"Common Language" used in mouse model research

Knockout (KO)

Mice generated by the genetic manipulation of ES cells to evaluate gene function on the basis of the complete elimination (null allele) or partially elimination of a candidate gene (that is, deletion of particular domain(s) of the encoded protein). A specific gene locus can also be targeted and rendered non-functional by inserting within it a selection cassette to disrupt the expression of the encoding locus.

Conditional KO (cKO)

Mice generated to ablate a target gene at a precise time and/or within a specific tissue, which allows the mouse to develop to a certain stage of development prior to gene inactivation. This approach utilizes Cre/LoxP or Flp/FRT technologies, which are site-specific recombination systems. This is the main method of genetic manipulation used to circumvent embryonic/neonatal lethality.

Inducible

Mouse models which allow for the temporal activation of genes in specific cells and tissues. Gene expression levels regulated by the administered agent are dosage-dependent. Common methods used to control gene expression in these mice are based on the tet-operon/repressor bi-transgenic system (cell-specific and temporal-specific with tetracycline as the inducible agent), and the estrogen receptor (ER) ligand-binding domain (cell-specific and temporal-specific induction of Cre-recombinase activity with tamoxifen as the inducible agent) (*47*).

Transgenic (Tg)

Transgenic mice contain genomic modifications due to insertion of one or more than one copy of a piece of DNA of foreign origin (usually an exogenous gene of interest). Creation of these mice, which ectopically express this gene of interest, enables researchers to study phenotypic defects *in vivo*. Examples of this approach include over-expression of a wild-type gene or expression of a dominant-negative form to diminish the normal function of a gene. There are a few downsides to using transgenes: 1) They can be relatively expensive to produce, and 2) Their expression pattern is controlled by a specific promoter whose activity may not fully recapture the endogenous gene levels regarding spatial and temporal patterning.

Gain-of-function (GOF)

"Gain-of-function" is another name for "Transgenic" and these mice are created to study the "gain-of-function" of a gene within the mouse. The idea is to force the ectopic expression of a gene during development and study its consequences; it addresses whether the over-expression of a gene-of-interest is sufficient to cause a developmental anomaly.

Loss-of-function (LOF)

"Loss-of-function" is another name for "Knock-out" and these mice are created to study the "loss-of-function" of a gene within the mouse. The elimination of a gene in this "knock-out" model system has been well established in mice.

Knock-in (KI)

Mice which integrate a reporter gene, such as GFP (green fluorescent protein), lacZ (β-galactosidase), or luciferase, into an existing gene to visualize its temporal and spatial expression pattern during development in heterozygous mice, which still contain a normal chromosome. In homozygous mice, the knocked-in reporter will interrupt both chromosomes, and thus this gene will be completely interrupted, just like a classic KO model. In addition, this technology will allow for the specific mutation of a targeted gene-of-interest to examine the effects that this mutation may have on gene expression, gene function, and subsequent development of the mouse. These models, combined with knockout mouse models, may be useful in the elucidation of specific mechanism(s) responsible for disease pathogenesis.

Practice and Application

Generation of transgenic mice (Fig. 1-1)

The common term "transgenic", when discussing transgenic mice, describes a particular genetic modification to the genome in which a copy (or more than one copy) of a piece of DNA of foreign origin is incorporated into the genome. This causes what is known as "ectopic expression" of a certain gene and is an efficient method for *in vivo* mouse studies varying from over-expressing a wildtype version of a gene of interest, to expression of a dominant-negative form to completely diminishing the normal function of the gene of interest.

Generation of transgenic mice includes three key steps: 1) generation of the transgene of interest (1-1A), 2) production of the transgenic mice (1-1B), and 3)

transgenic line screening by PCR (1-1B). In general, step two is performed by a mouse genetic core or a commercial company, and DNA PCR screening is a simple strict procedure using a pair of primers specific to the transgene. As shown in Fig.1-1B, a purified fragment of a transgene of interest is directly injected (by microinjection) into fertilized oocytes (more specifically, into the male pronucleus). The transgene then randomly inserts itself into the genome of the fertilized oocytes, which are then implanted into pseudo-pregnant females and are brought to term. Each oocyte will include differential transgene expression levels which is highly influenced by positional effect and the overall copy number of the transgene (how many copies were randomly inserted) (*48*).

Here we mainly focus on transgene preparation provided by the investigators using two different approaches, the classical transgene construct design and the BAC-clone recombineering technique.

Classical transgene construct design

The classical transgene includes 3 main components: 5' regulatory elements, a gene of interest and/or reporter gene and a poly A tail.

The 5' regulatory elements are required for transgene expression. These elements include tissue-specific promoters/enhancers or "Housekeeping gene" promoters (ubiquitous expression) such as chicken β-actin, cytomegalovirus (CMV) enhancer (CCAG or CAG promoter), or histone H4 promoter. In general, the promoter size is limited due to cloning difficulties.

The gene of interest and/or reporter gene includes a mini gene (containing both exons & introns), cDNA (lacking introns), anti-sense RNA (for blocking a gene of interest), or report genes (for tracking expression levels of the gene-of-interest). There are many reporter genes, but a few of the common reporter genes are: LacZ (from E. *coli*, encoding β-galactosidase), which can be visualized using histochemical (X-Gal staining) or immunocytochemical methods at single cell level which is particularly useful for cell-lineage tracing and defines Cre activities (see Section 3 for details). The

disadvantage of using this reporter is that it is less sensitive with a longer half-life. Another common reporter gene is luciferase (from the Firefly), which is extremely sensitive and particularly useful for quantifying expression levels but homogenization of the tissue is necessary for any analyses to be performed. Another set of common reporter genes includes the fluorescent proteins. These proteins are very sensitive, easily visualized, no substrates required, analysis performed in live tissue, isolation of cells using FACS, with different fluorophore colors: EGFP (green, from jelly-fish), EYFP (yellow), ECFP (cyan), and RFP (red, from reef coral). In particular, RFP is now very popular and there are different variants (such as DsRed and tdTomato). These new proteins have longer wavelengths, lower background, increased brightness and photo-stability. In addition, specific mono/polyclonal antibodies against DsRed-Express are available and they do not cross-react with EGFP and can be used for immunohistochemical analysis (see an example in Fig. 1-1C).

SV 40 PolyA sequences (poly A tail) are widely used for stabilizing the transgene for its translation in the cytoplasm.

Construction of the transgenic construct is amplified in bacteria using restriction enzymes and DNA ligases, and the final construct has to be released from the rest of the vector and purified. There are several limitations in building a construct via these methodologies. As a result, a new cloning technique has been developed: Recombineering (see below).

BAC-clone recombineering technique

Recombineering is a highly efficient method for genetically modifying DNA within *E.coli* and other bacteria. This technique incorporates *in vivo* homologous recombination-mediated genetic engineering and produces gene replacements, deletions, insertions, inversions, duplications and single and multiple point mutations, fusions and tags. Recombination, using this method, occurs efficiently with relatively short sequences of homology, and allows the recombinant DNA to be generated *in vivo*. A

benefit to recombineering is that it does not rely on restriction enzymes or DNA ligases in contrast to other genetic engineering methods. There are systems that are utilized within the bacteria and these are based on controlled expression of proteins that permit homologous recombination through homologies as small as 42 bp in size. The recombination proteins in these systems are inducible, meaning that they are transiently expressed to permit recombination and are not constitutively expressed. Recombinant clones are engineered to include a selective cassette such as antibiotic resistance for both positive and negative selection.

Bacterial Artificial Chromosome (BAC) transgenic mice express a variety of reporters, epitope tagged-proteins or Cre recombinase driven by specific promoters. A major benefit to the use of BAC cloning is that BAC's contain several hundreds of thousands of base pairs of regulatory sequences. These sequences act to regulate the endogenous genes more effectively than the shorter transgenes making BAC cloning an effective cloning system. In bacteria, such as E. coli, the BAC can easily undergo homologous recombination with a vector that has inserted into it the gene-of-interest between two homologous regions of the BAC. After homologous recombination occurs, the BAC will include the gene-of-interest cassette. This BAC is then microinjected into an oocyte. The oocyte is implanted into a pseudo-pregnant female mouse and the mice (approximately 20-30% of the offspring) that have incorporated the engineered DNA sequence, called "founders", are then backcrossed with wildtype mice. Of these new offspring, approximately 50% of them will inherit the BAC.

Conventional KO mouse model

A conventional KO mouse model is one in which a targeted gene or genetic region has been completely eliminated (ablated) creating a null allele, or has been functionally disrupted usually by partial elimination of the candidate gene – deletion of a particular domain of the gene important for functional activity (*48*). This can be carried out by either inserting a selection cassette (ex: neomycin-resistance) or a specific reporter gene (for tracing the expression pattern of the targeted gene) such as GFP or

lacZ into the gene locus. Deleting a gene in this manner yields a mouse model lacking the functional targeted gene. The production of a null allele causes gene ablation in all cells within the body and has been shown to be a very valuable approach in determining *in vivo* gene functions.

Creating a KO mouse (Fig. 1-2)

The construction of a conventional genetically modified knockout mouse requires the design and use of a targeting vector. Construction of the targeting vector will allow for either complete gene removal (gene ablation) or a specific exon is selected for removal that will disrupt the protein product for that gene. It is common for the construct to carry a selectable marker (such as a kanamycin/neomycin resistance gene) and for two regions of homology of the target gene to flank either end.

The targeting vector is introduced into a specific ES cell line such as 129Sv/EV ES cells, which will generate mice with a brown coat color. Electroporation is used to introduce the targeting vector into the ES cells and then these cells are plated out under optimal conditions for growth.

The ES cells are put through rigorous positive and negative selection methods to select for only those ES cells that have taken up the targeting vector through homologous recombination, in which nucleotide sequences are exchanged between the endogenous DNA and a very similar DNA fragment (the targeted DNA construct). A common method is to incorporate a neomycin resistance cassette into the gene area of interest within targeting vector (used for positive selection), and a TK (thymidine kinase) gene cassette placed outside the region of homology (used for negative selection). The neomycin cassette responds positively to administration of G418 (those cells that have not incorporated the neomycin cassette will die) and the TK cassette responds negatively to administration of Gancyclovir (those cells which have the incorporated TK cassette will die).

After the selection procedure, those ES cells that are left should theoretically be positively targeted cells. Due to the nature of a random incorporation of the targeted

gene construct into a chromosome, only one homologous recombination event occurs in each cell with approximately a 1% probability. Thus, more than 300 clones need to be screened in order to obtain one or possibly a few ES cells, which have undergone homologous recombination. Each positive clone is plated in two wells on separate 96-well plates and one set of the ES clones is frozen down while the other set is used for confirmation of correct homologous recombination via PCR and Southern Blot methods.

Once the positive clones have been identified, the second set of frozen clones is thawed, allowed to expand in culture and then used for blastocyst injection into 3.5-day-old mouse blastocysts, which are obtained from a different mouse strain such as C57/B6 mice with a black coat color.

A foster mother is chosen for uterine implantation of the blastocyst, and she is considered a pseudo-pregnant female as pregnancy is induced by mating her with a vasectomized male. The stimulus of mating elicits the hormonal changes needed to make the female mouse's uterus receptive.

The foster female mouse is allowed to come to term and among the mice that are born, there is the possibility for pure wildtype (WT) mice (black coat color), or chimeric mice that have a mixed genetic background (mixed black and brown coat colors). The cells in the chimeric mice come from two resources: the blastocyst (ex. C57/B6) and the engineered ES cells (ex. 129Sv/EV).

Male chimeric mice are then crossed back to female WT mice (black coat color) and then this backcross should give rise to mice that are heterozygous for the targeted allele (brown coat color, i.e. the entire genetic information comes from the engineered ES cell). These mice are then interbred to produce a homozygous gene targeted mouse.

It is important to understand that the reason that ES cells are used in producing gene targeted mice is because ES cells are considered pluripotent, meaning that they have full potential to give rise to any cell type within the body. A major problem for many transcription factors with this knockout approach is embryonic lethality whereby the neonates die prior to birth (usually during a critical developmental stage in which that gene is necessary for vitality). To circumvent this issue, a new genetic engineering

technique was developed which utilizes Cre/LoxP or Flp/Frt technologies (described later).

Knock-in (point mutation, reporter genes, and cre)

Knock-in mice are a powerful research model for all fields of research, including bone and craniofacial, and have multiple different uses. The knock-in method is also known as the exogenous gene introduction method in which anon-mouse gene is engineered into the genome and the protein functions of this specific gene are then analyzed. Usually, the ectopic gene is introduced at a specific gene locus, but it is also possible to introduce it in a form that disrupts the homologous gene in the mouse. Another term for the latter disruption method is called the knockout/knock-in method as one gene is being knocked out (ablated) due to another gene being expressed in its place, plus the gene structure is interrupted (see example presented in Fig. 1-3).

The most common uses of the knock-in mouse model:

Humanization: replaces a mouse gene with its human counterpart – could be a portion of the human gene or an entire gene. The human protein is then expressed under the mouse promoter and regulatory regions and it is a great model for studying the function of a specific gene in disease models.

Point Mutation: Using the knock-in method, it is also possible to introduce a point mutation into the sequence of a gene-of-interest causing the mouse to express a mutant protein instead of the wildtype protein. The mutation may cause abnormal protein function (a method to test protein functional domains) but it should not alter the expression pattern of the protein. The point mutation can be introduced as either a direct mutation or an inducible mutation (temporal- or tissue-specific). A great benefit to using this approach is that a point mutation model can be used in place of a knockout mouse model that causes embryonic lethality.

Reporter/Tag: This method introduces a reporter gene, such as LacZ, GFP, mCherry, YFP or DsRed, into the locus of a target gene in order to monitor the expression of that gene at the transcriptional level.

Conditional knockout mouse models

Conditional knockout mice are highly important in gene studies due to their ability to eliminate a gene-of-interest in a temporal- and tissue-specific manner. Due to the fact that the ablation of certain genes causes embryonic lethality, this model is utilized to circumvent this pertinent issue. The two major systems developed within the conditional knockout model are the Cre-Lox system and the Inducible system.

Cre-Lox system

The Cre-lox system was developed to facilitate the production of conditional knockout and reporter strain animal models. This method has been perfected in mice and is a widely used method of genetic manipulation in embryonic stem cells using site-specific DNA recombination through the interaction of a Cre-recombinase enzyme and LoxP sites to delete the gene in specific targeted tissues. The system was originally discovered in the P1 bacteriophage, which uses the innate Cre-lox recombination method to circularize and facilitate replication of its genomic DNA when reproducing (*49*). The use of this model also allows for gene deletion in the mature, adult mouse and is an alternative to studying gene expression embryonically (Figs. 1-4,5).

Craniofacial biology Cre-expressing mouse models

Different promoter-expressing Cre-recombinase mouse lines are known to be expressed in osteoblasts and chondrocytes and their progenitor cells (tables i & ii). Paired-related homeobox gene-1 (Prx1) is expressed in the early mesenchymal condensations that form the developing limbs and parts of the skull in the mouse, and has been well established to have a pertinent role in skeletal development (*50, 51*). Prx1-Cre has been shown to decrease RANKL in the calvaria and the developing limb when crossed with RANKL floxed mice (*51*) and caused short limbs, an open sternum and an open skull in TGFβR2 floxed mice crossed with Prx1-Cre mice (*52*). Prx1-Cre mice are readily available for purchase from Jax laboratories. The transcription factor Sox9 is expressed in osteo-chondroprogenitor cells and has a pertinent role in chondrogenesis

and endochondral bone formation. Sox9 activates chondrocyte-specific marker genes, such as Col2a1, Col11a1 and Aggrecan and severe chondrodysplasia develops if Sox9 is inactivated during or after mesenchymal condensation (*53*). Sox9 has also been shown to be highly expressed in proliferating chondrocytes in the condyle at E16.5 and has a crucial role in cartilage development. A study which ablated *Smo* (a positive effector in the Hedgehog signaling pathway) in chondrocyte progenitors when crossed with Sox9Cre, results in the normal formation of the condylar disk, but a failure of the disk to separate from the condyle itself (*54*). Information on this mouse model is available at Jax Laboratories and the International Knockout Mouse Consortium. Type II collagen, a major component of cartilaginous extracellular matrices, is highly expressed during chondrocyte differentiation (*55*). Osterix (Osx) Cre mice (OsxCre) is an tetracycline-inducible mouse line and has a GFP/Cre fusion protein active under the Osterix (Sp7) promoter (*56*). A recent study has shown that this mouse model actually has a phenotype itself at skeletally mature stages and in the absence of doxycycline administration before 12 weeks of age, it will display delayed growth. A second type of OsxCre mice is $OsxCre^{ERT2}$ which is tamoxifen-inducible and allows for the temporal induction of its activity and has been used for osteoblast lineage tracing *in vivo* (*57*).

The rat 2.3-kb Col1a1 promoter has been shown to be active in mature osteoblasts and odontoblasts. The temporal- and site-specific Cre/loxP system was used under the 2.3-kb Col1a1 promoter to inactivate *Osx* in osteoblasts (*58*). A 2.3-kb Col1a1-Cre^{ERT2} is also available and inducible Cre recombinase activity is shown to be detected in E18.5 and Day-18 long bone (limb) osteoblasts, ribs, vertebrae and calvaria (positive activity tested as these two time points) (Ref: Jax Laboratories <http://jaxmice.jax.org/strain/016241.html>). 2.3-kb Col1a1-Cre and 3.6 Kb Col1a1-Cre mice were bred to ROSA26 (R26R) mice to detect β-galactosidase expression evident in osteoblasts and for 3.6 kb promoter-driven Cre recombinase it was expressed in the bone marrow cells.

Common Cre lines for bone/tooth tissue (Ex: osteoblasts/odontoblasts/pulp cells)

Cre Mouse Line	Promoter	Cell Type Activity
Prx1Cre	Paired-related Homeobox 1	Mesenchymal Progenitor Cells
Sox9Cre	Sox9	Osteoprogenitor Cells
Col2a1Cre	Collagen 2 α1 chain	Osteoprogenitor Cells
OsxCre	Osterix	Osteoprogenitor Cells
$OsxCre^{ERT2}$	Osterix	Osteoprogenitor Cells
Col1a1 3.6kb Cre	Collagen 1 α1 chain	Osteoprogenitor Cells
Col1a1 3.2kb (Cre^{ERT2})	Collagen 1 α1 chain	Inducible-Cre for Osteoblast at any stage
Col1a1 2.3kb Cre	Collagen 1 α1 chain	Committed Osteoblast
OcCre	Osteocalcin	Mature Osteoblast

Common Cre lines for cartilaginous tissue (Ex: chondrocytes)

Cre Mouse Line	Promoter	Cell Type Activity
Prx1Cre	Paired-related Homeobox 1	Mesenchymal Progenitor Cells
Sox9Cre	Sox9	Chondroprogenitor Cells
Col2a1Cre	Collagen 2 α1 chain	Chondroprogenitor Cells
$Col2a1^{ERT2}$	Collagen 2 α1 chain	Chondroprogenitor Cells
$Col2a1^{ERT2}$	Collagen 2 α1 chain	Committed Chondrocyte
$Agc1^{ERT2}$	Aggrecan	Committed Chondrocyte
Col10a1	Collagen 10	Hypertrophic Chondrocyte
OcCre	Osteocalcin	Mature Osteoblast

Common Cre lines for osteoclasts

Osteoclasts are terminally differentiated cells that attach to the bone and secrete proteases, the primary protease of the organic component being Cathepsin K, which degrades the boney matrix. A common Cre-recombinase mouse line used for research in Osteoclasts is Cathepsin K-Cre (CtsK-Cre), which has been shown to delete the gene-of-interest in mature osteoclasts (*59, 60*). Another main Cre line specific to osteoclasts is the tartrate-resistant acid phosphatase (TRAP)-Cre. This Cre has been analyzed and shown to be active in long bones, vertebrae, ribs and calvaria but more specifically in osteoclasts (Ctsk-Cre and TRAP-Cre lines) and columnar proliferating and hypertrophic chondrocytes (TRAP-Cre line only) (*61*).

Common Cre lines for ameloblasts

A common Cre line used for studying gene expression in ameloblasts is K14-Cre and it has been verified in a study of conditional knockout (cKO) mice containing floxed p120 and the Cre recombinase linked to the keratin-14 promoter (K14-Cre p120-cKO) (*62*). Another study analyzed the role of FGFR1 in enamel and tooth development utilizing the K14-Cre mouse line demonstrating that there is a cell-autonomous requirement for FGF signaling in the dental epithelium during enamel formation (*63*).

Inducible systems

The inducible transgenic mouse model is an effective biological model for the temporal activation of a gene-of-interest in specific cells and tissues (for example, only within osteoblasts, ameloblasts or odontoblasts etc.). Expression levels are dosage-dependent (of the agent administered). There are certain advantages and disadvantages of using this system, which are important to understand before utilizing this method for research studies. Some of the more significant advantages include: 1) the ability to bypass issues with embryonic lethality within a specific genetic manipulation (seen in conditional knockout and conventional knockouts), 2) the fact that inactivation of a gene-of-interest can be studied at specific time points during different stages (in utero) during development or postnatally, and 3) the ability to induce the inactivation at any time point allows for the study of age-specific diseases. A major disadvantage of using this system is that endogenous gene deregulation or truncated protein expression can occur if a thorough analysis of the selected gene of interest and surrounding genetic material is not performed.

The two most widely used systems for inducible control of gene expression are the Tet-operon/repressor bi-transgenic system (tetracycline (Tc)-regulated) and the Estrogen Receptor (ER) ligand-binding domain system. (Cre-ER).

The tetracycline-regulated transgenic models are typically designed to activate the expression of the gene of interest in a temporal and tissue-specific manner. When the transgenic mouse carrying a Tc-regulated transactivation factor (put under a promoter of

choice) is mated with a mouse carrying a minimal promoter driving the expression of a specific gene-of-interest, the resultant offspring inheriting both transgenes can be treated with doxycycline and will then express the gene of interest. The treatment of doxycycline is the temporal control mechanism and the promoter of choice regulates the tissue-specificity.

The Estrogen Receptor (ER) ligand-binding domain system is a tamoxifen-inducible system which utilizes a fusion protein between Cre and a mutated form of the ligand binding domain of the estrogen receptor (Cre-ER^{TM}) that renders Cre activity tamoxifen (TM) inducible, allowing for conditional modification of gene activity in the mammalian neural tube *in utero*(*64*). The ER can also be fused to proteins other than Cre, such as transcription factors or kinases that are active in the nucleus. Cre can either be used to activate gene expression by removing stop cassettes from transgenes which are flanked by LoxP sites, or inactivate gene expression by removing the gene-of-interest flanked by LoxP sites (see example presented in Fig. 1-6).

These two systems are complementary in mouse models, being effective in cell-specific activation and inactivation of gene expression (*47*).

Discussion

By five years of age, approximately two to three percent of all infants and children are found to have craniofacial birth defects, and of the 6000 known hereditary syndromes, over 700 have either dental or craniofacial associated defects, and over 250 syndromes have associated clefting (*65*). The incorporation of genetically engineered mice into our research fields of interest (such as within the craniofacial and bone fields) has greatly improved and furthered our understanding of a variety of human development and disease states and will continue to aid in the development of many therapeutic approaches.

Prior to the use of mouse models, our understanding of the complexity of the human/mouse genome was still in its infancy. It wasn't until gene technology advanced

in mice that researchers were able to fully understand the complexity of these genes and how certain genes interact to produce a defined disorder/disease.

Mouse models have been utilized extensively to distinguish between genetic and environmental stimuli on craniofacial disorders. These models are important in diseases/abnormalities such as cleft lip and palate, tooth anomalies and cranial vault/skull malformations. Human craniofacial disorders are able to be recapitulated in the mouse and can be studied on a very intricate level which will ultimately lay the foundation for advancements in therapies.

CHAPTER III
POSTNATAL EFFECT OF OVEREXPRESSED DKK1 ON MANDIBULAR MOLAR FORMATION*

Overview

Dickkopf-related protein 1 (Dkk1) is a potent inhibitor of Wnt/β-catenin signaling. *Dkk1*-null mutant embryos display severe defects in head induction. Conversely, targeted expression of *Dkk1* in dental epithelial cells leads to the formation of dysfunctional enamel knots and subsequent tooth defects during embryonic development. However, its role in post-natal dentinogenesis is largely unknown. To address this issue, we studied the role of Dkk1 in post-natal dentin development using 2.3-kb Col1a1 *Dkk1*-transgenic mice, with the following key findings: (1) The *Dkk1* transgene was highly expressed in pulp and odontoblast cells during post-natal developmental stages; (2) the 1st molar displayed short roots, an enlarged pulp/root canal region, and a decrease in the dentin formation rate; (3) a small malformed second molar and an absent third molar; (4) an increase of immature odontoblasts, few mature odontoblasts, and sharply reduced dentinal tubules; and (5) a dramatic change in Osx and Nestin expression. We propose that Dkk1 controls post-natal mandibular molar dentin formation either directly or indirectly *via* the inhibition of Wnt signaling at the following aspects: (i) post-natal dentin formation, (ii) formation and/or maintenance of the dentin tubular system, (iii) mineralization of the dentin, and (iv) regulation of molecules such as Osx and Nestin.

