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CHARACTERIZATION OF THE ENAMEL ORGAN IN MUTANT MICE HARBORING AN OCULODENTODIGITAL DYSPLASIA-LINKED Cx43 MUTANT

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CHARACTERIZATION OF THE ENAMEL ORGAN IN MUTANT MICE HARBORING AN OCULODENTODIGITAL DYSPLASIA-LINKED Cx43 MUTANT

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ABSTRACT

Coordinated differentiation of the enamel organ is essential to enamel deposition and mineralization. This process is likely governed by Cx43-based gap junctional intercellular communication as oculodentodigital dysplasia (ODDD) patients harboring Cx43 mutants exhibit enamel defects. To assess the role of Cx43 in tooth development we employ an ODDD mouse model, *Gja1*^{Jrt}/+, which harbors a G60S Cx43 mutant and exhibits tooth abnormalities mimicking the human disease. Total Cx43 gap junction plaques were reduced in *Gja1*^{Jrt}/+ mouse incisors compared to wild-type littermate controls. Disorganized *Gja1*^{Jrt}/+ mouse ameloblasts and abnormal distribution of amelogenin were observed. A thin enamel layer became more apparent after tooth eruption suggesting enamel integrity is compromised. Mutant mouse incisors were longer with a thicker dentin layer, reflecting a mechanical stress response to the depleted enamel layer. Together, these data suggest that Cx43 gap junctions play a role in enamel organ function.

Keywords:

Ameloblast differentiation, amelogenesis, connexin, Cx43, enamel organ development, oculodentodigital dysplasia, mouse model of human disease

CO-AUTHORSHIP

All experiments were performed by Katharine Toth except for the following:

Chapter 2

Dr. Qing Shao performed the x-ray photographs shown in Figure 2.

DEDICATION

To my family for their love and support throughout my education.

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ABBREVIATIONS AND NOMENCLATURE

Am	ameloblasts
AMBN	ameloblastin
AMELX	amelogenin x-linked
BSA	bovine serum albumin
Cx	connexion
ENAM	enamelin
G138R	glycine to arginine substitution at amino acid position 138 of Cx43
G60S	glycine to serine substitution at amino acid position 60 of Cx43
GJA1	gap junction alpha 1
GJIC	gap junctional intercellular communication
I130T	isoleucine to threonine substitution at amino acid position 130 of Cx43
kDa	kilodalton
ODDD	oculodentodigital dysplasia
PCR	polymerase chain reaction
SC	supporting cells
WT	wild-type

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Multicellular organisms form networks of cells that share distinct and specialized traits. These specialized cells are organized into tissues and further into organs within a system. Most cells are able to communicate with their neighbours to coordinate their activities and maintain the survival of the organism through specialized intercellular channels collectively known as gap junctions. Gap junctions permit the passage of secondary messengers, ions, nutrients and metabolites between contacting cells (Cottrell and Burt, 2005; Neijssen et al., 2007). Gap junctional intercellular communication (GJIC) has multiple and diverse roles that include the regulation of cell signaling, communication, differentiation, proliferation, polarity and development (Evans and Martin, 2002; Neijssen et al., 2007; Vinken et al., 2006). For example, gap junctions have known roles in embryonic development (see Section 1.5) (Caveney, 1985; Elias and Kriegstein, 2008; Levin, 2002), and the differentiation and function of organs and tissues including the bone (reviewed in Section 1.9) (McLachlan et al., 2008; Stains and Civitelli, 2005), vasculature (Figueroa and Duling, 2009), brain (Bruzzone and Dermietzel, 2006), heart (Rohr, 2004; van Veen et al., 2001), and skin (Kelsell et al., 2001). Defects in gap junction structure and function are therefore implicated in a number of human pathologies and diseases (Cronier et al., 2009).