Introduction

Tooth formation begins with a series of reciprocal signaling interactions between the stomodeal ectoderm and the underlying neural-crest-derived ectomesenchyme (*66*). The first sign of tooth formation is the thickening of the oral epithelium. This is followed by the invagination of the epithelium into the underlying mesenchyme, which then condenses and forms a tooth bud. In the past two decades, many signaling molecules and transcription factors which are critical for these processes have been identified (*66-69*). However, critical factors required for tooth root formation, which occurs mainly post-natally, are largely unknown.

It is known that Wnt/β-catenin signaling in the dental epithelium is critical for dental patterning during multiple stages of early tooth development. The supportive evidence is that constitutive activation of β-catenin in the epithelium causes the formation of large malformed tooth buds and ectopic teeth (*18*). Alternatively, blocking Wnt/β-catenin signaling by the targeted expression of *Dkk1* in epithelial and underlying mesenchymal cells led to the formation of blunted molar cusps (*i.e.*, blocking the secondary enamel knots) (*18*). The role of Wnt signaling in the dental mesenchyme during tooth patterning is controversial. Chen *et al*. showed that the inactivation of β-catenin resulted in tooth arrest at the bud stage in both molars and incisors (*19*). In contrast, a new report showed that the genetic inactivation of β-catenin results in a splitting of the incisal placode. This inactivation forms 2 incisors *per* incisal placode in the lower jaw in approximately 50% of all mutant embryos (*70*).

Recently, Lohi *et al*. showed that Axin2 lacZ signal, which reflects the canonical Wnt signaling pathway, is expressed in dental pulp and developing odontoblast cells, but not in ameloblast cells post-natally (*20*), suggesting a potential role for canonical Wnt signaling in post-natal tooth formation.

Dkk1 (a secreted protein with 2 cysteine-rich domains, separated by a linker region) is expressed in the tooth and the limb during development (*16, 71-73*). Suomalainen and Thesleff showed that Wnt/β-catenin activity and *Dkk1* mRNA were detected in incisor mesenchyme (*74*). Dkk1 functions as an antagonist of canonical Wnt

signaling through two mechanisms: (1) by binding to the Lrp5/6 co-receptor to prevent its interaction with Wnt-Frzd complexes; and (2) by binding to the cell-surface receptor Kremen1 or Kremen 2 to promote the internalization of Lrp5/6 (*75*). Injections of *Dkk1* mRNA result in head induction in *Xenopus* embryos (*76*), and deletion of *Dkk1* leads to embryonic lethality with no anterior head structures in addition to exhibiting limb defects (*77*). Ectopic expression of *Dkk1* in K5-expressing epithelium blocks taste papilla development, causing a lack of innervation of the tongue (*78*).

Although *Dkk1* mRNA is expressed in the dental papilla, pre-odontoblasts, and odontoblasts (*16*), its function in post-natal tooth root formation is largely unknown. In this study, we attempted to address the function of Dkk1 in post-natal dentin formation using 2.3-kb Col1a1 *Dkk1*-transgenic (Tg) mice (*13*) and multiple approaches, including: radiography, μ-CT, histology, TRAP staining, immunohistochemistry, double-fluorochrome labeling for measuring dentin formation rates, and scanning electron microscopy (SEM) for determining dentin properties. Our results demonstrate that overexpression of *Dkk1* in pulp and odontoblast cells impaired mandibular molar dentin formation, suggesting that Dkk1 plays an active role in post-natal tooth formation either directly or indirectly through the Wnt/β-catenin signaling pathway.

Experimental Procedures

Generation of 2.3-kb Col1a1-Dkk1 transgenic mice

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at the institute. The 2.3-kb Col1a1 *Dkk1*-Tg mice were generated as described previously (*13*). A C57/B6 strain background was used in this study. The genotypes of the mice were determined by PCR analysis of genomic DNA extracted from tail biopsies. For the Col 1a1 *Dkk1*-transgene, the forward primer, 5′-CATCCCTGTGACCCCTCC-3′, and the reverse primer, 5′-CTCCAAACCACCCCCCTC-3′, were used to generate a PCR product of 150 bp.

Sample preparation and histological analyses of Dkk1 expression

Mandibular samples obtained from E16.5, P01, 1-week-, and 1-month-old mice were fixed in 4% paraformaldehyde in 4ºC overnight. These samples were then decalcified with 10% EDTA in a microwave, dehydrated, and embedded in paraffin. They were then sectioned (4-µm thick) and used in immunohistochemistry for Dspp (polyclonal antibody was kindly provided by Dr. Larry Fisher from NIDCR, National Institutes of Health, Bethesda, MD, USA), Dkk1 (polyclonal antibody, R&D Systems, Minneapolis, MN, USA), Osx (osterix monoclonal antibody, Abcam, Cambridge, MA, USA), Nestin (mouse monoclonal antibody, Santa Cruz, CA), and TRAP (tartrate-resistant acid phosphatase) staining. Finally, the slides were mounted with permount, and photographed under by light microscopy.

Analysis of dentin formation rate by double-labeling and imaging resin-cast odontoblast processes by scanning electronic microscopy (SEM)

To examine the dentin formation rate, we performed double-fluorescence labeling as described previously (*79*). Briefly, a calcein label (5 mg/Kg i.p.; Sigma-Aldrich, St. Louis, MO, USA) was administered to 20-day-old mice, followed by administration of calcein 7 days later. Mice were sacrificed 2 days after the second injection (1 mo old). The non-decalcified mandibles were embedded in resin (methylmethacrylate, MMA), sectioned, and photographed under epifluorescent illumination with a Nikon 800 microscope (Nikon, Melville, NY, USA). Furthermore, the surface of the same blot was polished with different diamond suspensions until smooth and scratch-free before being acid-etched and imaged by SEM as described previously (*80*). The surface was acid-etched with 37% phosphoric acid for 2 to 10 sec, washed twice with water followed by 5% sodium hypochlorite for 5 min, and washed again with water. After being air-dried, the samples were coated with gold and palladium, and examined by FEI/Philips XL30 Field emission environmental SEM.

Radiograph and micro-ct imaging of mandibles from 1-month-old Dkk1-Tg mice

Both the wild-type and *Dkk1*-Tg mandibles were radiographed with a Faxitron model MX-20 System (Faxitron X-Ray LLC, Lincolnshire, IL, USA), and scanned with a Micro-CT 35 (Scanco Medical AG, Bassersdorf, Switzerland).

Results

The 2.3-kb Col1a1-Dkk1 transgene is highly expressed in odontoblasts and pulp cells

To determine whether Dkk1 plays a role in odontogenesis, we first compared the Dkk1 expression patterns during dentinogenesis in WT and *Dkk1*-Tg mice starting from E16.5. Analysis of the immunohistochemical data from E16.5 molars showed that Dkk1 is largely undetected in both WT and Tg odontoblasts (Fig. 2-1A). Dkk1 was weakly expressed in WT newborn odontoblasts (Fig. 2-1B, left panel), but had a much higher expression level in newborn *Dkk1*-Tg pulp cells and odontoblasts within the molars at a low antibody concentration (Fig. 2-1B, right panel; 1:100 dilution). Higher expression levels were also observed in pulp and odontoblast cells of *Dkk1*-Tg 1-week old mandibular molars (Fig. 2-1C). A weak Dkk1 signal was detected in osteoblast cells, but a much higher level of Dkk1 was detected in Tg osteoblasts (Fig. 2-1D). A clear Dkk1 signal was detected in newborn and 1-week WT pulp, odontoblast, and ameloblast cells at a high antibody concentration (Fig. 2-5).

The 2.3-kb Col1a1-Dkk1 transgenic mice display a striking molar phenotype in the mandible

To address whether overexpression of *Dkk1* in mesenchymal cells changes the tooth phenotype, we first screened mandibular samples using x-ray and μCT (Fig. 2-2A). Unexpectedly, the 3rd molar in all *Dkk1*-Tg mice was missing in mice examined at the age of 2 wks (Fig. 2-6A) and 4 wks (Fig. 2-6B). The radiograph and μ-CT images obtained from the 1st molar also showed a malformed crown, which is reduced in size and has short roots and an enlarged pulp/root canal region. The 2nd molar is much

smaller than that in the age-matched control (Fig. 2-2A, *right panels*). Analysis of quantitative data showed that reduction of the full tooth, the root length, the ratio of tooth root/full tooth length, and dentin thickness in the *Dkk1*-Tg 1st molar is significant (Fig. 2-2B).

Since the tooth root is embedded in alveolar bone and the *Dkk1*-transgene is targeted to the osteoblast cells (Fig. 2-1D), we next asked whether there is an alveolar bone phenotype that could be responsible for the tooth phenotype observed within the *Dkk1*-Tg mice. There is mild bone loss in the *Dkk1*-Tg mice, shown by μCT and H&E staining assays (data not shown), although it is very unlikely that bone loss is the major cause of such a severe tooth phenotype.

To determine whether the overexpression of *Dkk1* changes the dentin ultrastructure, we next examined the first mandibular molar by SEM using an acid-etched resin-casting technique (Fig. 2-2C). When comparing the *Dkk1*-Tg first molars with the age-matched controls, we observed striking differences in the appearance of the dentin tubules, with the tubular processes being sharply reduced and disorganized. Analysis of quantitative data showed a significant reduction of dentinal tubule number in *Dkk1*-Tg mice (Fig. 2-2C, *middle panel*). We also measured the dentin apposition rate using a fluorochrome labeling assay (*81*). The distance between the 2 fluorochrome-labeled lines was used to calculate the dentin formation rate and was shown to be significantly reduced in the *Dkk1*-Tg mandibular molars (> 20%, Fig. 2-2D).

The above data indirectly reflect a critical role of Wnt/β-catenin signaling during post-natal molar dentin formation.

The 2.3-kb Col1a1-Dkk1 transgenic mice exhibit dentin erosion and expanded periodontal ligament (PDL)

As the striking tooth phenotype is displayed in the mandibular molars at 1-month of age, we further characterized the dentin phenotype using backscattered SEM imaging, which displayed numerous areas of dentin erosion in the *Dkk1*-Tg mice (Fig. 2-3A, *right panel*). To address the cause of dentin erosion, we re-examined Dkk1 expression in the

first molar. Unexpectedly, Dkk1 was detected in the PDL cells and the acellular matrix with the area of dentin erosion, which was filled with many cells (Fig. 2-3B, *right panel*). Since the osteoclast is the only cell that can resorb the mineralized tissue, we next examined the presence of osteoclast cells using a TRAP staining assay. The results showed that overexpression of Dkk1 significantly increased osteoclast numbers (Figs. 2-3C, 2-3D *right panels*). We also showed that there were few acellular cementoblasts in the *Dkk1*-Tg mice (Figs. 2-3B, 2-3C *right panel*), and the μCT image displayed an expanded PDL region (Fig. 2-3E, *right panel*). Analysis of these data, taken together, supports the notion that dentin erosion is caused by two factors: an increase in osteoclast number and a defect in acellular cementoblasts within *Dkk1*-Tg mice.

Overexpression of Dkk1 delays the maturation of dentinogenesis during post-natal development

To study the mechanism by which the targeted overexpression of *Dkk1* leads to the above dentin structural changes, we investigated the expression patterns of Osterix (Osx), a transcription factor which is critical for tooth formation (*79*), and Nestin, a specific marker for odontoblasts reflecting its neural-crest derivation (*82, 83*). Analysis of immunohistochemical data showed that Osx was mainly expressed in the nucleus of odontoblasts of WT molars (Figs. 2-4A, 2-4B *left panels*). Conversely, Osx was expressed in a much broader array of cells, including later pulp cells and early odontoblasts, and the *Dkk1*-Tg molars lacked a defined polarized odontoblast layer (Fig. 2-3C and Fig. 2-8, *right panels*). In addition, analysis of Nestin antibody staining data revealed a sharp reduction of Nestin expression levels in *Dkk1*-Tg odontoblasts (Fig. 2-4C, *right panel*). Analysis of these data suggests that Dkk1 regulates Osx and Nestin in post-natal dentin formation, and that the changes of these molecules could be partly responsible for the immature odontoblast phenotype in the *Dkk1*-Tg mice.

Discussion

Dkk1, primarily expressed in mesenchymal-derived tissues such as the dental papilla, pre-odontoblasts, and odontoblasts during tooth development, is speculated to be important for dental patterning and crown morphology (*16*). In this study, we directly targeted *Dkk1* overexpression in pulp odontoblasts to investigate its specific role during post-natal tooth (particularly root) development *in vivo*. Our main findings are: (1) The *Dkk1*-transgene, driven by the rat 2.3-kb Col1a1 promoter, whose activity is observed in both pulp (low level) and odontoblast (high level) cells (*84*) (Fig. 2-9), is mainly expressed in post-natal pulp and odontoblast cells; (2) *Dkk1*-Tg mandibular molars display a severe post-natal phenotype, including short roots, an enlarged pulp/root canal region, thin dentin, a considerable reduction in the dentin formation rate, a small malformed second molar, and an absent third mandibular molar; and (3) changes of Osx and Nestin expression in odontoblast cells, an increased number of immature odontoblasts, few mature odontoblasts, and a sharply reduced dentinal tubule number. Thus, we propose that Dkk1 controls post-natal mandibular molar dentin formation either directly or indirectly *via* inhibition of Wnt signaling in the following levels: (i) post-natal dentin formation, (ii) formation and/or maintenance of the dentin tubular system, (iii) mineralization of the dentin, and (iv) regulation of molecules such as Osx and Nestin.

The actual role of Wnt/β-catenin or Dkk1 in post-natal tooth (especially root) formation is not clear, since previous work mainly focused on tooth germ and crown formation during embryonic development. In this work, we directly target Dkk1 in pulp and odontoblast cells during the post-natal developmental stage. Analysis of our data clearly showed a striking dentin phenotype (see above), suggesting that Wnt/β-catenin signaling continuously plays a key role during post-natal dentin formation. Interestingly, the 2.3-kb Col1a1 *Dkk1*-transgene is active in pulp and odontoblast cells, while there is no sign of the 3rd mandibular molar in all *Dkk1*-Tg mice examined by radiograph, with 6 samples at the age of 2 wks and 6 samples at the age of 4 wks (Fig. 2-2). With an H&E staining assay, we noticed an empty cavity within the *Dkk1*-Tg mandibular alveolar bone

where the 3rd molar should be (data not shown), suggesting that the tooth germ was formed but its growth was arrested and then it was subsequently resorbed. Thus, we speculate that the high level of Dkk1 released from pulp cells either directly or indirectly (through the inhibition of Wnt/β-catenin signaling) blocks further development of the 3rd molar. At this stage, we do not know whether this inhibitory role is Wnt/β-catenin-dependent or –independent, or both. Future generation of an odontoblast-specific *Dkk1* knockout animal model would yield a clearer picture of the roles of Dkk1 in post-natal tooth development. In addition, we plan to cross the 2.3-kb Col1a1 *Dkk1*-Tg mice to the Top-Gal mouse line (*85*), to determine whether lacZ expression, which reflects Wnt/β-catenin signaling, is altered in the *Dkk1*-Tg tooth.

Tooth, unlike bone, is usually not resorbed by osteoclasts, likely because of the presence of anti-resorption factors residing in the PDL region and an intact acellular cementoblast layer for its protection (*86*). Here we showed severe dentin resorption on the *Dkk1*-Tg root surface (Fig. 2-3). This defect is most likely caused by an increase in osteoclast numbers and a defect in the acellular cementum. It is known that Wnt signaling up-regulates Opg in the mature osteoblast, which blocks Rankl-induced osteoclastogenesis (*87*). Overexpression of Dkk1 will increase osteoclast numbers *via* an inhibition of Wnt signaling.

In contrast to the severe tooth phenotype observed in the mandibular molars (Fig. 2-2), the maxillary molar phenotype is mild (Fig. 2-7); suggesting that regulation of tooth patterning is complex. Similar variation in tooth phenotype within other genetic animal models has also been reported. For example, Thomas *et al*. demonstrated that null mutations of both *Dlx-1* and *Dlx-2* homeobox genes display no maxillary molars, while the incisors and the mandibular molars develop normally (*88*). Denaxa *et al*. showed that a double knockout of homeodomain transcription factors *Lhx6* and *Lhx7* leads to molar agenesis, with incisors being largely unaffected (*69*).

In summary, 2.3-kb Col1a1 *Dkk1*-transgenic mice display a striking dentin phenotype, which occurs post-natally. We propose that Dkk1 in pulp and odontoblast cells directly or indirectly (though the inhibition of Wnt signaling) controls post-natal

mandibular molar development at three levels: (i) gene expression (through molecules such as Osx and Nestin) and cell maturation; (ii) dentin ultrastructure; and (iii) dentin mineralization.

CHAPTER IV

EFFECTS OF OVER-EXPRESSING DKK1 ON OSTEOBLAST AND OSTEOCLAST HOMEOSTASIS

Overview

Dickkopf-1 (Dkk1) is a potent inhibitor of the Wnt/β-catenin signaling pathway. Deletion of the *Dkk1* gene in mice leads to embryonic lethality, whereas transgenic mice over-expressing *Dkk1* in mature osteoblasts result in osteopenia and severe cranial vault abnormalities. The study of *Dkk1*-Transgenic (*Dkk1*-Tg) mice aims to determine the effect of over-expressing *Dkk1* in regards to calvarial osteogenesis by overexpressing the gene in mature osteoblasts following activation of the 2.3-kb Col1a1 promoter. *Dkk1*-Tg mice possess a markedly reduced calvarial bone density and multiple regions lacking any bone mineralization within the cranial vault, indicative of an osteogenic deficiency. Analyses performed by micro-computed tomography confirmed a >60% reduction of bone volume/total volume (bone fraction) within the parietal bone. The sagittal suture displays incomplete articulation of the parietal bones due to a combination of decreased mineralization and altered bone marker expression. Calvarial osteocytes are irregularly shaped, more rounded, non-spindle shaped, and abnormally small. Interestingly, there was a considerable increase in osteoclast cell number and activity as well as an unusual positioning of these cells on the endocranial surface of the calvarium. The expression of osteoblast/osteocytes cell markers *Dkk1*, *Sost*, *Dmp1*, *Bsp*, *Opn*, *Alp* and *Osx* were increased, while *Runx2* expression was unchanged. Markers of osteoclast activity: *Trap*, *CatK*, *Mmp-9* were significantly increased. Osteoclast differentiation and maturation markers: *RANKL* and *M-CSF* were increased, while expression of *OPG* was unchanged. Together, these data indicate that Dkk1 plays a role in calvarial osteogenesis by directly affecting osteoblast activity and indirectly increasing osteoclast number and activity.

Introduction

Dkk1, a soluble secreted Wnt antagonist, has been documented to play a key role in bone development and remodeling through its binding to the Lrp5/6 Kremen complex (*13, 28, 42, 89, 90*). The conventional deletion of the *Dkk1* gene in mice negatively affects their development resulting in embryonic lethality due to a lack of anterior head structures (*77*). Interestingly, with the ability to balance Wnt-signaling levels throughout development, the anterior head truncation could be rescued with the use of a doubleridge mouse, which reduced Lrp6 expression levels (*91*). Other work has shown that over-expression of *Dkk1* (driven by the 2.3-kb Col1a1 promoter) causes osteopenic effects in the skeleton (*13*).

Pivotal cells in bone formation, regulation and turnover include the osteoblasts and the osteoclasts. The osteoblasts are the cells responsible for bone formation whereas the osteoclasts are terminally differentiated, multinucleated cells responsible for bone resorption. These two cell types work in collaboration with one another to maintain bone homeostasis (*29*). To promote osteoclast differentiation osteoblasts secrete Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL), which is then free to bind to Rank, a protein expressed by osteoclasts (*30*). Osteoblasts also secrete macrophage-colony stimulating factor (M-CSF), which has shown an effect in osteoclast differentiation, but is not needed for the resorbing activity of osteoclasts (*31*). The counter-response to RANKL:RANK binding and osteoclast differentiation into a multinucleated osteoclast is modulated by the osteoblasts' expression of Osteoprotegerin (OPG), a decoy receptor. OPG binds to RANKL, preventing RANKL from binding RANK, thereby preventing osteoclastogenesis (*32*). Determination of the extent of normal bone resorption depends heavily on the ratio of RANKL:OPG. Once activated, osteoclasts stimulate resorption and degradation of the boney matrix, by expressing enzymes, such as Tartrate-Resistant Acid Phosphatase (TRAP), a glycosylated monomeric metallo-enzyme, Cathepsin K, a lysosomal cysteine protease, and Matrix Metallo-Proteinase-9 (Mmp-9), an extracellular matrix proteinase. These proteins are

secreted into the osteoclasts' lacunae, the space between the cell and their attachment site to the extracellular mineralized matrix, and degrade the matrix (*33*).

Bone formation is regulated by many different bone markers expressed through different pathways. Osterix (Osx) is a key player in bone formation whose expression has been previously documented to be under the control of Runx2. *Runx2* knockout (KO) mice lack bone formation completely and *Osx* KO mice are unable to form bone due to an arrest of osteoblast differentiation. Interestingly, an overexpression of both *Runx2* and *Osx* under the expression of the 2.3-kb Col1a1 promoter leads to an osteopenic phenotype in the long bones similar to that which is seen in 2.3-kb Col1a1 *Dkk1*-Tg mice. Osx is known to be pivotal in osteoblastic differentiation during the early stage of osteoblast differentiation, but during the later stages it is evident that Osx has an inhibitory function leading to an accumulation of immature osteoblasts. Osx is regulated by Runx2 as well as by the Bone Morphogenetic Protein (BMP) pathway. A potential interaction between the Wnt signaling pathway and the BMP pathway has been identified in our study that could lead to evidence of *Osx* expression regulation independent of Runx2.

Osteoclastic bone resorption has been indicated in patients with osteolytic bone diseases such as multiple myeloma (*34*). Speculations as to the driving force behind multiple myeloma lytic bone disease include both osteoblastic inhibition (such as with an over-expression of Dkk1) (*35*), and osteoclastic stimulation leading to an uncoupling of bone homeostasis. Dkk1 negatively regulates Wnt/β-catenin signaling and in turn functions as a critical regulator of bone physiology. Specific aspects of the Wnt/β-catenin signaling pathway have been studied within osteoblasts but a relatively scant amount of research has focused on the crosstalk of Wnt/β-catenin signaling and BMP signaling in osteoblasts. Studying the effects of Dkk1 on bone in the very serious and devastating lytic bone disease in humans (*36, 37*). Research has shown that there is a definitive link between patients with multiple myeloma and an over-secretion of Dkk1 from the patients' plasma cells (*35, 38, 39*). Elevated levels of Dkk1, present in the plasma and peripheral blood, were then subsequently correlated with the formation of

lytic bone lesions and the "punched out" bone lesions in the calvarium. Bone marrow biopsy specimens stained for Dkk1 exhibited very elevated levels, and also presented with an increased Dkk1 antibody staining within plasma cells adjacent to the bone (*36, 40*). Furthermore, Dkk1's involvement in multiple myeloma links the over-expression of the Dkk1 protein to a disruption in the balance between osteoblastic and osteoclastic activity. Patients with multiple myeloma have presented with markedly increased osteoclastic activity in contrast to a significantly diminished osteoblastic activity. The osteolytic lesions formed in a significant amount of patients with multiple myeloma seem to occur due to osteoclastic hyper-activation combined with osteoblastic inhibition.

The *Dkk1*-Tg mouse model used in this study proposes a method to more closely study the lytic bone lesions formed in multiple myeloma by bypassing the plasma cell discrepancy. Secreted Dkk1 from plasma cells adjacent to bone (osteoblastic/osteoclastic) cells is able to directly affect these cells whereas within the *Dkk1*-Tg mouse model, mature stage osteoblasts are secreting elevated levels of the Dkk1 protein, which is then able to act through autocrine/paracrine signaling on the osteoblasts. Other data presented with inconclusive evidence regarding the effects of Dkk1 on osteoclasts (*92*).