1.2 Connexins

The basic protein subunits of vertebrate gap junction are connexins (Cxs). There are 21 connexins in the human genome and 20 in the mouse, most of which can be paired as sequence orthologs (Table 1.1) (Cruciani and Mikalsen, 2005; Sohl and Willecke, 2004). The most common naming system of connexins is based on the molecular weight of the protein (Sohl and Willecke, 2003). For example, the Cx43 protein is named based on its predicted molecular weight of 43 kilodaltons (kDa).

There is much variation in the tissue expression of connexins. For example, keratinocytes within the human epidermis express Cx26, 30, 30.3, 31, 31.1, 32, 37, 40, 43, and 45 (Di et al., 2001) while neurons and supporting cells of the rodent nervous system express Cx26, 32, 36, 37, 40, 43, 45, and 46 (Rozental et al., 2000). In contrast to the broad expression of connexins in these tissues, liver hepatocytes express only Cx26 and Cx32 (Vinken et al., 2008). Connexin protein levels in these connexin-expressing tissues also vary during organ development (Evans and Martin, 2002).

Together, site-directed antibodies, electron microscopy and crystallography have been used to reveal the membrane topology and quaternary structure of connexins, as well as the arrangement of connexins within gap junctions (Figure 1.1) (Evans and Martin, 2002; Maeda et al., 2009; Pantano et al., 2008; Unger et al., 1999; Yeager and Harris, 2007). Each connexin contains four hydrophobic transmembrane regions, two extracellular loops, and one cytoplasmic loop, where both amino and carboxyl tails are exposed to the cytoplasm (Goodenough et al., 1996).

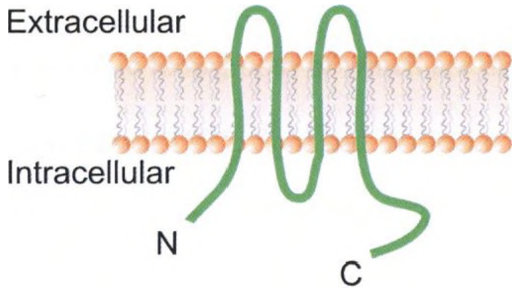
Table 1.1 Connexin orthologs in human and mouse. The human genome encodes 21 connexins while the mouse genome encodes 20 connexins. Dashed boxes indicate where orthologous connexins have not been identified. Modified from Cruciani and Mikalsen, 2006.

Human Connexins	Mouse Connexins
23	23
25	--
26	26
30	30
30.3	30.3
31	31
31.1	31.1
31.3	29
31.9	30.2
32	32
--	33
36	36
37	37
40	40
40.1	39
43	43
45	45
46	46
47	47
50	50
59	--
62	57

Figure 1.1 Gap junction structure. Each connexin contains four membrane-spanning hydrophobic domains, two extracellular and one intracellular loop, and cytoplasmic amino and carboxyl ends (A). Six connexins oligomerize to form a single connexon (B). At the plasma membrane, one connexon docks with another from a neighboring cell to form a gap junction channel (C), allowing for cytoplasmic continuity between cells.

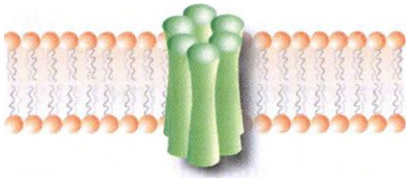
A

Connexin



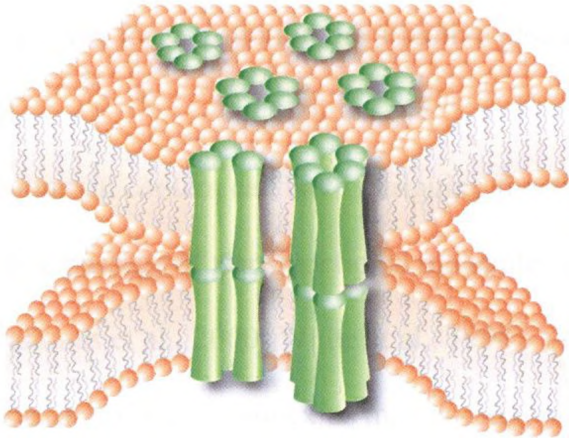
B

Connexon



C

Gap Junction Plaque



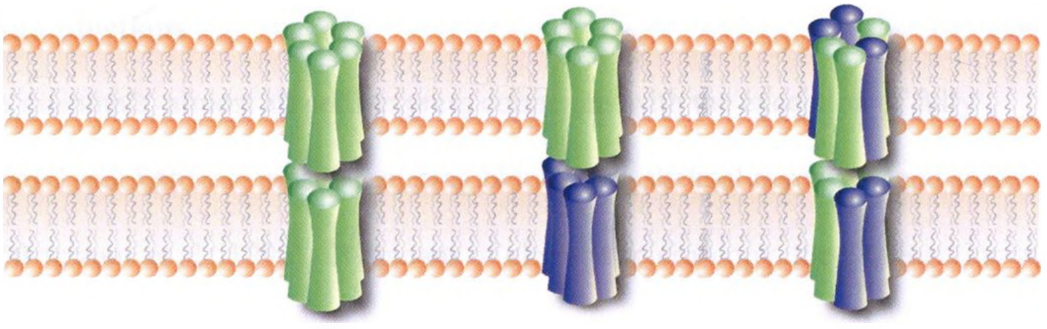
All members of the connexin family share structural similarity and amino acid sequence homology which is conserved across species (Goodenough et al., 1996; Sohl and Willecke, 2004). Connexin members vary mostly in the length of the carboxyl terminus, and the size of the cytoplasmic loops, both of which are thought to regulate the properties of the channel (John and Revel, 1991; Sohl and Willecke, 2004). Three cysteine residues in each extracellular loop are also highly conserved and interloop disulfide bonds are thought to play a role in protein docking between contracting cells (John and Revel, 1991).

1.3 Connexons and channel diversity

Six connexin subunits oligomerize to form a transmembrane (connexon) channel. Additionally, two connexons from apposing cells align and dock across the intercellular space to form a pore between cells. This 1.5 nm pore allows for selective continuity of ions, metabolites, and secondary messengers all of which are less than 1 kDa in size (Alexander and Goldberg, 2003). The connexin isoform composition of the channel can differ creating gated channels that allow for variation in the selective passage of molecules (Willecke et al., 2002).

Gap junctions of mixed connexin content can have intriguing functional consequences owing to the selective passage of transjunctional molecules (Brink et al., 2000; Cottrell and Burt, 2005; Valiunas et al., 2000; White et al., 1994). As most cell types express more than one connexin, different connexins may co-oligomerize within a connexon to form a mixed channel, as reviewed by Brink et al. (2000). Connexons composed of more than one connexin are termed heteromeric, while a connexon

Figure 1.2 Connexon and gap junction channel types. Homomeric connexons are composed of one connexin isoform and heteromeric connexons are composed of two or more different connexin isoforms. Homotypic gap junction channels are formed by two identical connexons and heterotypic channels are composed of two different connexons.



Connexon:	Homomeric	Homomeric	Heteromeric
Gap Junction Channel:	Homotypic	Heterotypic	Heterotypic

composed of only one connexin type is homomeric (Figure 1.2). Heterotypic channels are composed of two different connexons from apposing cells, while homotypic channels are composed of identical connexons for each apposing cell. Thus, the overall complexity of GJIC is dramatically affected by the assembly of both heteromeric and heterotypic channels (Brink et al., 2000).