Although Dkk1 appears to have a significant role in the balance of osteoblastic and osteoclastic activities, the precise role of osteoclastogenesis in multiple myeloma lytic bone disease still remains unclear regarding Dkk1 and the bone microenvironment. The role of Wnt/β-catenin signaling and the effect of Dkk1 on the Wnt/β-catenin signaling pathway regulating osteoclastic activity are still presently unknown. In this study we aimed to determine the effects of over-expressing *Dkk1* in mature osteoblasts to further study the effect on osteoclastic activity and bone homeostasis. The *Dkk1*-Tg mouse model allows for concentration on determining the relationship between Dkk1 expression and osteoblastic/osteoclastic activity as it is still unclear how the over-expression of *Dkk1* in osteoblasts affects osteoclastic activity. Here we present a hypothesis by which Dkk1 negatively regulates the Wnt signaling pathway, which then releases inhibitory activity on the BMP pathway stimulating *Osx* expression. Osx

expression at this later time point in differentiation then acts as a blocker of osteoblast differentiation from immature osteoblasts into mature osteoblasts. Osx expression also regulates *RANKL* and *M-CSF* expression increasing osteoclastogenesis.

Experimental Procedures

Animals

This study was conducted in accordance with federal animal care guidelines. All mice were maintained under guidelines established by Baylor College of Dentistry Institution of Animal Care and Use Committee (IACUC). For these studies, WT mice, 2.3-kb Col1a1 *Dkk1*-Tg mice (Amgen), and TOPGAL mice were used. Generation of the *Dkk1*-Tg mice has been described previously (*13*). Animals were housed under normal conditions with standard light cycles, and fed a standard rodent chow and tap water. Age groups included: P0, 2-wks, 4-wks, 12-wks, and 24-wks. Animals were genotyped as previously described.

Radiography and Microcomputed Tomography (μCT)

Radiographs were taken using Faxitron radiographic inspection unit (Model 8050, Field Emission Corporation, Inc), with digital capture image capability. μCT Analyses were performed using a Scanco Medical μCT 35 (Scanco Medical AG, Switzerland) of *Dkk1*-Tg and WT whole head samples from each age group using high resolution μCT scanning. Overall 3-D assessment will depict the shape/morphology of the calvarial bones, as well as allow for visualization and quantification of the quality of the calvarial bones by morphometric analyses. A comparable region between the WT and *Dkk1*-Tg parietal bones will be contoured (selected out) separately from the rest of the surrounding calvarial (cranial vault) bones to analyze in detail the bone quality and bone thickness. Measurements are made by running bone volume/density and bone trabecular morphometry scripts (μ-CT program) on various contoured regions. Measurements include: apparent density, cortical thickness, and average trabecular

thickness, number and separation.

FITC staining

Fluorescein isothiocyanate, a small molecular dye, will fill in the calvarial cells/fibers such as osteoblasts, osteocytes and osteoclasts, without staining the mineral matrix. Thus, the dye provides a visual representation of the organization of the bone. Immediately after harvest and dissection a coronally cut section of the skull that included the parietal bones was placed in EM fixative (0.5%gluteraldehyde, 2% paraformaldehyde in 0.05 M cacodylate-sodium buffer, pH 7.4) at room temperature (RT) and then processed and cut with a diamond blade saw (Buehler, Lake Bluff, IL) to obtain coronal section with 300-400 μm cross-sectional thickness. The sections were placed back into EM fixative for 24 hours at RT. The sections were then sanded down and polished to a final thickness of 30-50 μm for confocal imaging.

Analysis of bone formation rate by double-labeling

To examine the bone formation rate, we performed double-fluorescence labeling as described previously (*79*). Briefly, a calcein label (5 mg/Kg i.p.; Sigma-Aldrich, St. Louis, MO, USA) was administered to 20-day-old mice, followed by administration of calcein 7 days later. Mice were sacrificed 2 days after the second injection (1 mo old). The non-decalcified calvaria were embedded in resin (methylmethacrylate, MMA), sectioned, and photographed under epifluorescent illumination with a Nikon 800 microscope (Nikon, Melville, NY, USA).

Scanning Electron Microscopy (SEM) and resin-casted SEM

Selected calvarial bone tissues will be fixed in 70% ethanol and embedded in methyl-methacrylate (MMA, Buehler, Lake Bluff, IL). For backscattered SEM, the surface of the MMA embedded bone will be polished, and coated with gold, and examined by FEI/Philips XL30 Field emission environmental SEM (Hillsboro, OR, USA). For resin casted SEM, the above samples will be polished again to take away the previous coated materials followed by acid treatment for 5-10 seconds. Combinations of both assays will reveal morphological changes of the osteocyte, overall bone structure,

bone mineral and bone loss in WT mice and *Dkk1*-Tg mice. SEM will be performed within specified regions along the parietal bone.

Histology and immunohistochemical staining

Calvarial bones were cut coronally and sagitally (for different imaging), fixed in 4% paraformaldehyde (PFA), decalcified with 10% EDTA, and embedded in paraffin by standard histological procedures as previously described (*93*). Serial sections were cut (4-5 μm thick) and mounted on glass slides. Sections were then used for Hematoxylin and Eosin (H&E) staining, immunohistochemistry, TRAP staining and LacZ staining. DKK1 (Abcam, Cambridge, UK) immunostaining was performed on histological sections of the WT and *Dkk1*-Tg calvaria based on methods described previously (*94*). Polyclonal antibodies Sost, Dmp1, and Bsp were used for detection of osteocytes, osteoblasts and bone matrix. TRAP (Tartrate resistant acid phosphatase) staining was performed to detect TRAP+ osteoclasts that are actively resorbing bone. Whole mount B-galactoctosidase staining was performed to detect Wnt signaling on paraffin sections. The staining methods were performed according to the manufacturer's instructions.

Cell culture, RNA isolation and quantitative PCR (qPCR) analysis

To examine the effects of over-expressed *Dkk1* on the calvaria, primary calvarial osteoblasts were isolated from 3 sets of four-day-old WT and 3 *Dkk1*-Tg mice parietal bones. The osteoblasts were seeded at a concentration of 1 x 10^4 into 3 wells a 48 well plate per animals (total of 18 wells for 6 animals). Cells were cultured in alpha Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Media was changed every 2 days. After 4 days, the isolated primary osteoblast cells from *Dkk1*-Tg and WT mice were harvested for RNA isolation and quantitation analysis of gene expression by real time RT-PCR. RNA was isolated using RNeasy Mini Kit (Qiagen, 74106) according to the manufacturer's protocol. After isolation, 1 μg of total RNA treated with DNase and was reverse-transcribed in first strand cDNA using QuantiTect Rev. Transcription Kit

(Qiagen, 205311). Using 10 μl SYBR GREEN qPCR, reactions were performed in a 96 well optical reaction plate formatted in a CFX96 Bio-Rad real-time PCR (qPCR) machine with a standard reaction protocol. The transcript of GAPDH mRNA was employed as an internal control for RNA quality. For each gene, three independent RT-PCR reactions from the same reverse transcription sample were also performed for accuracy. The genes analyzed were osteoblast markers: *Dkk1*, *Dmp1*, *Bsp*; osteocyte markers: *Sost*, and osteoclast markers: *Trap*, *Cathepsin-K*, *Mmp-9*, *Rankl*, *Opg*, and *m-CSF*. *Gapdh* was used as the internal control.

Statistical analyses

The data analysis was performed with a student t-test. Each experiment was performed in triplicate experimentally and biologically. The quantified results are represented as the mean ± standard error of the mean (SE). We set $p < .05$ as the statistical significance level.

Results

Dkk1-Tg calvaria present with osteolytic-like lesions

Dkk1-Tg mice overexpress *Dkk1* under the 2.3-kb Col1a1 promoter (Fig. 3-1A) and show expression levels around 30-fold higher than wildtype (WT) control mice (Fig. 3-1B). *Dkk1*-Tg mice present with a relatively shortened stature (Fig. 3-2A) as well as a significant decrease in body weight (Fig. 3-2B). These mice present with osteolytic-like lesions within the calvarial bones, with most striking effects seen within the parietal bones, as determined with radiographic imaging (Fig. 3-2C). A diminished bone formation within the calvarial bones was also observed between littermate control and *Dkk1*-Tg calvaria at multiple different developmental as well as adult time points: P0, 4-week, and 12-week-old mice, as determined by analyses using micro-computed tomography (μ-CT) (Fig. 3-3A). The parietal bone phenotype was our focus for this study since it was the major constituent of the cranial vault consistently negatively

affected in *Dkk1*-Tg mice throughout development and into adulthood. The overall quality of the parietal bone was determined by selecting (contouring) out a single parietal bone for analysis using μ-CT software and assessing multiple parameters. The apparent density of the transgenic parietal bone was significantly lower than the WT parietal bone with greater than a 60% decrease in bone volume/total volume (bone fraction volume). There was also a significant decrease in apparent density and mineral density (Fig. 3-3B) showing a decrease in bone mineral density.

Defects in osteocyte morphology and bone quality

To further investigate the quality of the *Dkk1*-Tg calvarial bone and osteocyte cell morphology, FITC (Fluorescein isothiocyanate) labeling of the WT and *Dkk1*-Tg parietal bone was performed (Fig. 3-4A). FITC labeling confirmed the presence of osteolytic lesions interspersed with small bone-islands in the transgenic skull by detecting and staining cells but not staining mineralized material such as bone. Qualitatively, the stain displayed the osteocytes as having a substantial decrease in the number of dendritic processes radiating from the osteocytes as well as the presence of rounded osteoblast-like cells instead of spindle-shaped osteocytes. Scanning Electron Microscopy (SEM) showed a considerable qualitative decrease in parietal bone mineralization (Fig. 3-4B, C). The transgenic osteocytes/potential immature osteoblasts appear clumped together and abnormal in shape, with multiple cells sharing the same lacunae. The parietal bones, which abut on either side of the intervening sagittal suture, do not fuse in control mice normally, but rather they closely abut one another, whereas the *Dkk1*-Tg parietal bones are a significant distance from the anticipated abutment region (Fig. 3-4B). The cells reside in significantly under-mineralized areas of bone interspersed within the fibrous lesions. The matrix was poorly organized, lacking well-formed lacunae in which the cells reside. Also, the cell shape was altered from its normal osteocyte morphology, which can be visualized in the SEM acid-etched WT and *Dkk1*-Tg parietal bone imaging. The *Dkk1*-Tg cells are smaller and appear deflated (Fig. 3-4D).

The parietal sutures of the transgenic mice were also affected most likely due to a lack of bone mineralization but appear to align correctly from a developmental standpoint. In the WT murine cranial vault, the posterior frontal suture is the only suture that fuses, while the other cranial bone fronts closely articulate and align with one another. In the *Dkk1*-Tg mice, the posterior frontal suture fuses as normal, while the parietal bone abutment has a consistent gap where the fusion would normally have taken place (Fig. 3-5). It is assumed that the sagittal suture is wider due to a mineralization defect rather than a defect in suture development.

Significant alteration of Dkk1-Tg bone formation rate

The bone formation rate measured through the utilization of double calcein labeling methodology showed a decreased in bone formation on the endo-cranial surface and an increase in bone formation on the ecto-cranial surface (Fig. 3-6). The diminished bone formation on the endo-cranial surface is theorized due to the increase in the osteoclastic cell number and activity present on this bone surface as seen via the significant increase in tartrate resistant acid phosphatase positive (TRAP+) staining (Fig 3-7A). The increase in bone formation on the ecto-cranial surface is presumed to be present in a compensatory response to the increase in bone resorption on the endo-cranial surface.

Increase in osteoclast differentiation combined with an increase in resorptive activity

In 2-week and 4-week old *Dkk1*-Tg mice, there was a significant increase in TRAP+ staining on the endo-cranial surface of the parietal bones, suggesting sustained high levels of resorptive activity occurring in this area (Fig. 3-7A). *Dkk1*-Tg mice also exhibit a 9.5-fold increase in *Trap* mRNA expression, a 9.5-fold increase in *Cathepsin-K* mRNA expression and a 5.5-fold *Mmp-9* (matrix metalloproteinase 9) mRNA expression (Fig. 3-7B). Collectively, these data suggest that the *Dkk1*-Tg parietal bones possess an increase in osteoclastic activity of the mineralized regions. The osteoclast differentiation factors expression levels were then measured by reverse transcription real-time PCR (RT

qPCR) in the WT control and *Dkk1*-Tg mice parietal bones to determine if expression changes were evident. In the transgenic mice, we observed ~2.5-fold increase in *RANKL*, and a 1.8-fold increase in *M-CSF* but no concomitant increase in *OPG* mRNA expression (Fig. 3-7C).

Decrease in β-catenin nuclear translocation and activation of downstream effector genes in osteoblasts and osteocytes

To determine the effect of overexpression of Dkk1 on canonical Wnt signaling in calvarial parietal bone, *Dkk1*-Tg mice were crossed with Wnt reporter (TOPGAL) mice that express the lacZ gene encoding E. coli β-galactosidase under the control of three Lef1/Tcf consensus binding sites linked to a minimal promoter (*85*). Expression of the transgene-specific, E. coli β-galactosidase staining was greatly increased in parietal bone from 2-week-old *Dkk1*-Tg mice compared to those in WT littermates shown as whole mount B-gal staining (Fig. 3-8A) and parietal bone sectioning (Fig. 3-8B). LacZ expression was seen prominently in osteoclasts (previously documented to be increased due to the increased activity (*95*)) within the *Dkk1*-Tg parietal bone and was severely reduced/absent in the osteocytes. In the WT littermates, lacZ expression was seen in osteocytes and osteoblasts.

Significant alterations in bone marker gene expression as well as a maintenance of osteoblasts in an immature State

Histologically, hematoxylin and eosin (H&E) stained sections of transgenic calvaria as compared to wildtype calvaria, displayed fibrous-like tissue regions lacking mineralization, which were interspersed between under-mineralized islands of trabecular bone (Fig. 3-9A). Interestingly, the calvarial osteoblasts lining the regions of bone showed a significant increase in Osx protein expression (Fig. 3-9B). *Osx* expression increase was confirmed via RT qPCR, whereas the expression of *Runx2* was unchanged (Fig 3-10). Along with this data, protein expression of Bone sialoprotein (Bsp) and Osteopontin (Opn) were increased providing evidence of immature osteoblast markers

and immature bone formation (Fig. 3-11). On the contrary, there was a significant decrease in Osteocalcin (Ocn) expression, a well-known mature osteoblast marker. This data was further confirmed with RT qPCR, with a 3.5-fold increase in *Bsp* expression, a 2.9-fold increase in *Opn* expression, a 3.2-fold increase in *Alp* expression and a 5-fold decrease in *Ocn* expression (Fig. 3-12).

Interestingly, even though the *Dkk1*-Tg parietal bone presented with disrupted mineralization and a decreased bone density, the osteocytes displayed an increase in Sclerostin (Sost) expression (Fig. 3-13A) as well as Dentin Matrix Protein (Dmp1) (Fig. 3-13B) as shown by immunohistochemical staining (IHC). This data was further confirmed with real-time PCR (qPCR) from purified mRNA extracted from isolated and homogenized WT and *Dkk1*-Tg paired parietal bones showing ~3-fold increase in *Sost* mRNA expression, ~4.5-fold increase in *Dmp1* expression (Fig. 3-14).

Discussion

In search for potential roles of Dkk1 in craniofacial bone development, we studied the 2.3-kb Col1a1 *Dkk1*-Tg mice, which exhibits striking osseous defects in the calvaria. *Dkk1*-Tg mice over-express *Dkk1* in immature and mature osteoblasts due to the nature of the 2.3-kb Col1a1 promoter and its expression-dependent manner in osteoblasts around E14.5 (previously reported using R26R reporter mice) (*96*). Primary parietal bone osteoblasts isolated from *Dkk1*-Tg mice used in this study had an average of a 30-fold increase in *Dkk1* mRNA expression (Fig. 3-1).

The main observation in the calvarial bones was an osteopenia-like phenotype with a woven bone appearance. The parietal bones displayed a decreased mineral density, compared to WT controls, and regions of the bone were interspersed with fibrous-like regions of cells in which there is a complete absence of mineralization. Interestingly, the osteoblast cell density was increased as shown using an Osx expression marker, but overall bone formation was reduced. Most of the osteoblasts can be characterized as immature osteoblasts due to the increase in immature osteoblast marker

expressions of Opn and Bsp. Mature osteoblasts are known to express *Ocn* and this was reduced 5-fold in *Dkk1*-Tg osteoblasts. Furthermore, in *Dkk1*-Tg mice there was a significant increase in Osx protein expression. An increase in Osx could explain the increase in immature osteoblast-specific markers such as Bsp, Opn and Alp (*97*). This model could explain a role of Dkk1 expressed in osteoblasts under the 2.3-kb Col1a1 promoter in immature and mature osteoblasts.

Due to the similarity in phenotype of the 2.3-kb Col1a1 *Osx*-Tg mice (*44*) to the 2.3-kb Col1a1 *Dkk1*-Tg mice, our data may connect the gap to a potential link between Dkk1 and Osx. A major difference in the two mouse models is that there is no change in osteoclastic activity in *Osx*-Tg bone, but this analysis was performed on the femur bone (long bone), which is formed through endochondral ossification. *Dkk1*-Tg mice cortical femur bone was also not noted to show a change in osteoclast number or activity (*13*). This can be assumed to be a mechanistic difference between endochondral and intramembranous ossification pathways as we were able to show that the alveolar bone (formed through intramembranous ossification) did present with an increase in osteoclast cell number and activity (*98*). Therefore, we hypothesize that an increase in *Dkk1* in the immature to mature transition osteoblast stage increases Osx activity, which then maintains the cells in a proliferative immature undifferentiated state. At the stage that the Col1a1 promoter is active, Osx does not have this function, as its major function is in early pre-osteoblastic cells.

Ocn is a very late and specific osteoblast marker and it is usually expressed around E18.5. With the significant decrease in Ocn expression in *Dkk*1-Tg parietal bone, this demonstrates the characteristic decrease in the number of cells in a mature osteoblastic state. Osteoblast differentiation is inhibited and immature osteoblasts are accumulated (*99*). These data suggest that proper doses of certain proteins are required to promote osteoblast differentiation and bone formation. The increase in Sost and Dmp1 which are known mature osteocyte markers could be increased due to a compensatory response of those osteoblasts that were already matured prior to the activation of the transgenic promoter.

Since bone development and homeostasis requires a balance between formation and resorption we hypothesize that there is an increase osteoclast function due to the prior knowledge that immature osteoblasts act in a potentially pro-osteoclastogenic manner (*100*). Additionally, it has been documented that osteoblasts serve a dual role, either in support of osteoclastogenesis or in support of bone formation, and these two roles may be performed by the same lineage of cells at different stages of their maturation.

The osteoclasts showed an overall increase in TRAP production, as well as increased *Mmp-9* and *Cathepsin K* expression. These data confirm the increase in osteoblast maturation and activity. The imbalance of RANKL:OPG ratio likely results in increased osteoclast maturation and the increased resorptive activity observed. The increased RANKL secretion may also repress any feedback inhibition that may occur in bone homeostasis not allowing for a let up in bone resorption. Multiple myeloma cells were hypothesized to increase the secretion of RANKL from osteoblasts and *Dkk1*-Tg mice also show an increase a 2.5-fold increase in RANKL secretion. This increase in RANKL secretion allows for an increase in osteoclast maturation and activation. Also, it is noted that the increase in TOPGAL staining for β-catenin signaling is increased in osteoclasts. As Dkk1 is a negative inhibitor of the Wnt signaling pathway, the location of the wnt signaling shows that Dkk1 is blocking the signaling in osteoblasts (which are dispersed around the edges of the woven bone) but Wnt activity is still very strong in the osteoclasts. Rather, the increase in RANKL leads to a subsequent increase in osteoclastogenesis. Therefore, in the transgenic animals, over-expression of Dkk1 is able to enhance the levels of RANKL expression, which has also been confirmed in other studies (*32*). This hypothesis is supported by data showing that Dkk1 significantly increased *RANKL* mRNA expression in mouse primary parietal bone osteoblast cells in contrast to comparative WT control cells (Fig. 6B). There is an uncoupling of the bone remodeling activities in favor of bone resorption with an increase in osteoclast-mediated destruction. It must be mentioned though, that there is the potential for Dkk1, which is expressed under the 2.3-Col1a1 promoter and secreted by osteoblasts into the

surrounding environment to target pre-osteoblastic cells lining the ossifying bone fronts in a paracrine manner.

Our mouse model partially recapitulates the multiple myeloma tumor micro-environment with regards to secreted Dkk1 as a key regulator and its direct effects on osteoblast proliferation and maturation and its indirect effect on osteoclast maturation and activation. This mouse model is a potentially excellent viable mouse model to study the effects of Dkk1 specifically targeting the lytic bone lesions, which occur later in adult life.

We hypothesize that due to previous studies suggesting a crosslink between Wnt signaling and BMP signaling that Dkk1 acts as a Wnt signaling antagonist and this then removes the block that Wnt would normally have on BMP signaling and subsequent suppression of Osx at this time point during osteoblast differentiation (*101*). The increase in Osx expression maintains the osteoblasts in an immature state. Osx has also been shown to regulate *Rankl* and *M-csf* expression as well as *Sost* expression (*102-104*) (Fig 3-15).

CHAPTER V

CONCLUSION

Dkk1 has an important role in osteogenesis and odontogenesis, two very important developmental processes. When *Dkk1* is over-expressed in immature and mature odontoblasts and osteoblasts disruptive changes in odontogenesis, osteoblastogenesis, and osteoclastogenesis.

Role of Dkk1 in Odontogenesis

Upon studying *Dkk1*-Tg mouse tooth development, our main findings were: (1) The 2.3-kb Col1a1 promoter is known to be active, and therefore regulating over-expression of *Dkk1* in pre-odontoblasts and odontoblasts prior to the late bell stage (E16.5). At this stage, the inner enamel epithelium sends signals to the cells adjacent to them lining the dental papilla to become odonotoblasts. Therefore the cells are mostly within the pre-odontoblastic to odontoblastic transitional stage during the activation of the transgene; (2) *Dkk1*-Tg mandibular molars display a severe post-natal phenotype, including short roots, an enlarged pulp/root canal region, thin dentin, a considerable reduction in the dentin formation rate, a small malformed second molar, and an absent third mandibular molar; and (3) changes of Osx and Nestin expression in the odontoblasts, an increased number of immature odontoblasts, few mature odontoblasts, and a sharply reduced dentinal tubule number. The increase in expression of Osx within the cells lining the dentin within the root canal could explain the role of Osx expression at this time-point in keeping the cells in a pre-odontoblastic immature stage and increasing the proliferation of these cells rather than allowing the cells to differentiate into mature polarized odontoblasts. The lack of mature polarized odontoblasts effects the maturation of the dentinal tubules, the thickness of the dentin (which is shown to be reduced in *Dkk1*-Tg molar root dentin) and the reduction in the dentin formation rate.

The actual role of Wnt/β-catenin or Dkk1 in post-natal tooth (especially root) formation is not clear, since previous work mainly focused on tooth germ and crown formation during embryonic development. In this work, we directly target Dkk1 in odontoblastic cells during the post-natal developmental stage. Analysis of our data clearly showed a striking dentin phenotype, suggesting that Wnt/β-catenin signaling continuously plays a key role during post-natal dentin formation.

Thus, we propose that Dkk1 controls post-natal mandibular molar dentin formation either directly or indirectly *via* inhibition of Wnt signaling in the following levels: (i) post-natal dentin formation, (ii) formation and/or maintenance of the dentin tubular system, (iii) mineralization of the dentin, and (iv) regulation of molecules such as Osx and Nestin.

It was noted that the alveolar bone expresses the *Dkk1*-transgene in a similar pattern as does the calvarial bone and therefore as well as it harboring an increased number of osteoclasts which plague the alveolar bone surface. Interestingly, there was an increase in the number of osteoclasts that were present along the external surface of the root lining the dentinal surface adjacent to the PDL. There also appears to be a disruption in the formation of the layer of acellular cementum. Acellular cementogenesis is necessary at the root surface to allow for the anchorage of PDL fibers providing a strong attachment of the tooth to the alveolar bone (*105*). Future studies are needed to concentrate the effect of Dkk1-mediated disruption of acellular cementum.