1.4 Gap junctions

In mammals, almost all cells express connexins with the exception of fully differentiated spermatozoa, most circulating blood cells, skeletal myocytes, and some neurons (Bruzzone et al., 1996). By means of electron microscopy, the gap junction was originally characterized as a close apposition of the plasma membranes where the intercellular space narrows from 25 nm to 2-4 nm (Revel and Karnovsky, 1967). It was later revealed that each of these regions of close apposition, termed plaques, contained up to thousands of intercellular channels (Goodenough and Revel, 1970; Loewenstein, 1981; Makowski et al., 1977). Further biochemical analysis has led to a widely accepted model of gap junctions as transmembrane structures where each cell contributes connexons which connect with apposing connexons across the intercellular space (Figure 1.1) (Goodenough and Revel, 1970; Makowski et al., 1977; Unger et al., 1999).

1.5 GJIC in physiology and development

Gap junctions can be regulated by many factors such as pH, phosphorylation, voltage, calcium, and other molecular signals (Levin, 2002). Because of the potential for the regulation of information flow, GJIC can play a key role in cell physiology and

embryonic development. GJIC is known to play a role in rapid syncitial communication in cardiac tissue (Kimura et al., 1995; Severs, 1999) and in the brain (Budd and Lipton, 1998), as well as synchronizing hormonal secretion in glands (Meda, 1996).

During embryonic patterning, GJIC has a known role in regulating the flow of molecular determinants into distinct cellular compartments (Bruzzone et al., 1996; Levin, 2002). Antibodies, antisense oligonucleotides, and connexin knock-out mice have been used as molecular tools and animal models to determine the functional roles of connexins in embryonic development (Becker and Davies, 1995; Becker et al., 1992; Lee et al., 1987). Aberrant expression of connexins can lead to developmental defects, such as cardiac structural and functional abnormalities (Liu et al., 2006), and even embryonic lethality (Levin, 2002), revealing a critical role of connexins during these developmental processes.

1.6 Connexin knock-out mouse models

Connexin knock-out mice reveal the importance of GJIC in tissue development, differentiation, and function, as recently reviewed by Dobrowolski and Willecke (2009). To date, most of the 20 connexin family members have been individually ablated in mice (Dobrowolski and Willecke, 2009). The phenotype of a connexin knock-out with tissue-restricted expression is generally less severe than a knock-out of a connexin with a wider endogenous tissue distribution. For example, Cx50 has a confined expression to the lens of the eye. Cx50 knock-out mice develop cataracts, indicating that this connexin is necessary for lens homeostasis (White and Paul, 1999). In contrast, when the widely-expressed Cx43 is ablated, the phenotype includes severe cardiac defects which lead to

lethality at birth (Reaume et al., 1995). To circumvent lethal Cx43 knock-out phenotypes, conditional knock-outs have been generated to examine tissue-specific Cx43 function. Cx43-targeted knock-outs have been generated in endothelial cells (Liao et al., 2001), smooth muscle cells (Liao et al., 2007), Sertoli cells (Sridharan et al., 2007), neural crest and neural tube cells (Liu et al., 2006; Xu et al., 2006), to name a few. Each conditional Cx43 knock-out revealed a tissue-specific role of Cx43 in development and differentiation.

1.7 Connexin gene mutations in human disease

Mutations in the genes encoding connexins are known to cause several diseases. Deafness and skin disease are caused by mutations in Cx26, Cx30, Cx30.3, Cx31, and Cx43 (Kelsell et al., 2001; Kelsell et al., 1997; Richard et al., 2003; van Steensel, 2004). X-linked Charcot-Marie Tooth Disease is correlated with over 300 mutations in the gene encoding Cx32, leading to the demyelination of peripheral nerves (Bergoffen et al., 1993; Scherer et al., 1995; White and Paul, 1999). Congenital cataracts can occur as a consequence of mutations in the genes encoding Cx46 and Cx50 (Mackay et al., 1999; Shiels et al., 1998). Currently 62 mutations in the gene encoding Cx43 are known to cause oculodentodigital dysplasia (ODDD) (Paznekas et al., 2009). At the cellular level, many of these connexin mutants traffic inefficiently, but nevertheless can assemble into non-functional channels or channels that are severely compromised (Krutovskikh and Yamasaki, 2000; McLachlan et al., 2005; Zhou and Griffin, 2003).