Interestingly, the 2.3-kb Col1a1 *Dkk1*-transgene is active in pulp and odontoblast cells, while there is no sign of the 3rd mandibular molar in all *Dkk1*-Tg mice examined by radiograph, with 6 samples at the age of 2 wks and 6 samples at the age of 4 wks (Fig. 2-2). With an H&E staining assay, we noticed an empty cavity within the *Dkk1*-Tg mandibular alveolar bone where the 3rd molar should be (data not shown), suggesting that the tooth germ was formed but its growth was arrested and then it was subsequently resorbed. Thus, we speculate that the high level of Dkk1 released from pulp cells either directly or indirectly (through the inhibition of Wnt/β-catenin signaling) blocks further development of the 3rd molar. At this stage, we do not know whether this inhibitory role

is Wnt/β-catenin-dependent or –independent, or both. Future generation of an odontoblast-specific *Dkk1* knockout animal model would yield a clearer picture of the roles of Dkk1 in post-natal tooth development. In addition, we plan to cross the 2.3-kb Col1a1 *Dkk1*-Tg mice to the Top-Gal mouse line (*85*), to determine whether lacZ expression, which reflects Wnt/β-catenin signaling, is altered in the *Dkk1*-Tg tooth.

The severe dentinal resorption on the *Dkk1*-Tg root surface shows a severe defect that could be due to the increase in osteoclast cell number, maturation and activation. Another alternative explanation could be due to the fact that the defect in the acellular cementum promotes dentinal erosion and ablation. It is known that Wnt signaling up-regulates Opg in the mature osteoblast, which blocks Rankl-induced osteoclastogenesis (*87*). Over-expression of *Dkk1* increases osteoclast cell number and their subsequent activation via an inhibition of Wnt signaling and an increase in RANKL secretion.

In summary, 2.3-kb Col1a1 *Dkk1*-transgenic mice display a striking dentin phenotype, which occurs post-natally. We propose that Dkk1 in pulp and odontoblast cells directly or indirectly (though the inhibition of Wnt signaling) controls post-natal mandibular molar development at three levels: (i) gene expression (through molecules such as Osx independently from Runx2 (*106*)) and cell maturation; (ii) dentin ultrastructure; and (iii) dentin mineralization.

Role of Dkk1 in Cranial Vault Osteogenesis

Initial studies of 2.3-kb Col1a1 *Dkk1*-Tg mice dentinal phenotype and alveolar bone phenotype lead to further investigation of other bone defects such as the striking osseous defects within the calvarial bones. *Dkk1*-Tg mice over-express *Dkk1* in immature and mature osteoblasts (as well as within the pre-odontoblasts/odonotoblasts) due to the nature of the 2.3-kb Col1a1 promoter.

The *Dkk1*-Tg presentation of the osteopenic-like phenotype within the calvaria with the presence of a woven bone appearance and the decreased mineral density, confirmed the presence of a mineralization defect. The increase in density of immature osteoblasts (displayed through use of an Osx expression marker) as well as a significant

disruption in various bone markers confirmed an osteoblastic deficiency. Mature osteoblasts markers such as *Ocn* was significantly reduced in *Dkk1*-Tg calvarial bone, in contrast to an increase in immature bone markers such as Bsp, Opn and Alp (*97*). This model could explain a role of Dkk1 expressed in osteoblasts under the 2.3-kb Col1a1 promoter in immature and mature osteoblasts.

Due to the similarity in phenotype of the 2.3-kb Col1a1 *Osx*-Tg mice (*44*) to the 2.3-kb Col1a1 *Dkk1*-Tg mice, our data may connect the gap to a potential link between Dkk1 and Osx. A major difference in the two mouse models is that there is no change in osteoclastic activity in *Osx*-Tg bone, but this analysis was performed on the femur bone (long bone), which is formed through endochondral ossification. *Dkk1*-Tg mice cortical femur bone was also not noted to show a change in osteoclast number or activity (*13*). This can be assumed to be a mechanistic difference between endochondral and intramembranous ossification pathways as we were able to show that the alveolar bone (formed through intramembranous ossification) did present with an increase in osteoclast cell number and activity (*98*). Therefore, we hypothesize that an increase in *Dkk1* in the immature to mature transition osteoblast stage increases Osx activity, which then maintains the cells in a proliferative immature undifferentiated state. At the stage that the Col1a1 promoter is active, Osx does not have this function, as its major function is in early pre-osteoblastic cells.

Ocn is a very late and specific osteoblast marker and it is usually expressed around E18.5. With the significant decrease in Ocn expression in *Dkk*1-Tg parietal bone, this demonstrates the characteristic decrease in the number of cells in a mature osteoblastic state. Osteoblast differentiation is inhibited and immature osteoblasts are accumulated (*99*). These data suggest that proper doses of certain proteins are required to promote osteoblast differentiation and bone formation. The increase in Sost and Dmp1 which are known mature osteocyte markers could be increased due to a compensatory response of those osteoblasts that were already matured prior to the activation of the transgenic promoter.

Since bone development and homeostasis requires a balance between formation and resorption we hypothesize that there is an increase osteoclast function due to the prior knowledge that immature osteoblasts act in a potentially pro-osteoclastogenic manner (*100*). Additionally, it has been documented that osteoblasts serve a dual role, either in support of osteoclastogenesis or in support of bone formation, and these two roles may be performed by the same lineage of cells at different stages of their maturation.

The osteoclasts showed an overall increase in TRAP production, as well as increased *Mmp-9* and *Cathepsin K* expression. These data confirm the increase in osteoblast maturation and activity. The imbalance of RANKL:Opg ratio likely results in increased osteoclast maturation and the increased resorptive activity observed. The increased RANKL secretion may also repress any feedback inhibition that may occur in bone homeostasis not allowing for a let up in bone resorption. Multiple myeloma cells were hypothesized to increase the secretion of RANKL from osteoblasts and *Dkk1*-Tg mice also show an increase a 2.5-fold increase in RANKL secretion. This increase in RANKL secretion allows for an increase in osteoclast maturation and activation. Also, it is noted that the increase in TOPGAL staining for β-catenin signaling is increased in osteoclasts. As Dkk1 is a negative inhibitor of the Wnt signaling pathway, the location of the wnt signaling shows that Dkk1 is blocking the signaling in osteoblasts (which are dispersed around the edges of the woven bone) but Wnt activity is still very strong in the osteoclasts. Rather, the increase in RANKL leads to a subsequent increase in osteoclastogenesis. Therefore, in the transgenic animals, over-expression of Dkk1 is able to enhance the levels of RANKL expression, which has also been confirmed in other studies (*32*). This hypothesis is supported by data showing that Dkk1 significantly increased *Rankl* mRNA expression in mouse primary parietal bone osteoblast cells in contrast to comparative WT control cells (Fig. 6B). There is an uncoupling of the bone remodeling activities in favor of bone resorption with an increase in osteoclast-mediated destruction.

Our mouse model partially recapitulates the multiple myeloma tumor micro-environment with regards to secreted Dkk1 as a key regulator and its direct effects on osteoblast proliferation and maturation and its indirect effect on osteoclast maturation and activation. This mouse model is a potentially excellent viable mouse model to study the effects of Dkk1 specifically targeting the lytic bone lesions, which occur later in adult life.

We hypothesize that due to previous studies suggesting a crosslink between Wnt signaling and BMP signaling that Dkk1 acts as a Wnt signaling antagonist and this then removes the block that Wnt would normally have on BMP signaling and subsequent suppression of Osx at this time point during osteoblast differentiation (*101*). The increase in Osx expression maintains the osteoblasts in an immature state. Osx has also been shown to regulate *Rankl* and *M-csf* expression as well as *Sost* expression (*102-104*) (Fig 3-15). An increase in *Dkk1* in the transition of immature to mature osteoblasts increases Osx expression within these cells. Osx then has the capability to maintain the cell in a proliferative immature state that is incapable of differentiating into a mature osteoblast. During the developmental stage that the 2.3-kb Col1a1 promoter is active, Osx is not expressed highly within the late stage pre-osteoblasts/early mature osteoblasts, as its major function is within the early pre-osteoblastic cells. With the significant decrease in Ocn expression (an specific late osteoblast marker) in *Dkk*1-Tg parietal bone, this can explain the decrease in the number of cells in a mature osteoblastic state. Osteoblast differentiation is inhibited and immature osteoblasts are accumulated (*99*). DKK1 significantly increased RANKL expression in osteoblasts inducing an uncoupling of the bone remodeling activities in favor of bone resorption with an increase in osteoclast-mediated destruction.

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APPENDIX A

FIGURES

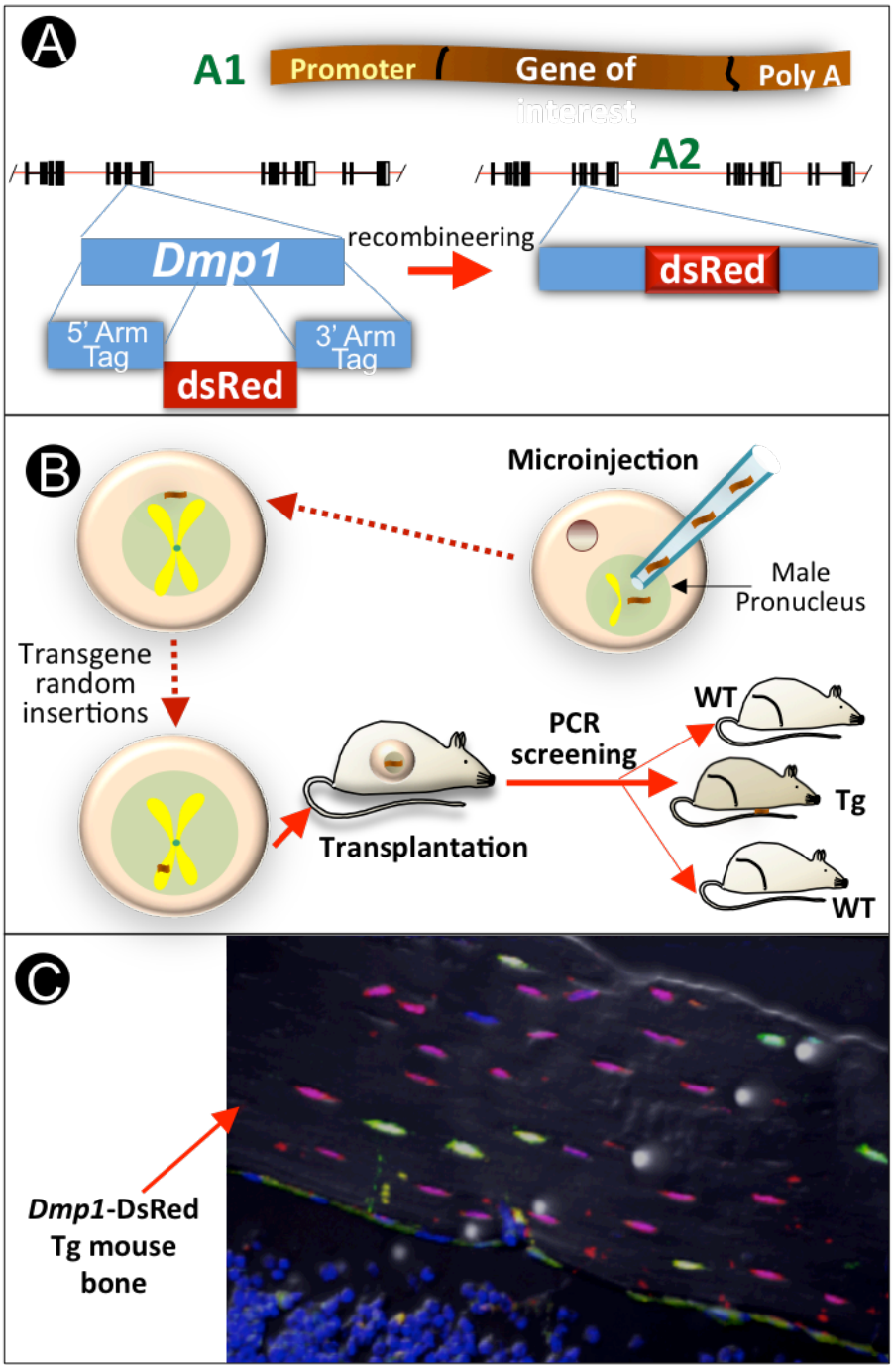


Figure 1-1. Generation of a transgenic mouse. The first step is to produce a transgene construct which is achieved by two different approaches: The conventional approach is to use restriction enzymes to build the complete transgenic unit together, including a tissue specific promoter (the size is less than 10 kb in most cases), a gene-of-interest (such as GFP reporter), and a polyA tail (to keep the targeted gene stable for translation when it is expressed) (**A1**). The common problem with this method is that the transgene expression level is affected by the insertion site plus surrounding genes' influence. The second method is recombineering (recombination-mediated genetic engineering, (**A2**). The advantages to using this technique are: 1) there is no need for positioned restriction sites and thus the transgene can be inserted into a ~200 kb (150-350 kb ranges) BAC clone; and 2) the expression level of the transgene is identical to that of the endogenous gene. **B.** The transgene generated can be injected into male pronucleus of an oocyte (the size is not an issue), which will then be randomly inserted into any site of the chromosome. The oocyte with the transgene is then implanted into a psuedo-pregnant female foster mouse and the offspring either contain the transgene in their genome (transgenic) or do not (wildtype, WT). **C**. A confocal view of expressions of the *Dmp1*-DsRed (signal in pink in osteocytes) generated in **A2**. Data was generously provided by Dr. Stephen E. Harris (UT Health Science Center At San Antonio, TX USA)

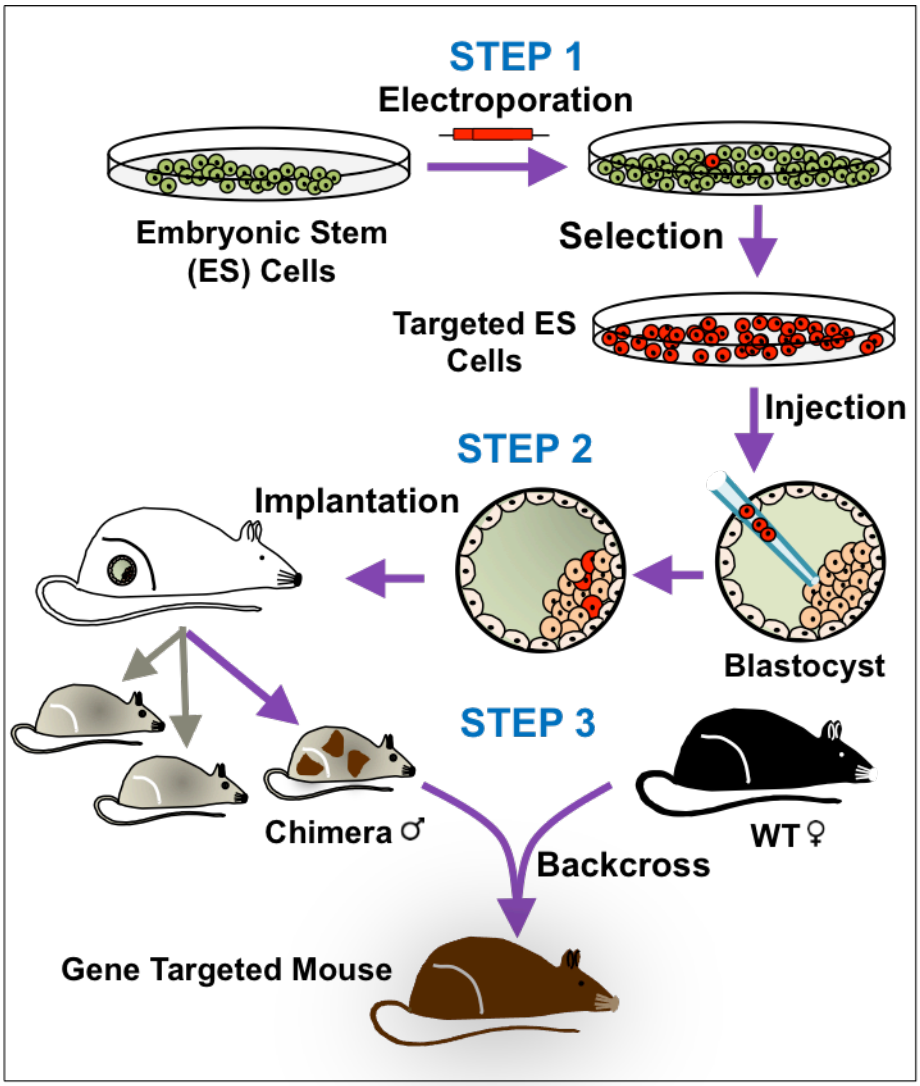


Figure 1-2. Generation of gene targeted mice using embryonic stem (ES) cells. Step 1: The designed targeting vector is electroporated into ES cells and targeted ES cells are selected for through antibiotic selection. Southern blot and PCR analyses are performed to confirm homologous recombination. Step 2: The targeted ES cells are injected into a blastocyst and then implanted into a pseudo-pregnant foster female mouse. Step 3: A chimeric male of the offspring is backcrossed to a wildtype (WT) female to ultimately create a gene-targeted mouse (germline transmission).

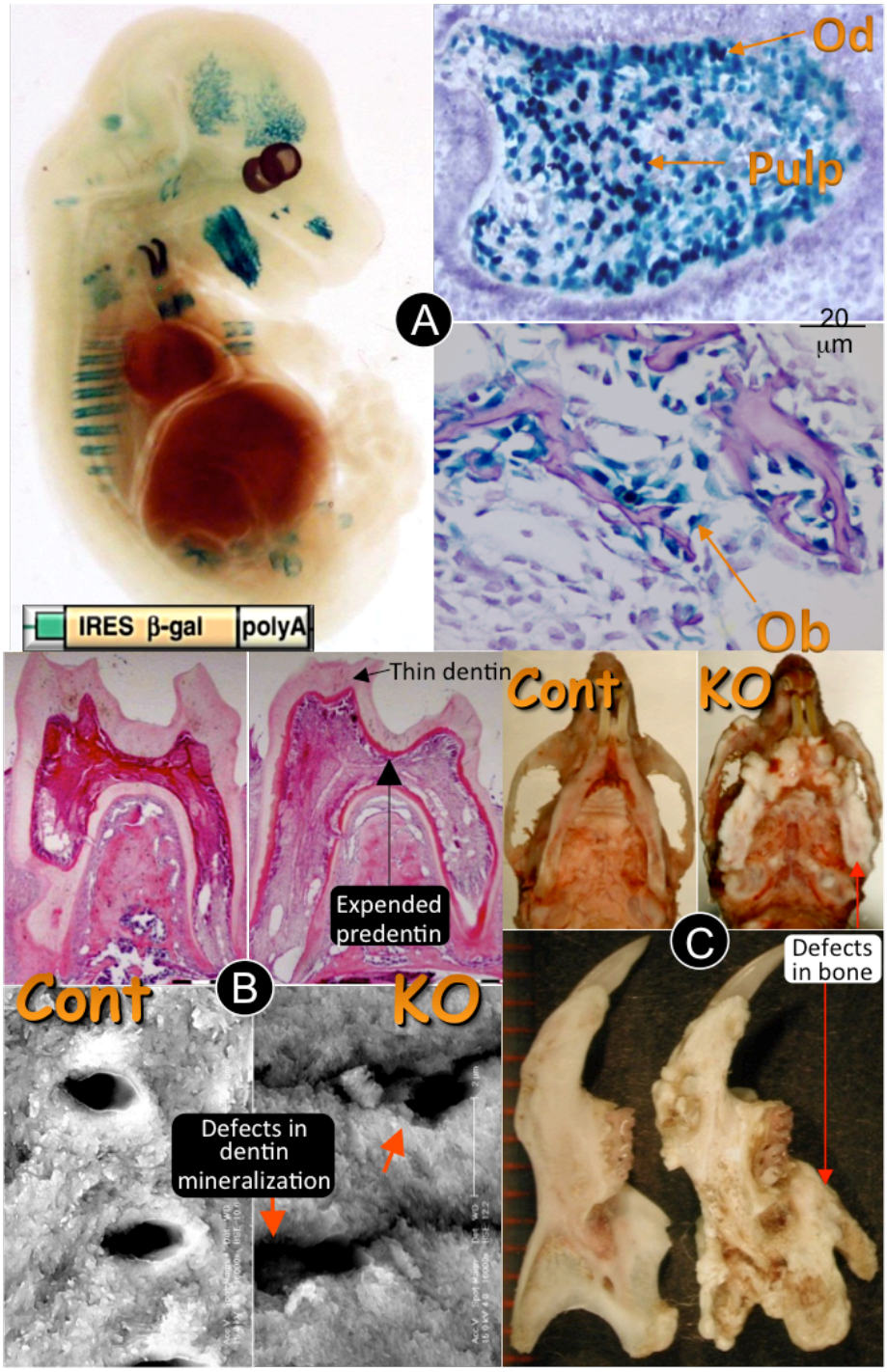

Od
Pulp
A
20 μm
IRES β-gal
polyA
Ob
Thin dentin
Cont
KO
Expended predentin
B
C
Defects in bone
Cont
KO
Defects in dentin mineralization

Figure 1-3. *Dmp1*-lacZ knock-in mouse line: A useful model for studying gene expression and function in craniofacial development. A. A whole-mount X-Gal stain of an E15.5 Dmp1-lacZ knock-in embryo (left panel) and two tissue sections stained by X-Gal: newborn incisor (upper right), and alveolar bone (lower right), show high lacZ expression in the pulp, odontoblast (Od), and osteoblast (Ob) cells (shown in blue). A lacZ reporter gene was used to replace exon 6 of the Dmp1 gene. The expression of lacZ reflects endogenous Dmp1 expression. Data is adapted from Feng *et al*. (2003)(*107*). **B**. H&E stained images revealed thin dentin and enlarged predentin in this knockout (KO) 1st molar (right), and backscattered SEM images displayed sharp reduction of mineral surrounding dentin tubules (white indicating high mineral content) plus structure changes in the KO dentin (lower right), suggesting DMP1 is critical for dentin mineralization. Data is adapted from Ye *et al.* (2004)(*108*). **C**. Photograph images showed severe defects in the KO alveolar bone morphology, indicating an essential role of DMP1 in bone remodeling (right images). Data is adapted from Zhang *et al*. (2011)(*109*).

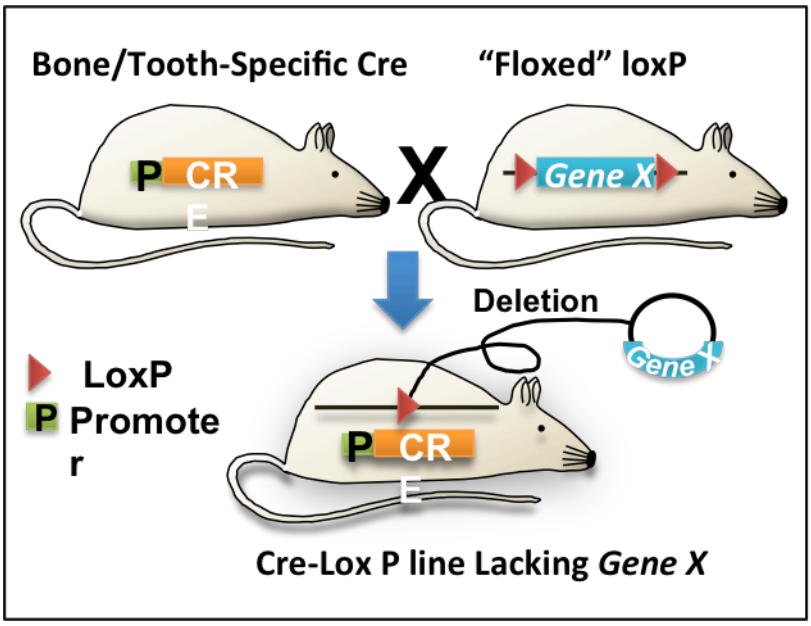


Figure 1-4. Creation of Cre-loxP bone/tooth-specific conditional knockout model. Mice expressing Cre-recombinase under a bone/tooth specific promoter are crossed with a "floxed" mouse that has lox sites flanking the gene of interest. The resultant offspring are Cre-lox mice in which the Cre-recombinase enzyme has looped out (eliminated) the gene of interest from the genome. This knockout event occurs only in the tissue in which the promoter is active (ex: bone or tooth) and does not affect gene activity in other tissues.