1.7.1 Oculodentodigital dysplasia

Not surprisingly given the widespread expression of Cx43 within multiple tissues, ODDD affects multiple developmental processes. The diagnostic phenotype of ODDD involves craniofacial abnormalities, including narrow nose and nostrils, defects of the digits including syndactyly of the fourth and fifth fingers, ocular defects including small eyes and glaucoma, and dental defects (detailed in Section 1.7.2) (Paznekas et al., 2009). In later life, neurologic defects including spastic gait, hyperreflexia and incontinence are often reported in patients (Loddenkemper et al., 2002). Further neurologic phenotypes are noted as the patient reaches the second decade of life, including weakness of the speech muscles, bladder disturbances, spastic weakness of the lower extremities, ataxia, and seizures (Loddenkemper et al., 2002). Conductive hearing loss and poor hair growth are also characteristics of this disease (Paznekas et al., 2009).

ODDD is typically of autosomal-dominant inheritance although a few autosomal-recessive cases (Paznekas et al., 2003; Richardson et al., 2006) and sporadic cases (Debeer et al., 2005) have been reported. The disease has been mapped to the *GJA1* gene which encodes Cx43 (Paznekas et al., 2003). Currently, 62 mutations in the *GJA1* gene have been identified in patients with ODDD (Figure 1.3). These mutations typically manifest as amino acid missense substitutions in the polypeptide backbone of Cx43, although frame-shifts occur at two mutation sites (van Steensel et al., 2005; Vreeburg et al., 2007).

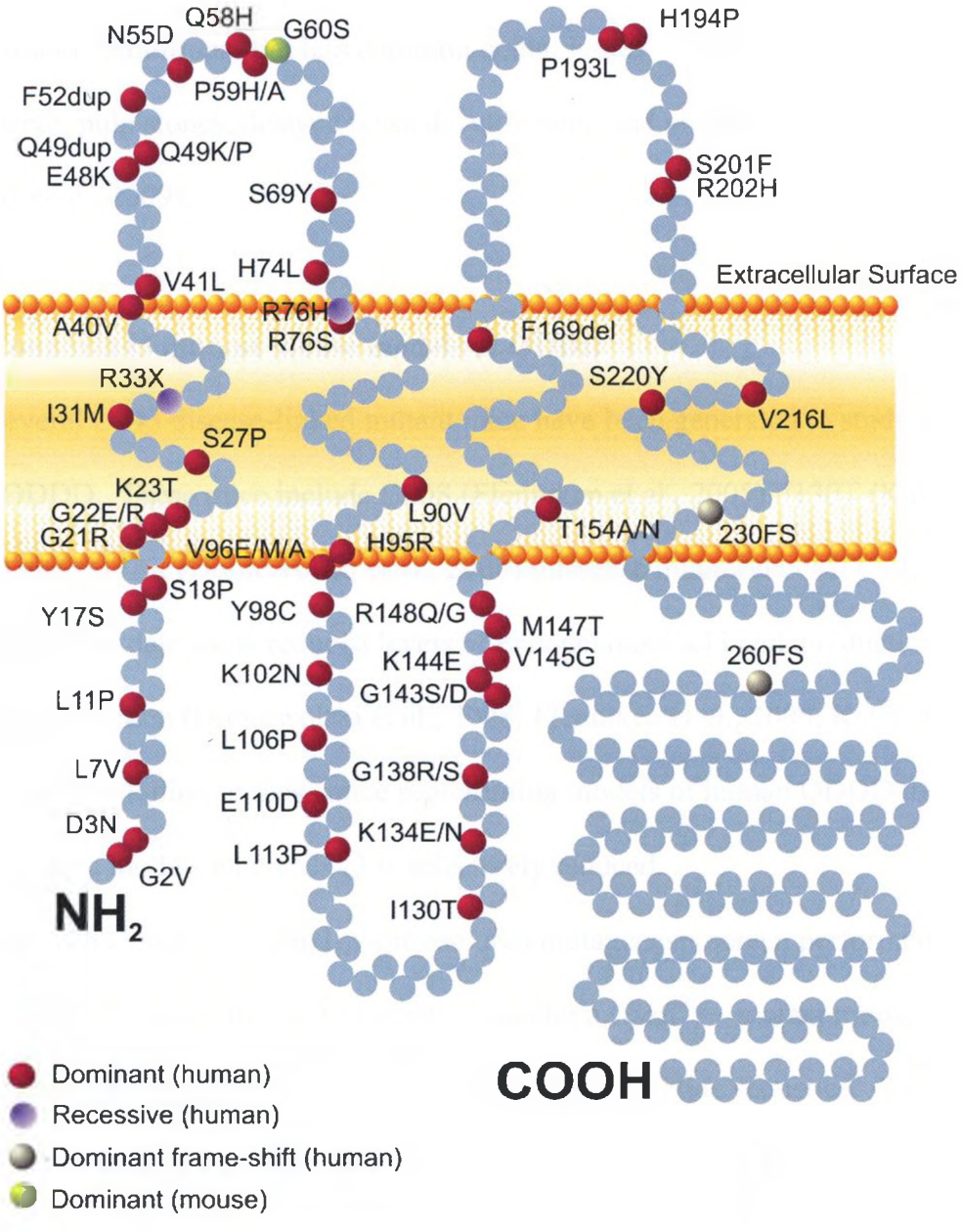
In vitro studies on many ODDD-linked mutations have revealed defects in mutant Cx43 transport and channel function (Beahm et al., 2006; Flenniken et al., 2005; Gong et al., 2007; Gong et al., 2006; Lai et al., 2006; McLachlan et al., 2005; Roscoe et al., 2005;