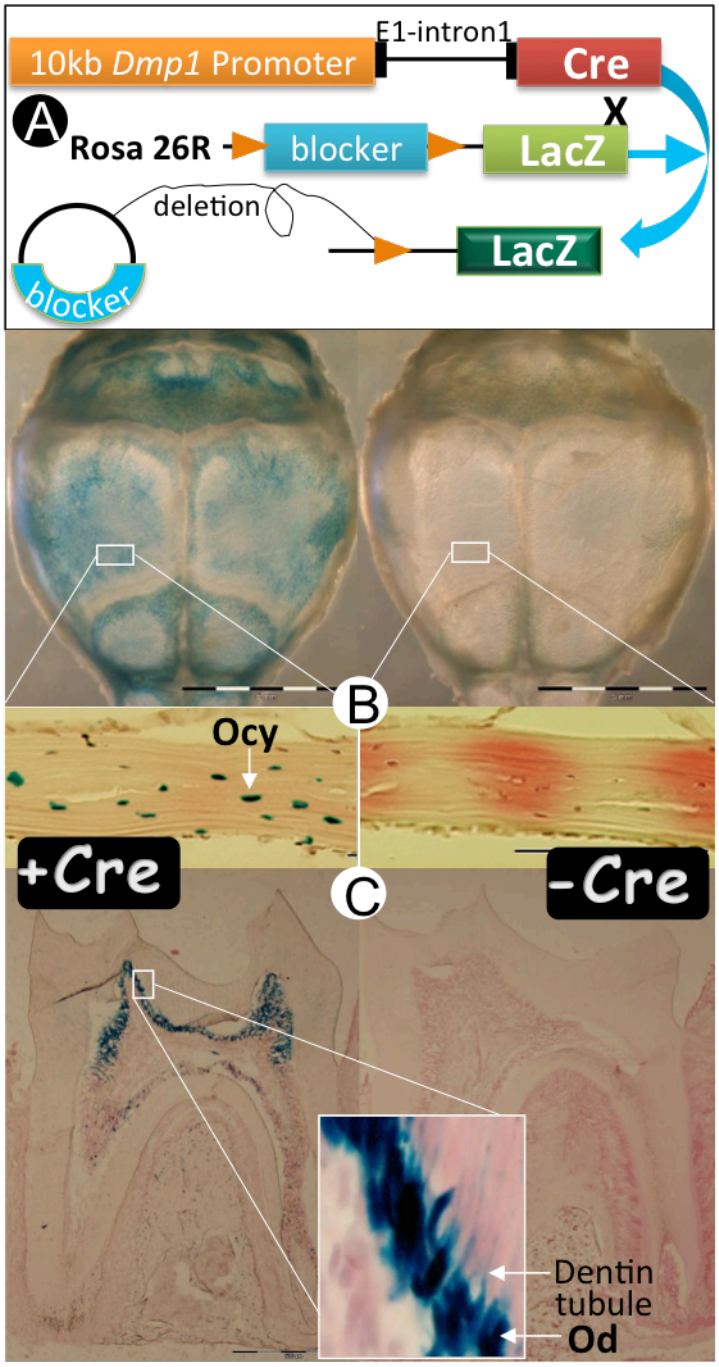

E1-intron1
10kb Dmp1 Promoter
Cre
A
X
Rosa 26R
blocker
LacZ
deletion
LacZ
blocker
B
Ocy
+Cre
C
-Cre
Dentin
tubule
Od

Figure 1-5. Cre-loxP model utilizing lacZ staining in craniofacial development. The 10-kb *Dmp1*-Cre transgenic mouse line is highly active in osteocytes (Ocy) and odontoblasts (Od). **A.** The 10-kb *Dmp1*-Cre mouse line (1-month), containing a 10-kb promoter plus a 4-kb intron and Cre cDNA, is crossed to the Rosa 26R-lacZ mouse line (a ubiquitous gene), in which a stop cassette or "blocker" is inserted into the promoter region with two loxP sites to prevent lacZ activation. After crossing, the Cre removes the stop cassette and activates the lacZ, whose expression reflects the Cre specificity; **B.** Whole mount skull staining with X-Gal (a substrate of β-Galactosidase) showed strong blue staining in the calvaria (upper left) and in the osteocytes (Ocy) (lower left) compared to the litter mate control containing ROSA26R only (right images). **C**. A strong blue signal is detected in the odontoblast (Od) layer with a weaker signal in the dentinal tubules (left) compared to the littermate controls (right). The data are adapted from Lu et al. (JDR. 2007)(*110*).

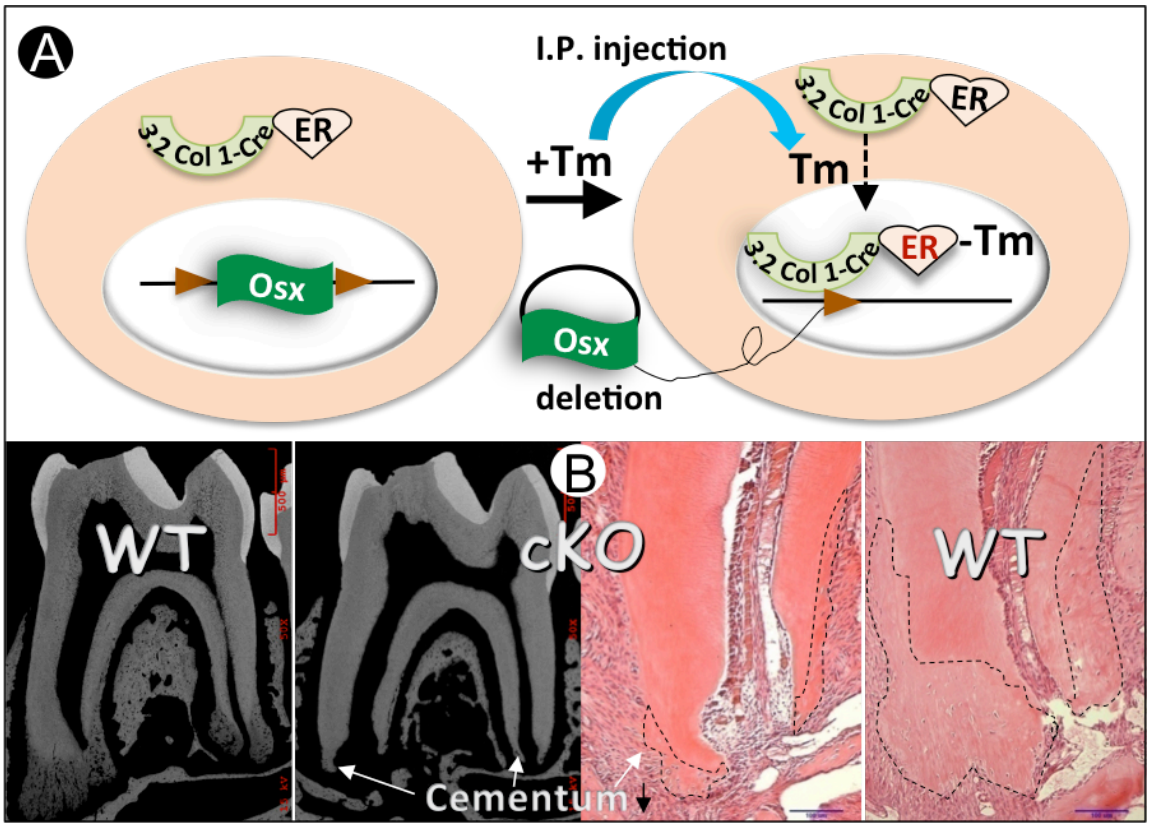


Figure 1-6. Tamoxifen (Tm), an antagonist of estrogen, induced tissue specific conditional knockout (cKO) of *Osx* (Osterix) in cementum. **A**. Osx is a transcriptional factor, which is expressed in all mesenchymal derived hard tissues (including cellular cementum) and the conventional Osx KO is lethal, whereas cementum is formed after birth. To study the role of Osx in cementogenesis, a 3.2 kb Col1-Cre ER mouse line is crossed to an Osx-floxed mouse line. In the absence of Tm the Cre will not be activated (left panel). In the presence of Tm (Intraperitoneal injection, i.p.) the Cre will delete Osx in the nucleus (right panel). **C**. Backscattered SEM images displayed a sharp reduction of cellular cementum sizes in the Osx cKO cementum, which is confirmed by H&E stain. WT, wildtype control. The data are adapted from Cao *et al*. (2012)(*111*).

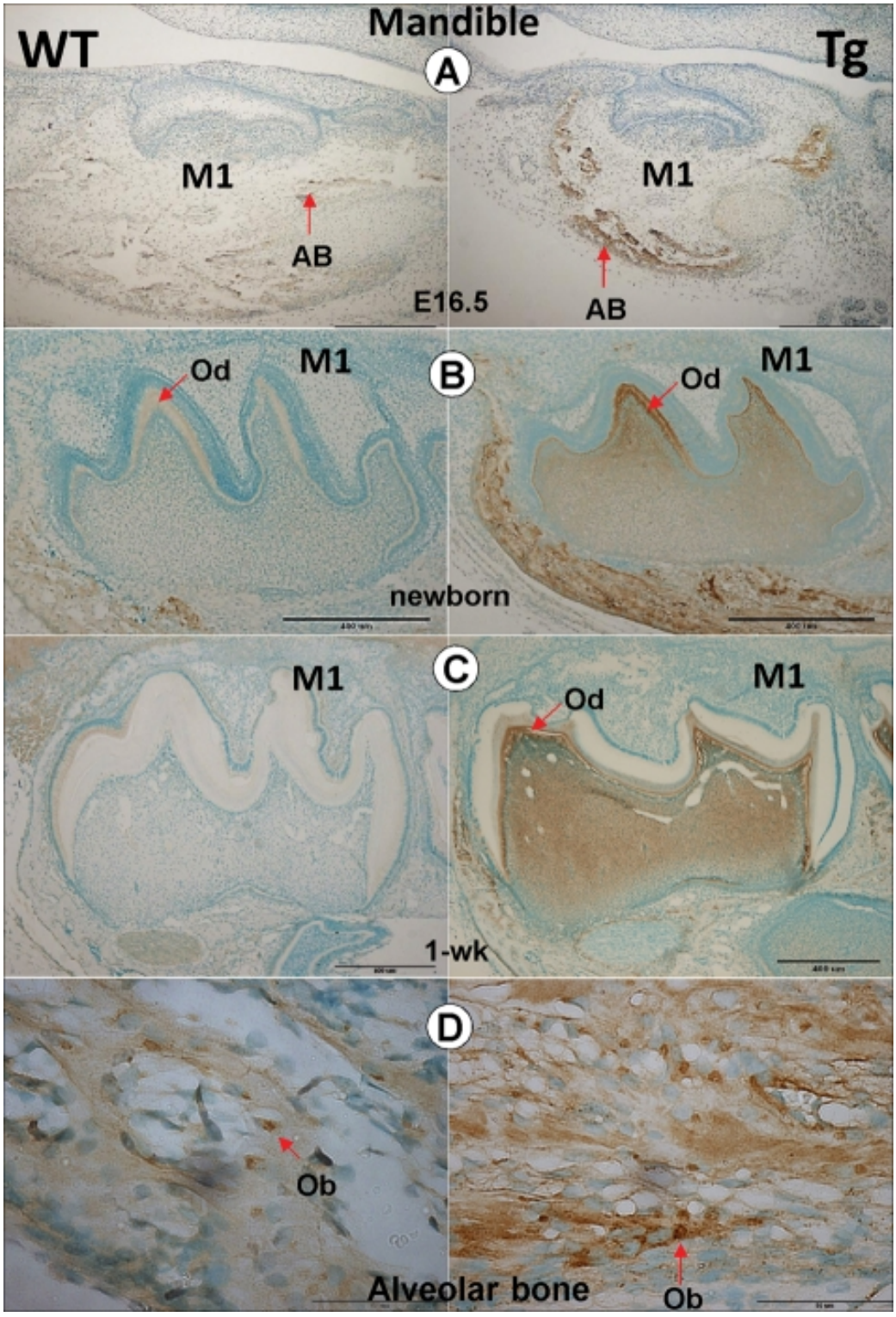

WT
Mandible
Tg
A
M1
AB
E16.5
M1
AB
Od
M1
B
Od
M1
newborn
M1
C
Od
M1
1-wk
D
Ob
Alveolar bone
Ob

Figure 2-1. Expression Patterns of the 2.3-kb *Col1a1 Dkk1*-Transgene (Tg) During Tooth Development. Immunohistochemical staining showing Dkk1 expression patterns in both WT and Tg mice during different stages of mouse tooth development. (**A**) At E16.5 Dkk1 is highly expressed in the Tg mouse alveolar bone (AB) with no signal in the molars (*right panel*) compared to the age-matched control (*left panel*). (**B**) In the Tg newborn first molars, Dkk1 is highly expressed in the pulp and odontoblast cells (*right panel*) compared to the age matched control (*left panels*). (**C**) In 1-week-old *Dkk1*-Tg mouse molars, the Dkk1 signal was maintained high in both pulp and odontoblast cells. (**D**) In the newborn alveolar bone the Dkk1 signal was detected in both WT (*left panel*) and *Dkk1* Tg (*right panel*) osteoblast cells.

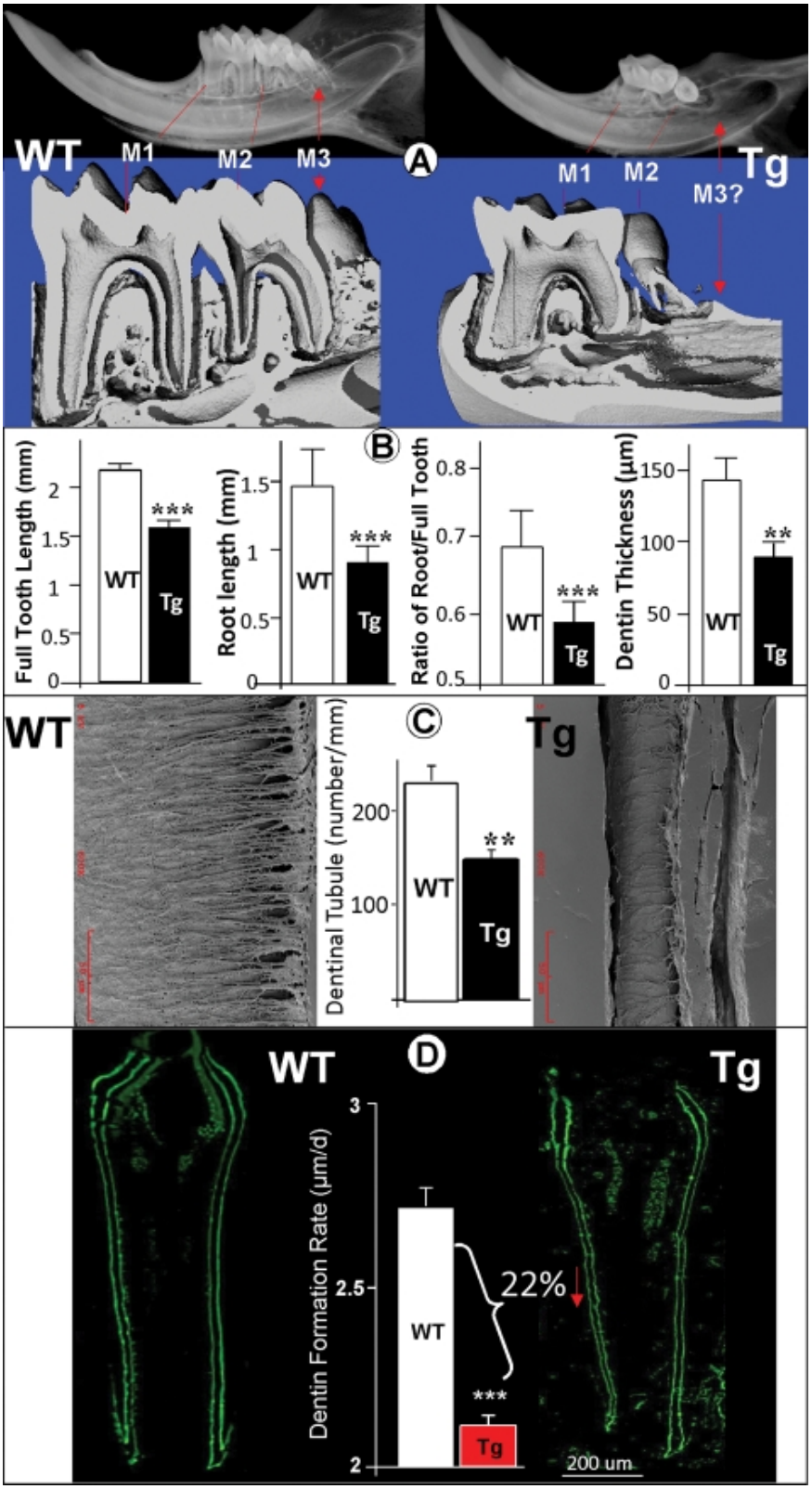

WT
M1
M2
M3
A
M1
M2
M3?
Tg
B
Full Tooth Length (mm)
2
1.5
1
0.5
0
***
WT
Tg
Root length (mm)
1.5
1
0.5
0
***
WT
Tg
Ratio of Root/Full Tooth
0.8
0.7
0.6
0.5
***
WT
Tg
Dentin Thickness (μm)
150
100
50
0
**
WT
Tg
WT
C
Dentinal Tubule (number/mm)
200
100
**
WT
Tg
Tg
WT
D
Tg
Dentin Formation Rate (μm/d)
3
2.5
2
22%
***
WT
Tg
200 um

Figure 2-2. *Dkk1*-Tg mice display a severe mandibular molar defect. (**A**) Representative radiograph (*upper panels*) and μCT (*lower panels*) images reveal a severe molar phenotype in a 1-month-old *Dkk1*-Tg mandible, including an expanded pulp chamber and root canal region in the 1st molar, a small malformed 2nd molar, and lacking a 3rd molar; (**B**) Quantitative data based on radiographs (see Sfig. 2C for the measurement method) shows a significant reduction of dentin root in the *Dkk1*-Tg 1st molar (data are mean±SEM from n=4 samples, **p<0.01; ***p <0.001 by Student's t-test). The 1st molar dentin thickness was calculated based on SEM images obtained from 4 WT and 4 Tg 1-mo-old samples. (**C**) Images of an acid-etched resin-embedded 1-month 1st molar with a striking difference between the well-organized WT dentin tubules (*left panel*) and the irregular dentin structure in *Dkk1*-Tg mouse (*right panel*). Quantitative data showed a significant reduction of dentin tubule numbers in *Dkk1*-Tg mouse (*middle panel,* data are mean±SEM from n=4 samples, **p<0.01). (**D**) 1-month 1st molar fluorochrome-labeled sections of the lower first molar from WT (*left panel*) and *Dkk1*-Tg mice (right panel) unveiled a reduction of the dentin mineralization rate in the Tg mice. Scale bar=200 μm. Quantitative analysis shows a significant difference in the dentin appositional rate between the WT and Tg first molar (data are mean±SEM from n=4 samples, ***p<0.001 by Student's t-test).

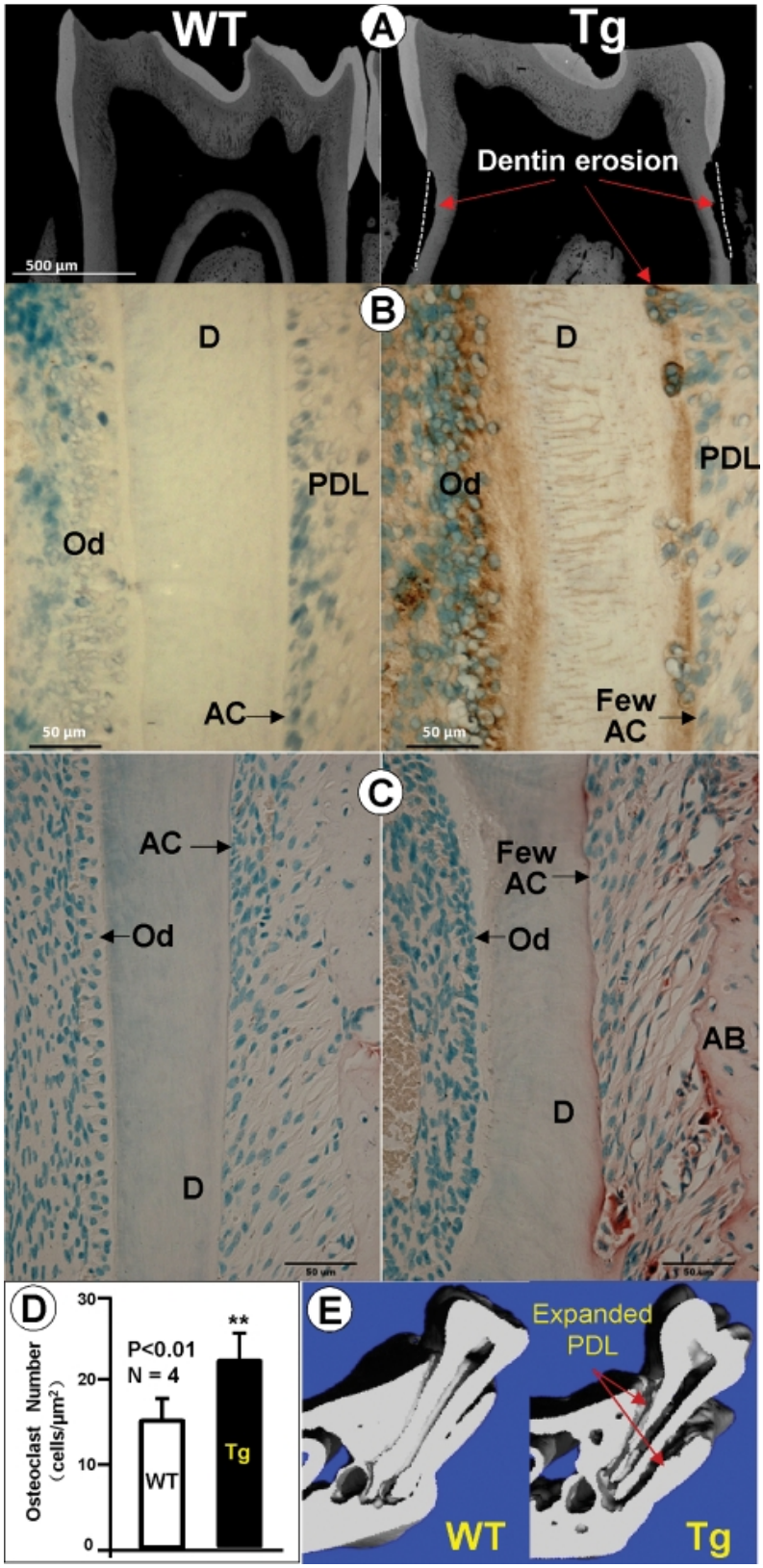

A
WT
Tg
Dentin erosion
500 μm
B
D
PDL
Od
AC
50 μm
D
PDL
Od
Few
AC
50 μm
C
AC
Od
D
Few
AC
Od
AB
D
D
P<0.01
N = 4
**
Osteoclast Number
(cells/μm²)
30
20
10
0
WT
Tg
E
Expanded
PDL
WT
Tg

Figure 2-3. Dentin Erosion in the *Dkk1*-Tg Lower First Molar. (**A**) Backscattered SEM images displaying a reduced dentin thickness and areas of dentin erosion in *Dkk1*-Tg 1-month-old molars (*right panel*). (**B**) Immunostained images show a strong Dkk1 expression in pulp/odontoblast cells plus a weak expression of Dkk1 in the *Dkk1*-Tg PDL and acellular cementoblasts in the 1-month-old molars. The number of acellular cementoblasts appears to be reduced (right panel) compared to the age-matched WT control (*left panel*), exposing the dentin to osteoclast erosion. (**C**) TRAP (Tartrate resistant acid phosphatase) stained images showing more TRAP+ cells in the *Dkk1*-Tg mouse AB with a few in the eroded dentin and PDL region (*right panel*). A majority of the PDL region was occupied by fibrous-like cells with few acellular cementoblast cells in the *Dkk1*-Tg mouse. Dkk1-Tg mice appear to be lacking a polarized odontoblast layer. (**D**) Quantitative TRAP staining data showing a significant increase in osteoclast number in the *Dkk1*-Tg mouse (data are mean ± SEM from n=6 replicates, ***$p<0.001$ by Student's t-test). (**E**) The μ-CT images confirmed the expanded PDL region and eroded root surface in the *Dkk1*-Tg mouse (right panel, arrows). AC: Acellular Cementoblasts; D: Dentin; Od: Odontoblast; PDL: Periodontal Ligament).

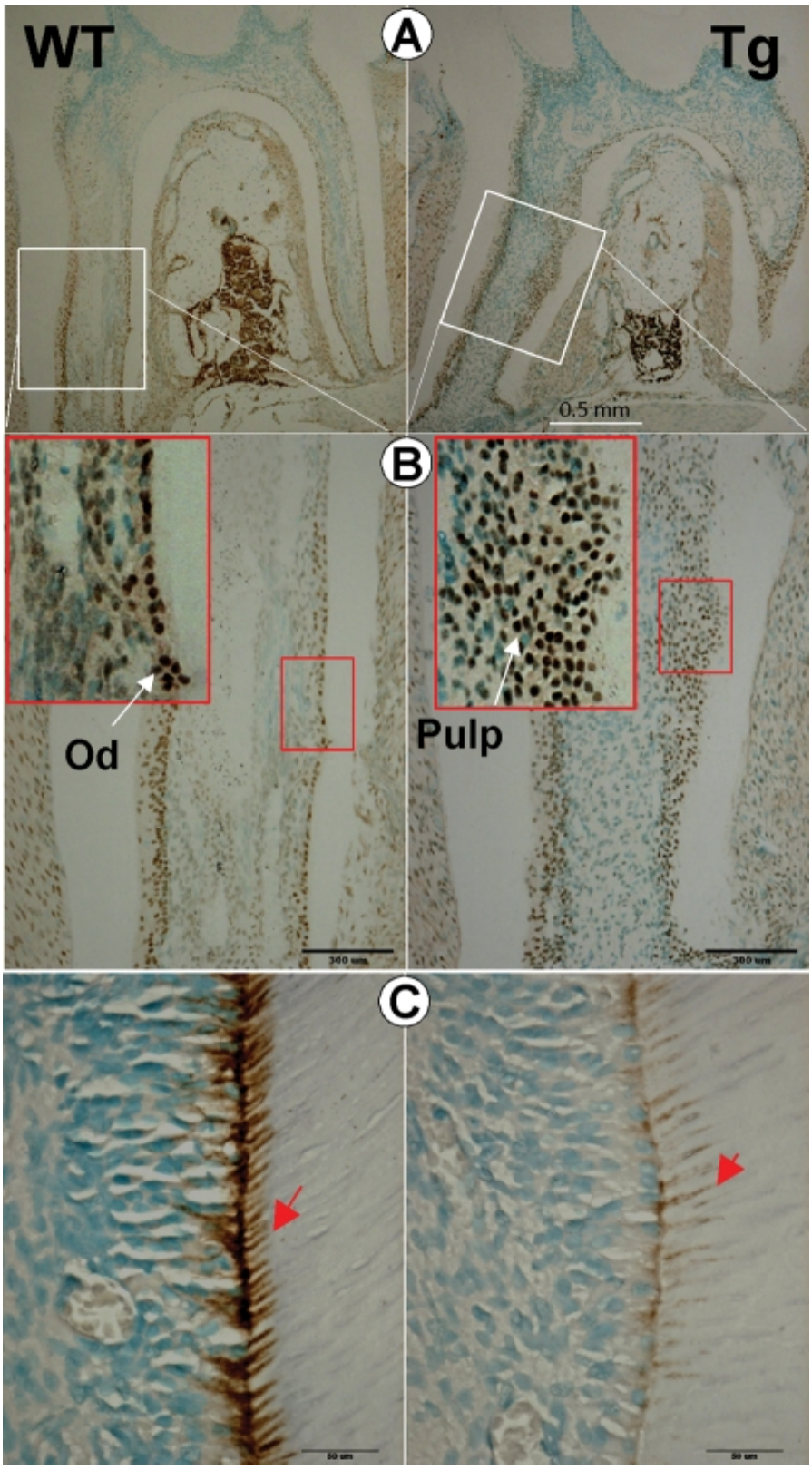

WT
A
Tg
0.5 mm
B
Od
Pulp
C

Figure 2-4. *Dkk1*-Tg mice have an increased expression of Osx in immature molar odonotblasts. (**A-B**) Immunohistochemical data showed that Osx was mainly expressed in odontoblast (Od) layer in the wild type (WT, *left panels*), whereas Osx was widely expressed in immature odontoblast cells in *Dkk1*-Tg pulp (*right panels*). (**C**) Immunohistochemical data revealed a marked reduction of nestin in *Dkk1*-Tg dentin (*right panel*) compared to the WT (*left panel*). Pd, predentin.

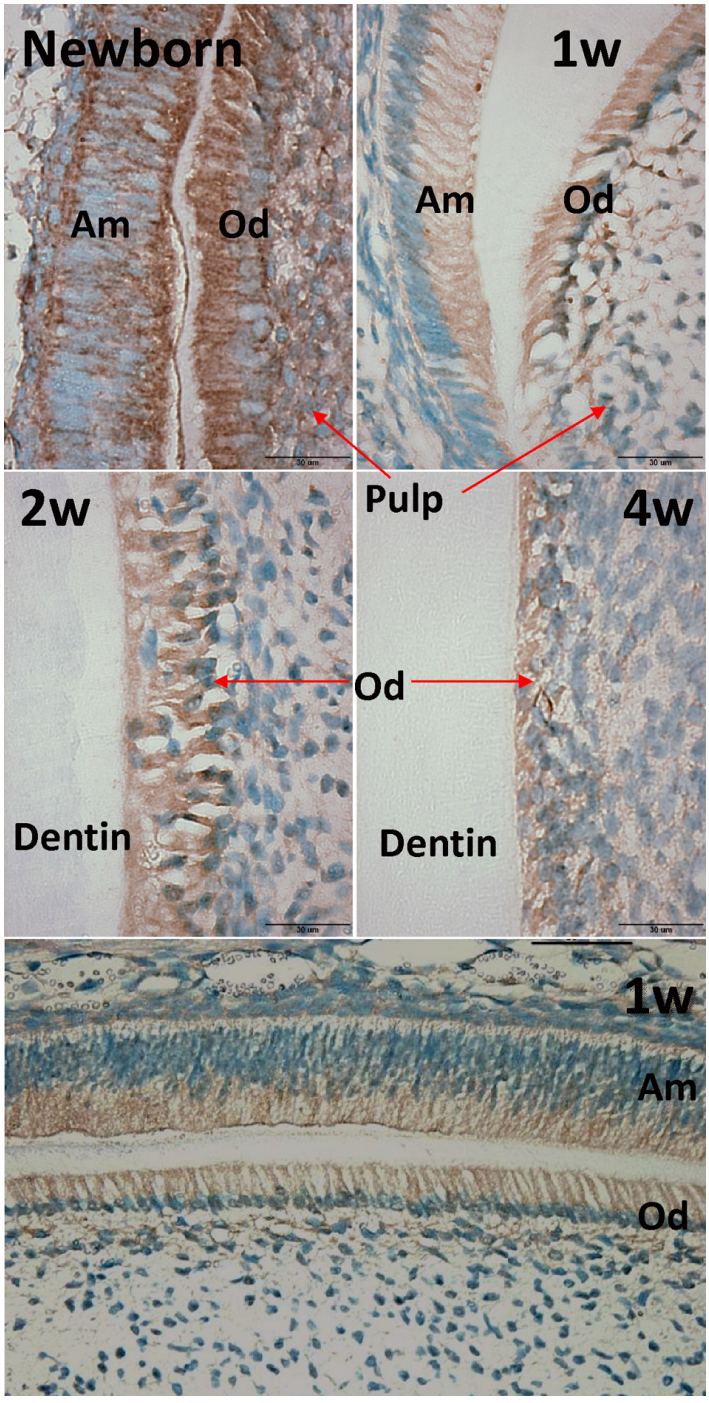


Figure 2-5. Dkk1 expression pattern during post-natal tooth development. At a higher concentration (1:25), Dkk1 is expressed in ameloblast (Am), odontoblast (Od), and pulp cells of lower molar and incisors.

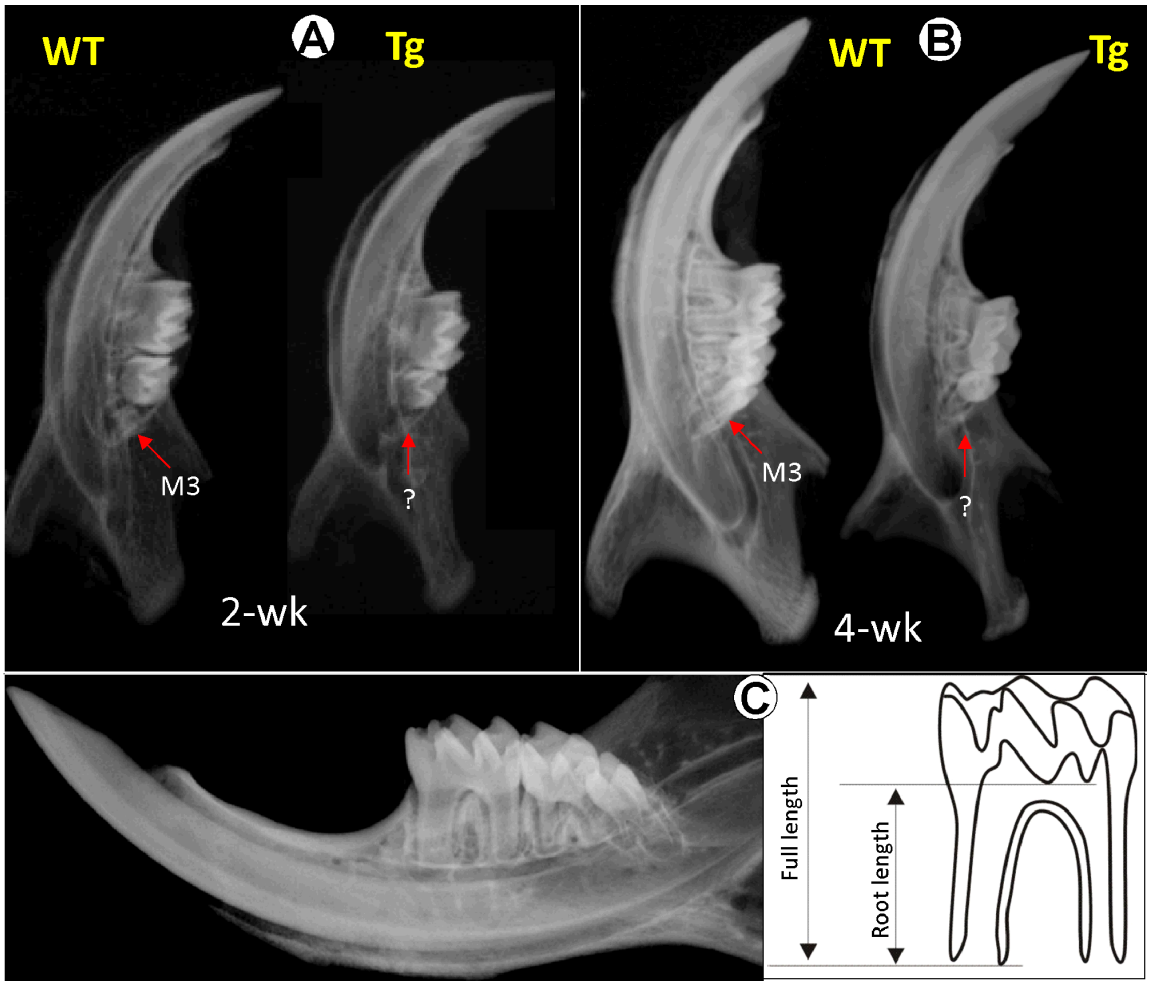


Figure 2-6. Radiograph image displays absence of 3rd mandibular molars in *Dkk1*-Tg mice. (A) Representative radiographs of 2-week-old Tg lower jaws, revealing lack of 3rd molar in the *Dkk1*-Tg mandible (6/6). (B) Representative radiographs of 1-month-old lower jaws displaying no signs of 3rd molar (6/6). (C) A representative radiograph of 1-month-old lower jaw which was used for measurement of tooth length (left panel). A schematic 1st molar based on this x-ray shows how the full tooth length and the tooth root length were calculated: full tooth length—the distance between the top of the crown and the bottom of the root; and root length—the distance between the top of the pulp chamber and the bottom of the root.

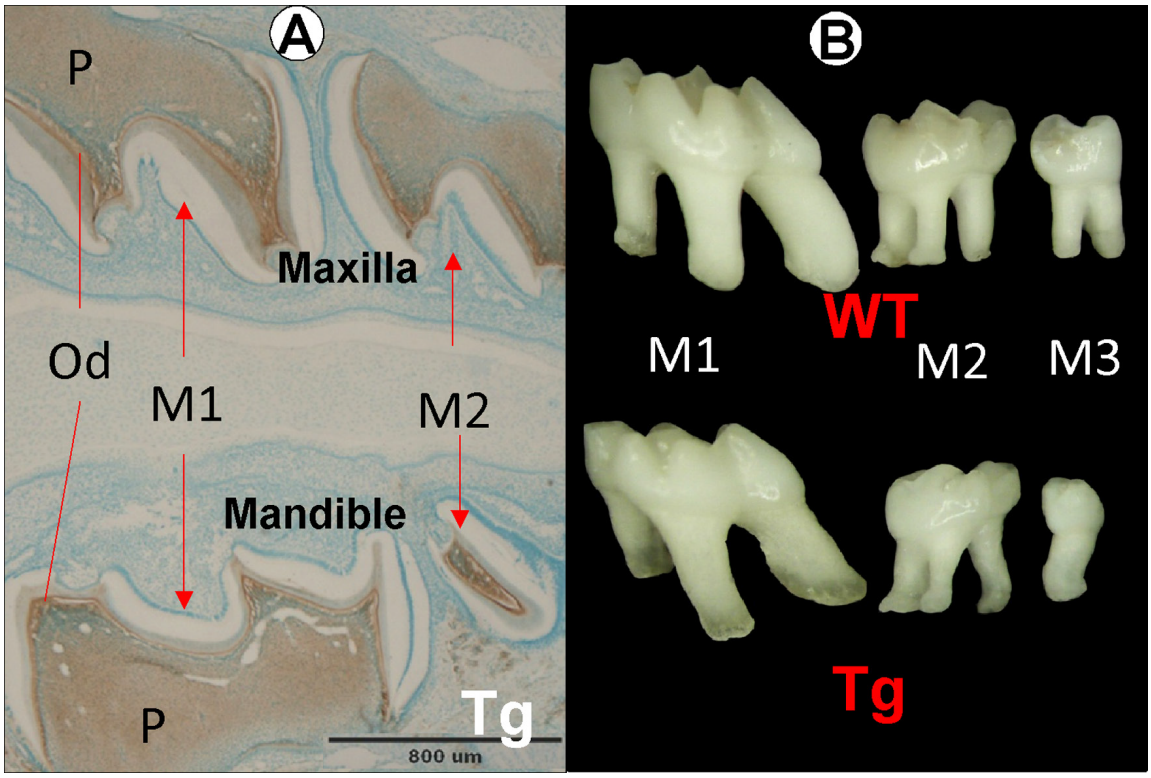


Figure 2-7. Relatively mild maxillary molar phenotype present in *Dkk1*-Tg mice. (A) With a polyclonal antibody against Dkk1 (1:200), a high Dkk1 signal is detected in Tg pulp (P) and odontoblast (Od) cells obtained from both the maxilla and the mandible. (B) Representative photographs of the upper extracted molars from WT (upper panel) and *Dkk1*-Tg mice (lower panel) reveal a minor defect in the *Dkk1*-Tg maxillary molars, including a reduction in the root diameter of the 2nd molar and the absence of one tooth root in the 3rd molar.

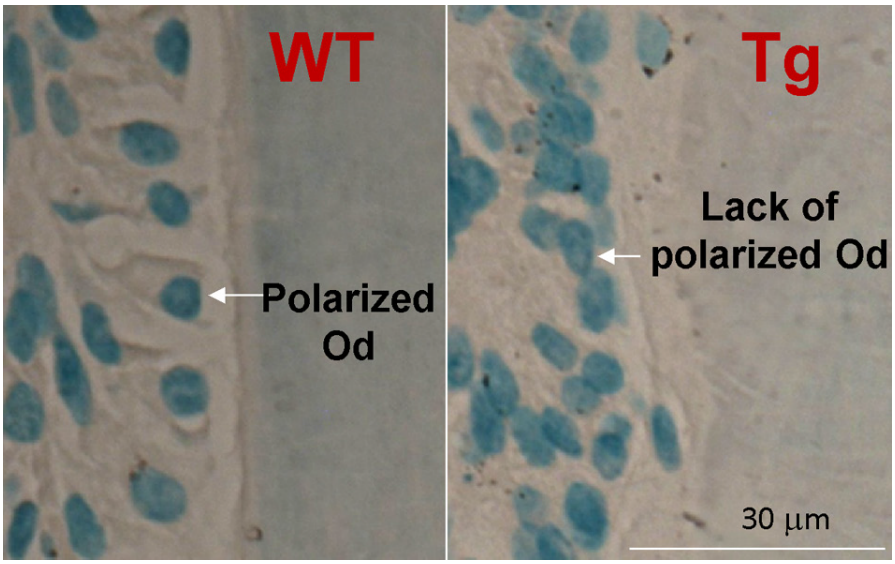


Figure 2-8. Unpolarized odontoblasts (Od) in the *Dkk1*-Tg mandibular molar. 1-month-old 1st molar showing unpolarized odonotoblasts, suggesting that an over-expression of Dkk1 inhibits odontoblast cell maturation.

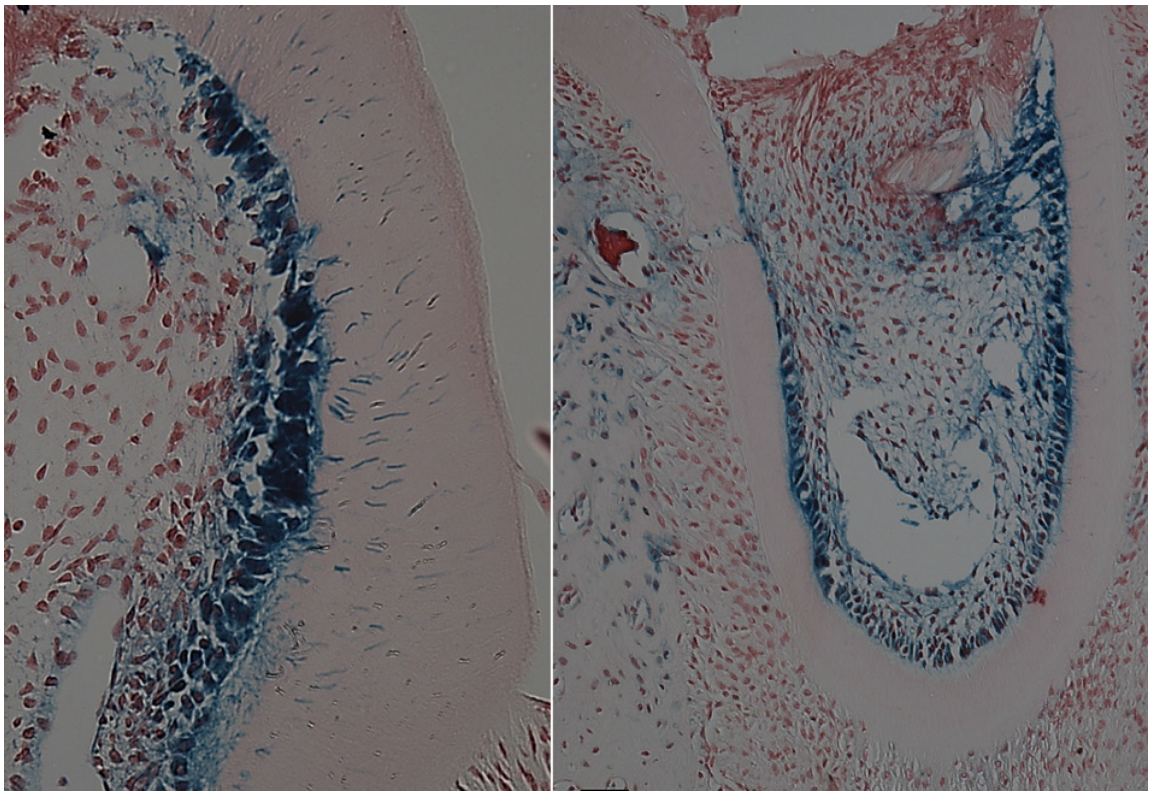


Figure 2-9. 2.3-kb Col1a1 promoter activity in both pulp and odontoblast cells. To document activity of the 2.3-kb Col1a1 promoter, we crossed the 2.3-kb Col1a1-Cre mouse (generously provided by Dr. Barbara Kream from the University of Connecticut) to a Rosa 26 lacZ report mouse line (purchased from Jackson Lab, West Grove, PA, USA). The offspring (3 wks old) was sacrificed, and a lower jaw was isolated for β-Gal staining overnight, followed by H&E count staining. The blue-stained signal, reflecting the 2.3-kb Col1a1 promoter activity, is observed in both pulp and odontoblast cells in the crown (left panel) and the tooth root (right panel).

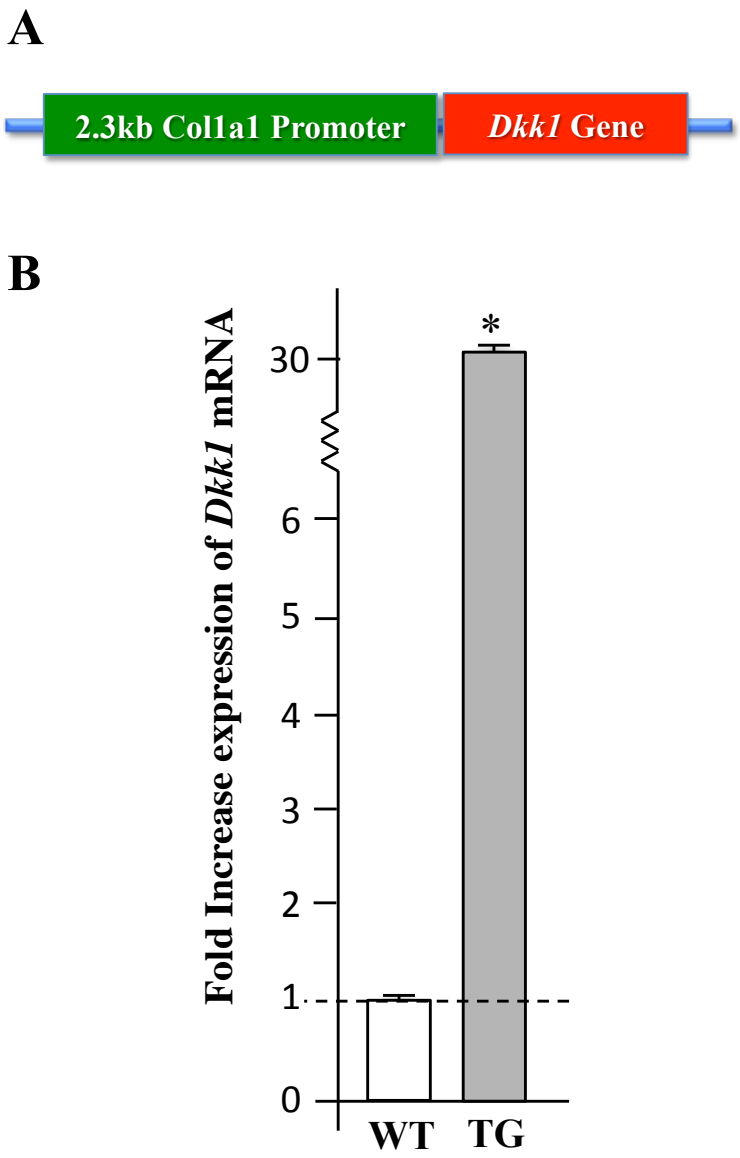


Figure 3-1. Schematic of *Dkk1*-Tg mice promoter construct and relative expression levels of the *Dkk1*-transgene. A) Schematic of the DNA construct used to generate 2.3-kb Col1a1 *Dkk1*-Tg mice. **B)** *Dkk1* mRNA expression in parietal bones of 4-day old calvaria evaluated using RT qPCR (real time). Wild-type control mice expression levels were set to 1, and relative expression levels of *Dkk1*-Tg mice are shown. *P < 0.01 (showing a significant difference); WT, n = 3; *Dkk1*-Tg, n = 3.