Seki et al., 2004; Shibayama et al., 2005). In general, ODDD-linked Cx43 mutants are able to transport to the cell membrane and assemble into gap junction plaques (Roscoe et al., 2005; Seki et al., 2004), however in many cases populations of the mutants are trapped in the Golgi apparatus and endoplasmic reticulum and fail to properly traffic to the cell membrane (Gong et al., 2006; McLachlan et al., 2005). Despite the fact that a large fraction of most Cx43 mutants reach the cell membrane, they frequently exhibit reduced channel function or no function at all (Beahm et al., 2006; Lai et al., 2006; Shibayama et al., 2005). Furthermore, all Cx43 mutants tested *in vitro,* as well as *in vivo* studies of the G60S mutant, have been dominant-negative to the function of wild-type Cx43 (Beahm et al., 2006; Flenniken et al., 2005; Gong et al., 2007; McLachlan et al., 2005; Roscoe et al., 2005). This suggests that, despite a presumed 50% expression of wild-type Cx43 in patients, these individuals are likely functioning with much less than 50% of normal GJIC.

1.7.2 Tooth phenotype of ODDD

The principal dental anomalies associated with ODDD include enamel hypoplasia in both the primary (deciduous) and permanent dentition, often resulting in many cavities and early tooth loss (Paznekas et al., 2003). Because these defects occur in both sets of human dentition, it is evident that the mechanism of the ODDD tooth phenotype is underlying the entire development of the tooth and is not due to a temporary disturbance in the development process. We will further investigate the mechanisms behind the ODDD dental disturbance in this thesis.

Figure 1.3 Location of Cx43 mutations associated with ODDD. To date, 62 mutations have been linked to ODDD. Red balls represent the amino acids in the Cx43 polypeptide sequence that are affected by dominant mutations in the *GJA1* gene. Purple balls represent the two known amino acids that are affected by autosomal recessive mutations. Grey balls represent the amino acid origin of two frame-shifts that are affected by dominant mutations. The green ball at position 60 represents the amino acid substitution found in the $Gja1^{Jrt}/+$ mouse model of ODDD. Adapted from Laird, 2006.


Q58H
N55D
G60S
P59H/A
F52dup
Q49dup
Q49K/P
E48K
S69Y
H74L
V41L
R76H
A40V
R76S
R33X
I31M
S27P
K23T
G22E/R
G21R
V96E/M/A
S18P
Y17S
Y98C
R148Q/G
M147T
K102N
K144E
V145G
L11P
G143S/D
L106P
L7V
G138R/S
E110D
D3N
K134E/N
L113P
G2V
I130T
NH2
H194P
P193L
S201F
R202H
Extracellular Surface
F169del
S220Y
V216L
L90V
T154A/N
230FS
H95R
260FS
COOH
Dominant (human)
Recessive (human)
Dominant frame-shift (human)
Dominant (mouse)

Another common dental phenotype in ODDD patients is smaller teeth (microdontia) (Paznekas et al., 2003). As dentin comprises the bulk of the tooth our studies look further at the development of this tissue in the presence of an ODDD-linked mutant protein. Additional but less common dental issues in ODDD patients include missing teeth, pulp stones, delayed tooth development, and abnormal patterning of teeth (Paznekas et al., 2009).

1.8 Connexin43 mutant mouse models of ODDD

Several Cx43 disease-linked mutant mice have been generated to study the role of Cx43 in ODDD. These mice include G60S (Flenniken et al., 2005), I130T (Kalcheva et al., 2007) and G138R (Dobrowolski et al., 2008) missense mutations. All Cx43 mutant mice generated to date show reduced levels of endogenous Cx43 and a reduction in overall Cx43 function (Dobrowolski et al., 2008; Flenniken et al., 2005; Kalcheva et al., 2007). Thus, in addition to these mice representing models of human ODDD they also represent mouse models where Cx43 is selectively reduced.

Through a random *N*-ethyl-*N*-nitroso urea mutagenesis screen performed by the team headed by Dr. Janet Rossant (Toronto, Canada) a *Gja1*^{Jrt}/+ mutant mouse was identified that harbors a G60S mutation in the Cx43 polypeptide sequence. While this mutation is not specifically matched to a known human patient mutation, it lies in close proximity to reported human mutations in the first extracellular loop of the protein (Figure 1.3). This mutant mouse is used extensively in the current study.

The *Gja1*^{Jrt}/+ mouse has a phenotype that is similar to the clinical presentation of patients with ODDD. *Gja1*^{Jrt}/+ mice exhibit syndactyly in the limbs, craniofacial

abnormalities, and enamel defects as well as ocular defects, cardiac dysfunctions, and skeletal abnormalities (Flenniken et al., 2005). The G60S mutation is autosomal-dominant with a 1:1 ratio of mutant to wild-type Cx43, matching the genetics of most ODDD patients. Initial study of the *Gja1*^{Jrt}/+ mouse by Flenniken et al. (2005) revealed Cx43 localized to the plasma membrane as well as within the Golgi apparatus and endoplasmic reticulum compartments. Cx43 gap junction plaques were greatly reduced by over 50% in the mutant mouse myocardium and granulosa cells (Flenniken et al., 2005), indicating a dominant-negative function of the mutant protein. *In vitro* functional studies of this mutant in granulosa cells revealed reduced electrical conductance and minimal dye transfer compared to normal cells (Flenniken et al., 2005).

The *Gja1*^{Jrt}/+ mouse has been used to examine the role of Cx43 within cells and tissues including cardiomyocytes (Manias et al., 2008), keratinocytes (Langlois et al., 2007), mammary gland (Plante and Laird, 2008), and bone (previously reviewed in section 1.6) (McLachlan et al., 2008). This mouse matches the mutant protein dosage of patients and will continue to be an important knock-down model for the study of Cx43 in other tissues affected by this mutation.