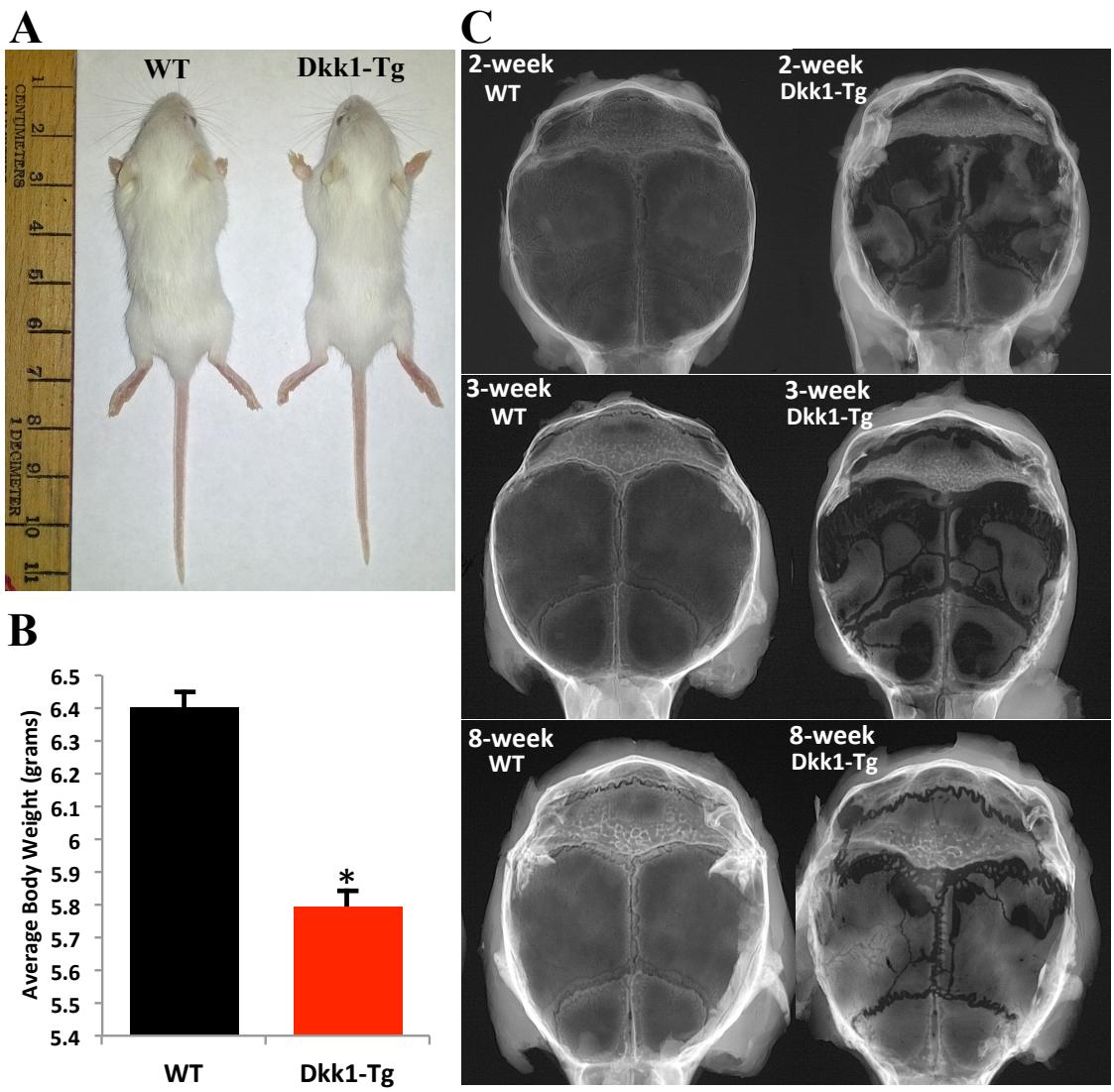


Figure 3-2. Calvarial osteolytic lesions develop in 2.3-kb Col1a1 *Dkk1*-Tg mice. **A)** Full body photographs displaying overall decrease in body size in 2-week-old *Dkk1*-Tg mice compared to WT control animals. **B)** Graphical display comparing average body weight of Tg and WT animals: WT: 6.4g; Dkk1-Tg: 5.8g. (n = 8 per group; *p < .05) **C)** Relative to non-transgenic wildtype (WT) littermates, radiographs of 2-week, 3-week and 8-week 2.3-kb Col1a1 *Dkk1*-transgenic mice exhibit significant areas of reduced radiodensity within the calvarial bones (specifically in the parietal bone).

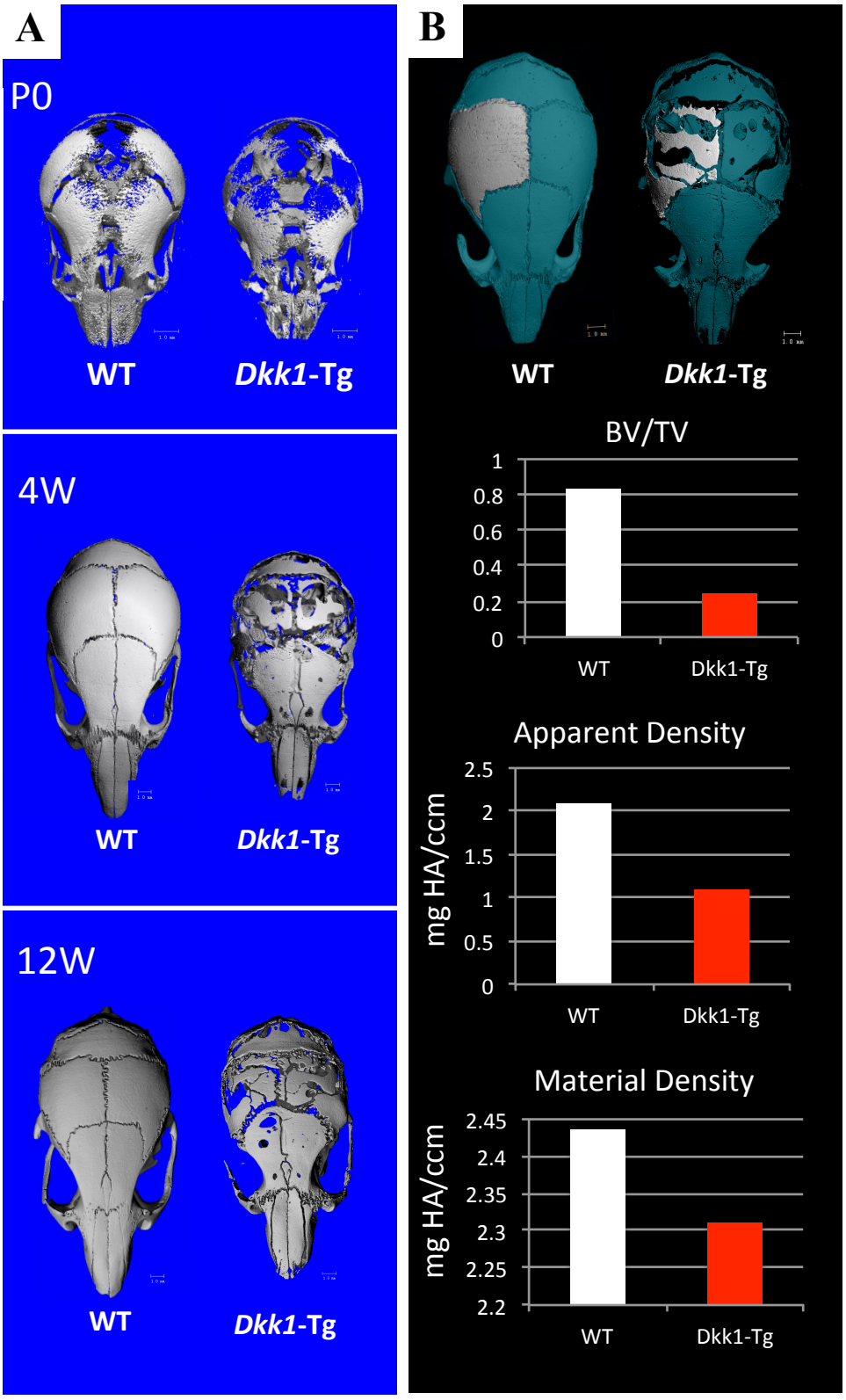

A
P0
WT
Dkk1-Tg
4W
WT
Dkk1-Tg
12W
WT
Dkk1-Tg
B
WT
Dkk1-Tg
BV/TV
1
0.8
0.6
0.4
0.2
0
WT
Dkk1-Tg
Apparent Density
mg HA/ccm
2.5
2
1.5
1
0.5
0
WT
Dkk1-Tg
Material Density
mg HA/ccm
2.45
2.4
2.35
2.3
2.25
2.2
WT
Dkk1-Tg

Figure 3-3. Microcomputed tomography (μ-CT) displays mineralization deficiency in calvarial bones. **A)** μ-CT imaging displays *Dkk1*-Tg calvarial bone mineralization defects (most strikingly in the parietal bones) at P0, 4-weeks, and 12-weeks. **B)** Images of individually contoured (selected) out parietal bones of 4-week-old WT and *Dkk1*-Tg parietal bones using μ-CT software presents with greater than a 60% decrease in Bone Volume/Total Volume (bone volume fraction), as well as a reduction in Apparent Density and Material Density.

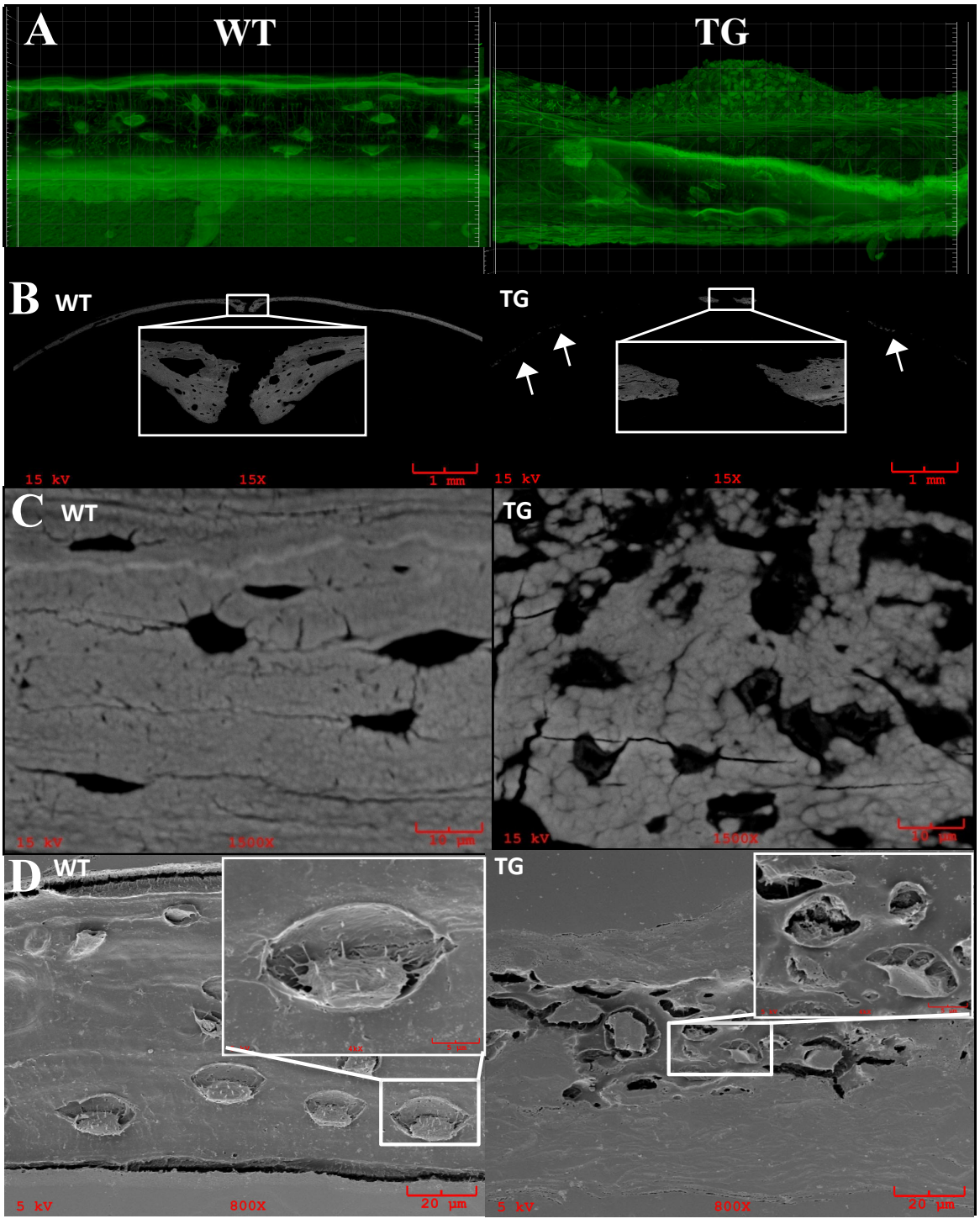

A
WT
TG
B
WT
TG
15 kV
15X
1 mm
15 kV
15X
1 mm
C
WT
TG
15 kV
1500X
10 µm
15 kV
1500X
10 µm
D
WT
TG
5 kV
800X
20 µm
5 kV
800X
20 µm

Figure 3-4. Decreased dendritic processes, misshapen osteocytes, and decreased mineralization in *Dkk1*-Tg parietal bone. A) FITC (Fluorescein isothiocyanate) labeled parietal bone regions showing osteocytes with decreased dendritic processes in 4-week-old 2.3-kb Col1a1 *Dkk1*-Tg mice. **B)** Backscatter-SEM imaging revealing a non-continuous 4-week-old parietal bone with non-abutting parietal bones in the sagittal suture with the bone regions pointed to with white arrows, compared to WT littermate controls. **C)** Enlarged 4-week-old WT parietal bone region and *Dkk1*-Tg parietal bone displaying a mineralization defect and malformed osteocytes within the bone matrix. **D)** 4-week-old Acid-Etched SEM images of WT parietal bone and *Dkk1*-Tg parietal bone further confirming the presence of defective osteocyte canaliculi and regions of poor mineralization as well as the accumulation of osteocytes in small regions.

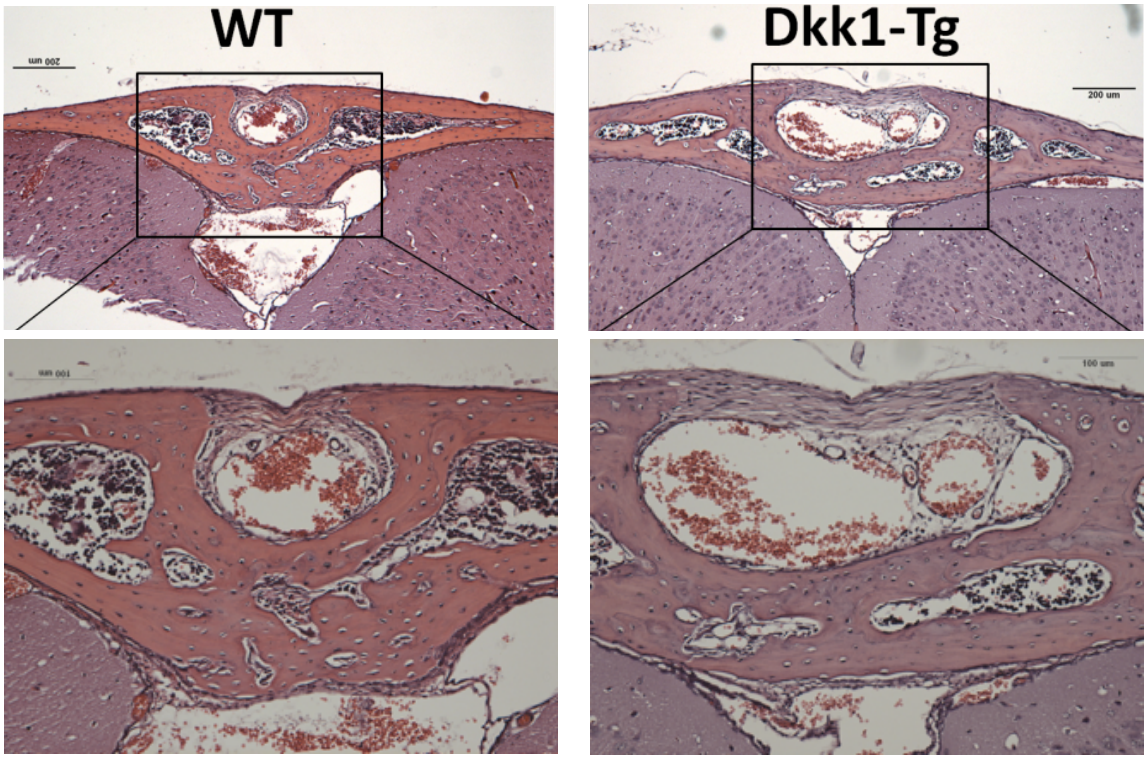


Figure 3-5. Normal suture fusion of *Dkk1*-Tg posterior frontal bones. Posterior frontal bone suture fusion (left images – enlarged on bottom) occurs in normal wildtype calvarial development. *Dkk1*-Tg (right images – enlarged on bottom) posterior frontal bones fuse normally with no inadequacy in suture fusion.

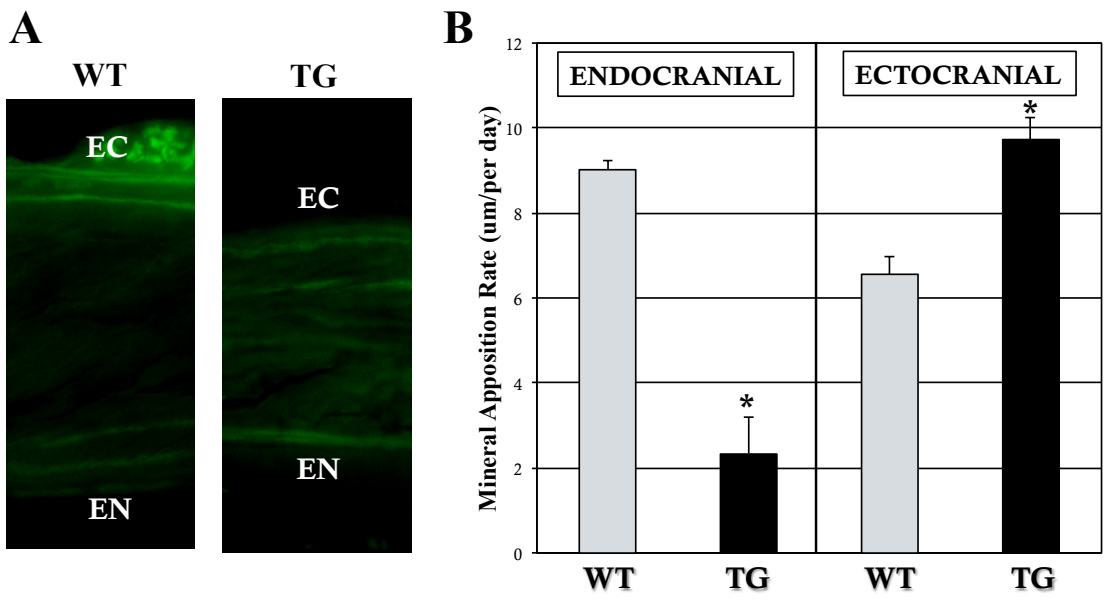
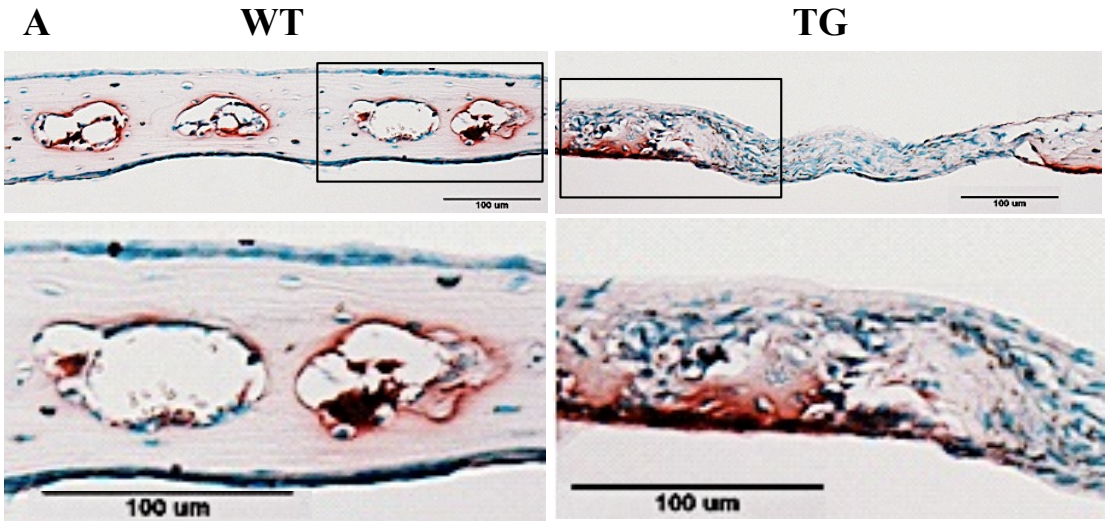


Figure 3-6. Increase in ecto-cranial bone formation and a decrease in endo-cranial bone formation in Dkk1-Tg parietal bone. A) Double calcein labeling shows differential bone formation when comparing the ecto-cranial and endo-cranial surfaces. B) Graphical display of the endo-cranial surface (left two bars) showing bone resorption with relatively no bone growth. Endo-cranial bone formation rate was decreased approximately 4-fold. Graphical display of the ecto-cranial surface (right bar graphs) showing an increase in bone production. Ecto-cranial bone formation rate was increased approximately 0.5-fold. *$P < 0.05$ (showing significant difference); WT, n = 3; *Dkk1*-Tg, n = 3.


A
WT
TG
100 um
100 um
100 um
100 um

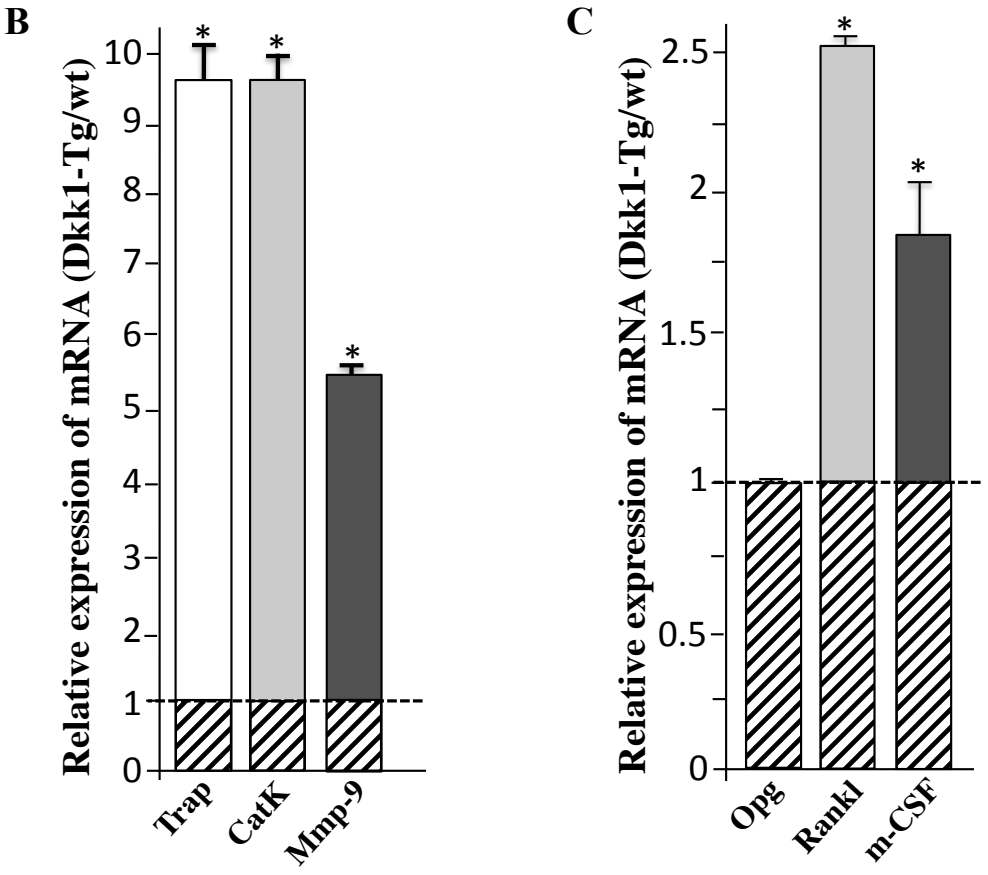

B
Relative expression of mRNA (Dkk1-Tg/wt)
0
1
2
3
4
5
6
7
8
9
10
*
*
*
Trap
CatK
Mmp-9
C
Relative expression of mRNA (Dkk1-Tg/wt)
0
0.5
1
1.5
2
2.5
*
*
Opg
Rankl
m-CSF

Figure 3-7. Increase in *Dkk1*-Tg osteoclast maturation and activity. A) TRAP stained WT control and *Dkk1*-Tg parietal bones display an increase in TRAP+ stained osteoclasts on the endo-cranial surface of *Dkk1*-Tg parietal bones. **B)** In *Dkk1*-Tg parietal bones, both *Trap* and *Cathepsin-K* mRNA expression increased 9.5 fold and *Mmp-9* expression increased 5.5 fold. **C)** *Opg* gene expression was unchanged compared to the WT control bones, but *Rankl* expression was increased 2.5 fold and *M-csf* expression was increased 1.8-fold. Relative expression of mRNA relative to a *Gapdh* internal control. *Dkk1*-Tg mRNA gene expressions compared to a normalized WT control (WT = 1) mRNA gene expression for all genes tested. *Dkk1-Tg* levels are shown. *P < 0.01 (showing significant difference); WT, n = 3; *Dkk1*-Tg, n = 3.

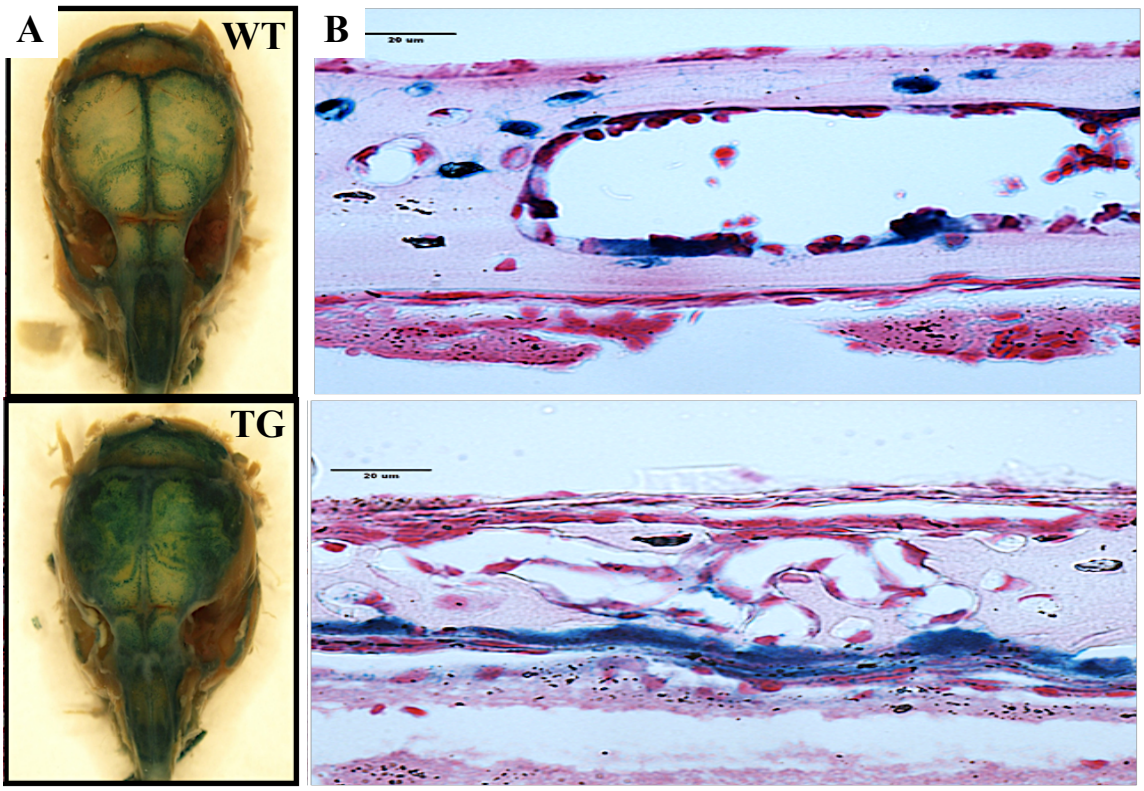


Figure 3-8. Wnt/β-catenin signaling in *Dkk1*-Tg mice: Increased in osteoclasts and decreased in osteoblasts and osteocytes. Dkk1-Tg;TOPGAL 2-week parietal bones. WT parietal bone shows distinct β-galactosidase staining in osteoblasts, osteoclasts and osteocytes. Dkk1-Tg parietal bone shows β-galactosidase staining in the osteoclasts but not within the osteocytes or osteoblasts.

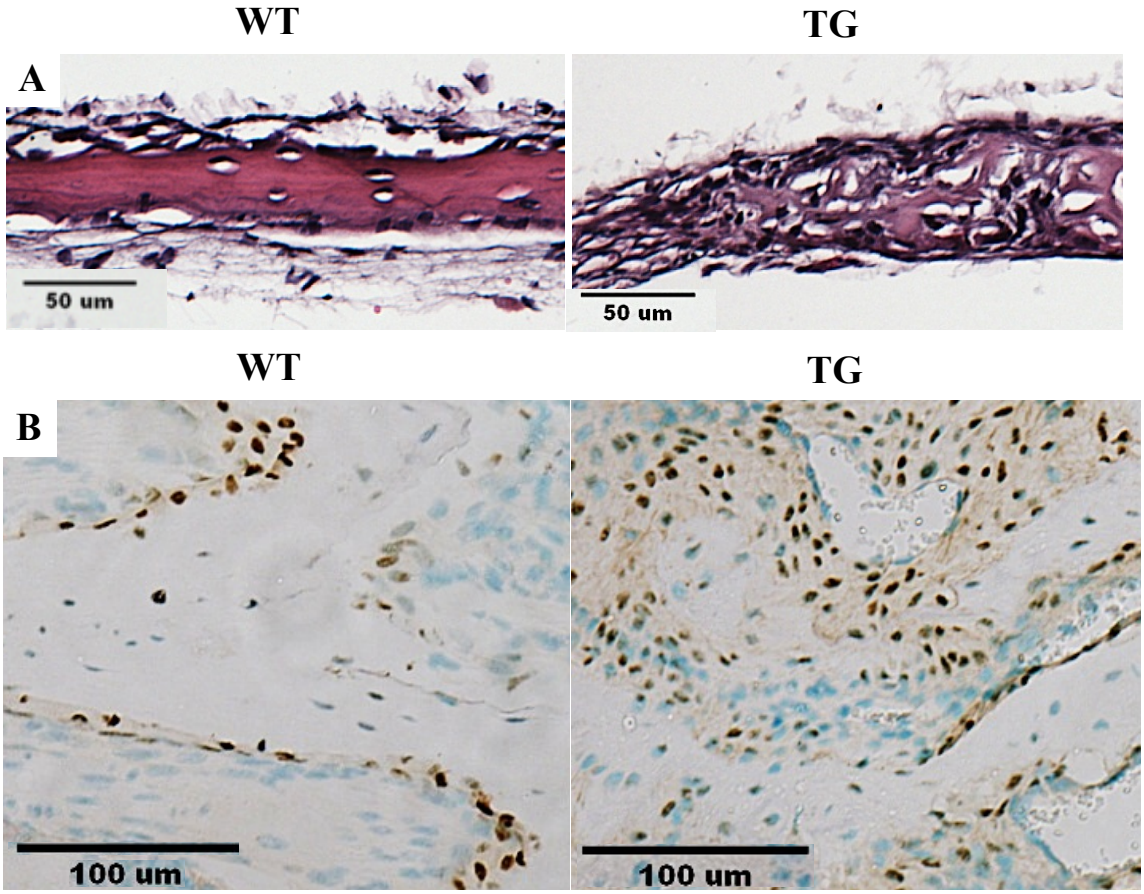


Figure 3-9. Increase in trabeculation in the under-mineralized bone as well as an increase in Osx: an osteoblast marker. A) 2 week-old WT (left) and *Dkk1*-Tg (right) parietal bone H&E staining showing the disrupted bone matrix with interspersed fibrous-like tissue between the woven bone trabeculae, **B)** 2-week-old WT (left) & *Dkk1*-Tg (right) immunohistochemical (IHC) staining showing increased Osx expression in the immature osteoblasts that accumulate within the immature bone adjacent to the partially mineralized areas of parietal bone.

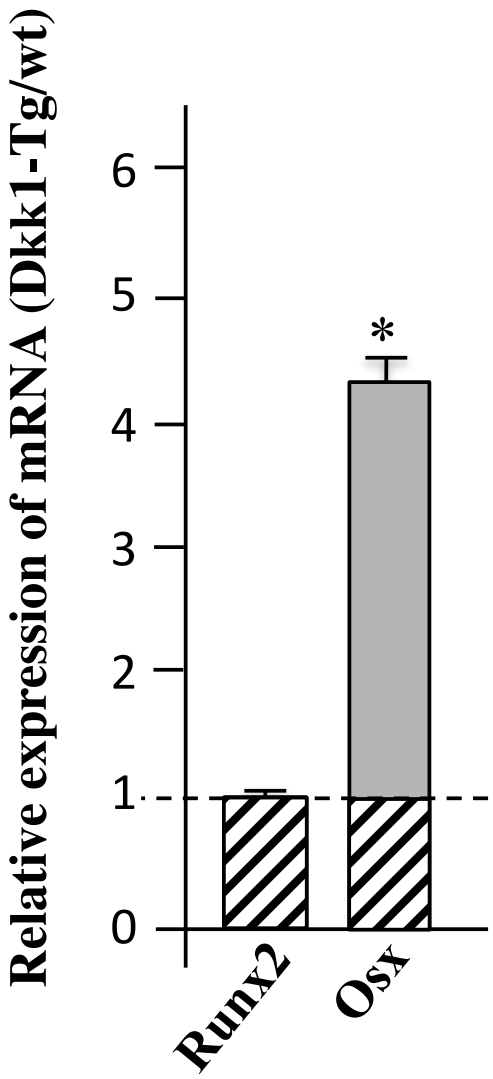


Figure 3-10. Increase in *Osx* gene expression independent of *Runx2*. Real-time PCR performed on 2-week parietal bone showing no change in *Runx2* gene expression, but an unexpected approximately 4.5-fold increase in *Osx* gene expression. Relative expression of mRNA relative to a *Gapdh* internal control. *Dkk1*-Tg mRNA gene expressions compared to a normalized WT control (WT = 1) mRNA gene expression for all genes tested. *Dkk1-Tg* levels are shown. *P < 0.01 (showing significant difference); WT, n = 3; *Dkk1*-Tg, n = 3.

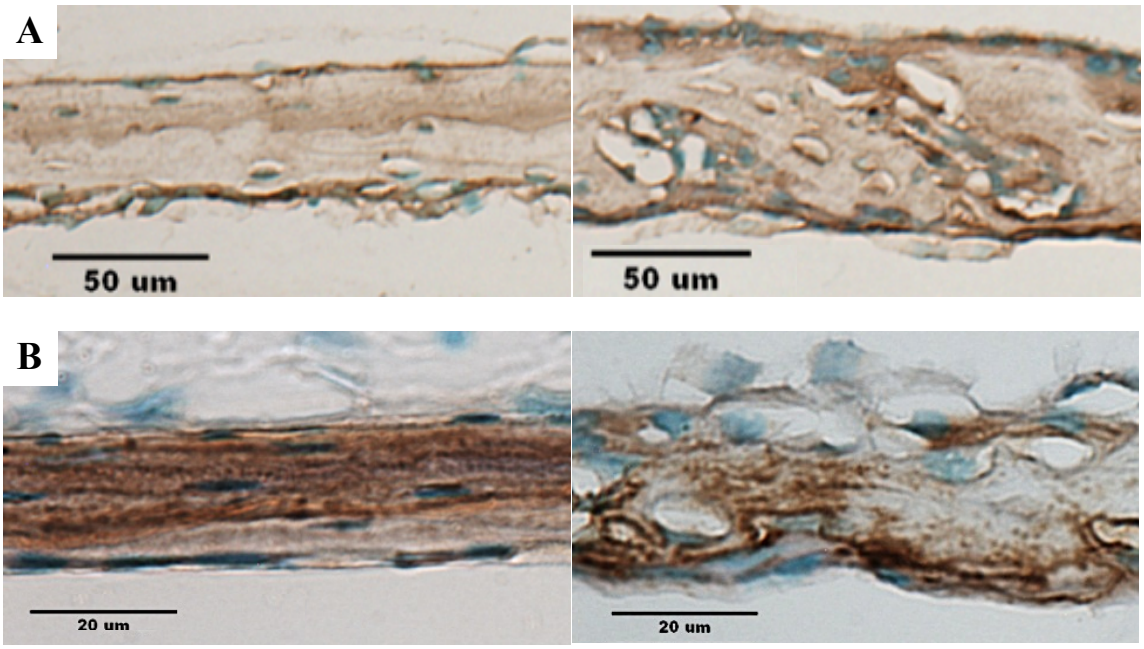


Figure 3-11. An increase in immature osteoblast markers Bsp and Opn in *Dkk1*-Tg parietal bone. A) 2-week-old WT (left) & *Dkk1*-Tg (right) immunohistochemical (IHC) staining showing increased Bsp expression in the immature mineralized matrix. **B)** Increased Opn expression in the mineralized matrix in *Dkk1*-Tg (right) compared to WT (left).

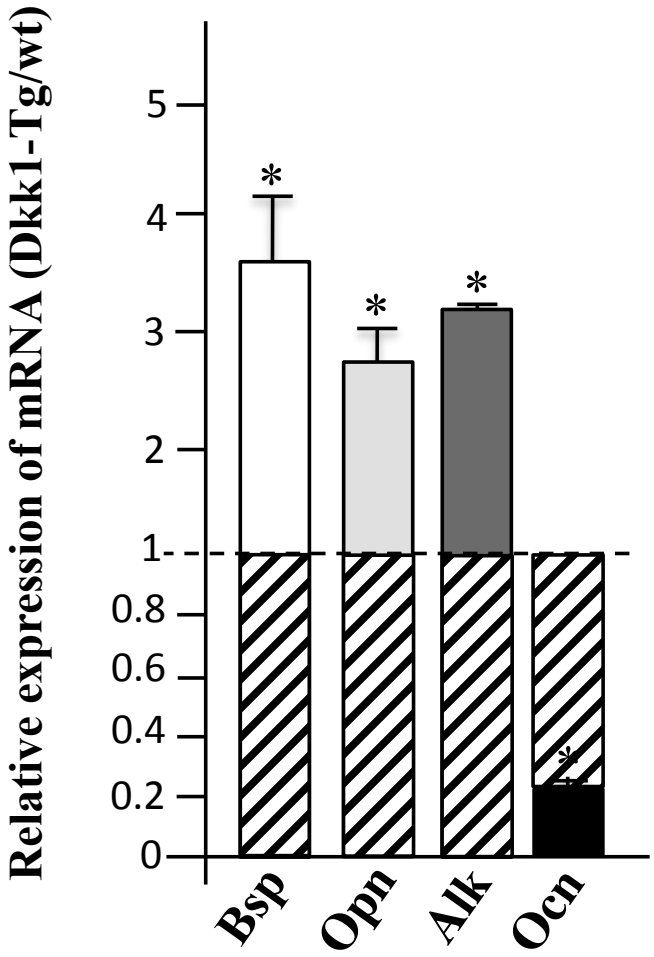


Figure 3-12. An increase in expression of immature osteoblast markers: *Bsp*, *Opn* and *Alp*, along with a decrease in expression of *Ocn*, a mature osteoblast marker within the *Dkk1*-Tg parietal bone. A) Real-time PCR performed on 2-week parietal bone showing approximately a 3.0-fold increase in *Bsp* expression, a 2.8-fold increase in *Opn* expression, a 3.2-fold increase in Alp expression and a 5-fold decrease in *Ocn* expression. Relative expression of mRNA relative to a *Gapdh* internal control. *Dkk1*-Tg mRNA gene expressions compared to a normalized WT control (WT = 1) mRNA gene expression for all genes tested. *Dkk1-Tg* levels are shown. *P < 0.01 (showing a significant difference); WT, n = 3; *Dkk1*-Tg, n = 3.

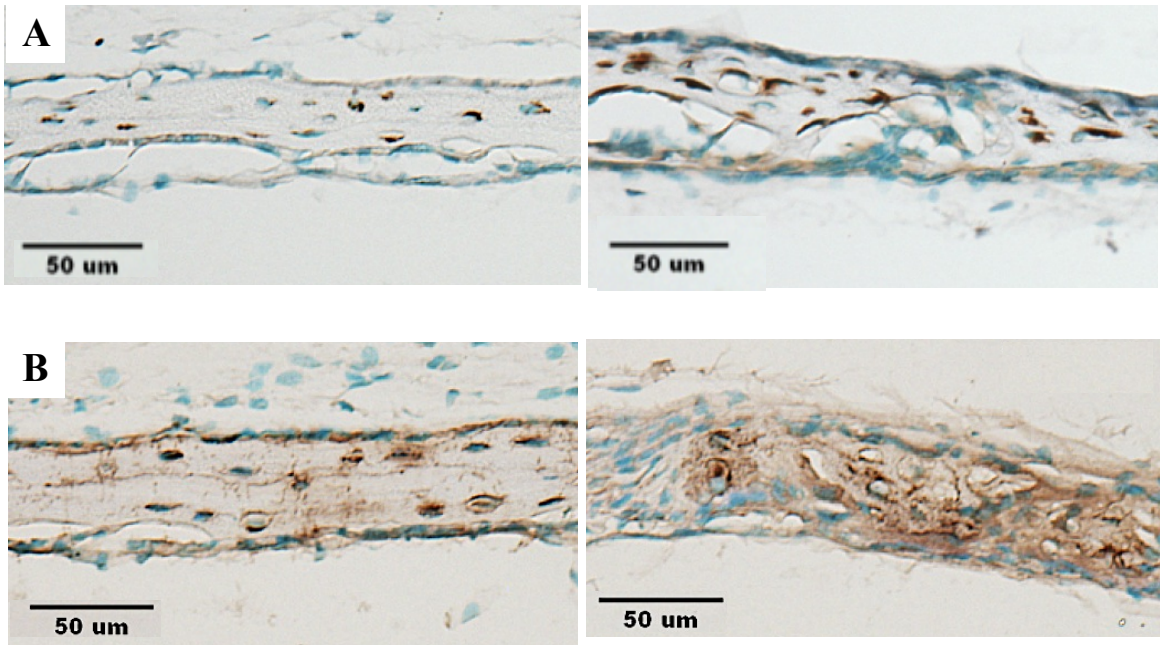


Figure 3-13. Increase in parietal bone Sost and Dmp1 in *Dkk1*-Tg calvaria. A) 2-week-old WT (left) & *Dkk1*-Tg (right) immunohistochemical (IHC) staining showing increased Sost expression in the immature osteocytes. **B)** Increased Dmp1 expression in the mineralized matrix in *Dkk1*-Tg (right) compared to WT (left), with no expression in the fibrous-like mesenchymal tissue which lacks any mineralization.

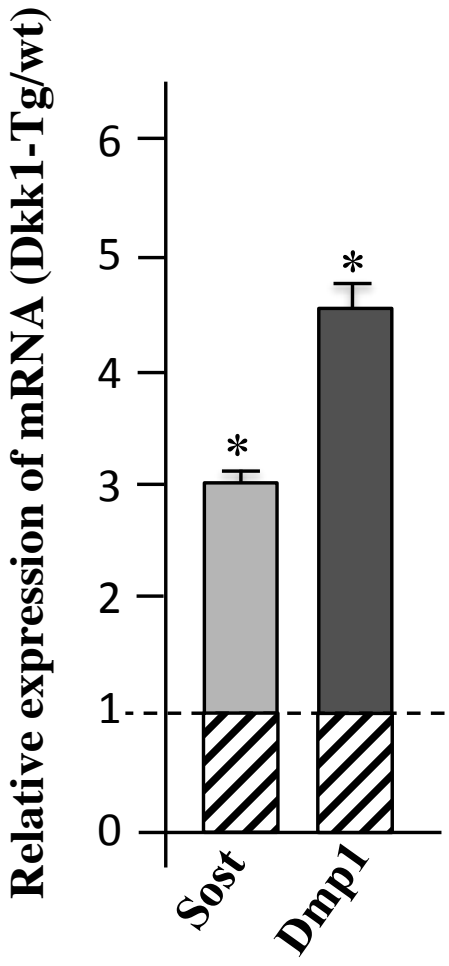


Figure 3-14. Increase in *Sost* and *Dmp1* gene expressions in *Dkk1*-Tg primary parietal bone assay. Real-time PCR performed on 2-week parietal bone showing a 3.0-fold increase in *Sost*, and a 4.5-fold increase in *Dmp1*. Relative expression of mRNA relative to a *Gapdh* internal control. *Dkk1*-Tg mRNA gene expressions compared to a normalized WT control (WT = 1) mRNA gene expression for all genes tested. *Dkk1-Tg* levels are shown. *P < 0.01 (showing significant difference); WT, n = 3; *Dkk1*-Tg, n = 3.

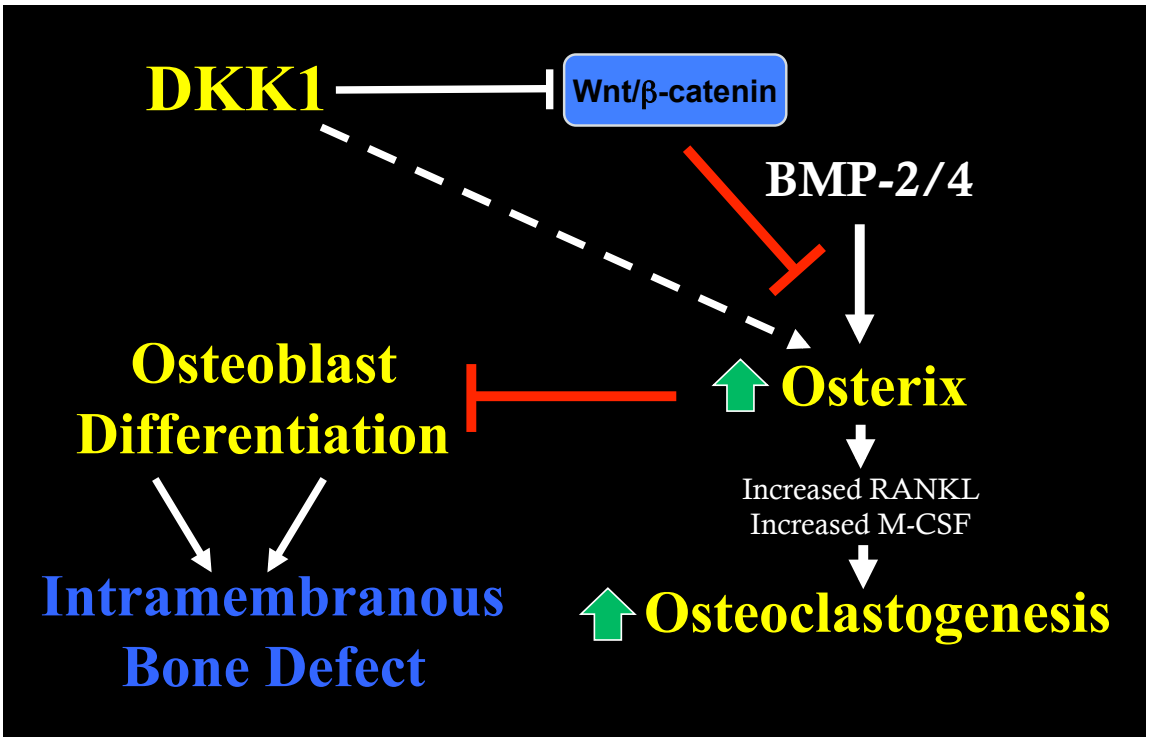


Figure 3-15. Working Hypothesis: Dkk1 indirectly induces Osterix expression. Dkk1 directly inhibits the Wnt/β-catenin signaling pathway impeding downstream bone formation. Our hypothesis supports the theory that Wnt signaling has an inhibitory effect on BMP regulation of Osterix expression. Dkk1, being a Wnt inhibitor, is able to regulate Osterix expression through its suppression Wnt activity thereby removing the block on BMP induced Osterix expression. Osterix then regulates RANKL and M-CSF expression to promote osteoclastogenesis and prevents the differentiation of immature osteoblasts to mature osteoblasts promoting an osteogenic defect.