Since the generation of the *Gja1*^{Jrt}/+ mouse, other mouse models of naturally occurring human ODDD-linked mutations have been generated. The human Cx43 cytoplasmic loop mutant I130T was introduced into the mouse genome using gene targeting by the team of Glenn Fishman (Kalcheva et al., 2007). More recently, a cre-recombinase mediated introduction of the human cytoplasmic loop Cx43 G138R mutation was generated by the team of Klaus Willecke (Dobrowolski et al., 2008). Both of these mutant mouse models appear to reliably mimic the phenotype of ODDD;

however, the mice that harbor these mutations may also exhibit additional morbidities not related to ODDD. For example, the Cx43 I130T mouse presents with spontaneous and induced ventricular tachyarrhythmias (Kalcheva et al., 2007) which are rarely seen in ODDD patients. Similarly, the Cx43 G138R mouse carries a high heart-related mortality (Dobrowolski et al., 2008) which is not observed in the human population. The *Gja1*^{Jrt}/+ mouse used in our studies, although not a mutation reported in the human population, exhibits few morbidities not associated with ODDD.

1.9 GJIC in differentiation and mineralization

By means of connexin mutant and knock-out mouse studies, GJIC has been implicated in the regulation of tissue differentiation. ODDD-linked Cx43 mutants have been used to study the role of Cx43 in many tissues including the differentiation of the mammary gland (McLachlan et al., 2007; Plante and Laird, 2008) and the bone (McLachlan et al., 2008). For example, Cx43 is expressed in bone cells including osteoblasts, osteocytes, and osteoclasts (Jones et al., 1993; Stains and Civitelli, 2005). McLachlan et al. (2005) found that when dominant-negative Cx43 mutants G21R, G138R, and G60S were expressed in neonatal calvarial osteoblasts they failed to impede differentiation, as assessed by alkaline phosphatase activity levels, or the state of mineralization. It was suggested that the lack of mutant Cx43 effect *in vitro* in osteoblasts already committed to differentiation may not accurately reflect *in vivo* embryonic bone development or the abnormalities observed in ODDD patients and mouse models. In other studies a clear requirement for Cx43 in bone development was shown as the Cx43 null mouse exhibits delayed ossification in many bones (Lecanda et

al., 2000). In addition, osteoblast-specific deletion of Cx43 leads to reduced bone mass and osteoblast dysfunction (Castro et al., 2002; Castro et al., 2003). The difference in these in vitro and in vivo studies may reflect the time of the Cx43 mutant being introduced into cells that are part of the osteoblast lineage.

McLachlan et al. (2008) went on to study the skeletal abnormalities of ODDD by looking *in vivo* at osteoblast differentiation in cells isolated from calvaria of the Cx43 G60S mutant mouse, which shows decreased bone density, mineral content, and mechanical strength, as well as delayed ossification of the craniofacial bones (Flenniken et al., 2005). Late stage differentiation markers osteocalcin and bone sialoprotein were inhibited in osteoblasts differentiated from the G60S mouse, where GJIC measures were decreased by over 60% (McLachlan et al., 2008). Thus, it seemed that the severity with which Cx43 mutations affect osteoblast differentiation is dependent on the developmental time frame when the mutation is present. Similarly to these studies on Cx43 in embryonic bone mineralization, we chose to look at ameloblast cells that were derived from the G60S mouse lineage. In our studies, we wished to gain knowledge of the ability of mutant Cx43 expressing cells to differentiate to form and mineralize enamel.

1.10 Mammalian tooth development

Mammalian tooth development has generally been divided into three distinct yet overlapping stages: initiation, morphogenesis and cell differentiation (Kollar and Lumsden, 1979; Thesleff and Hurmerinta, 1981). During the initiation phase of tooth development, the tooth regions within the jaw and tooth type are established. The determination of tooth identity as incisor, canine, premolar or molar occurs at this stage

and is suggested to be pre-specified by the origin of neural crest cells (Ruch, 1995; Sharpe, 1995). The human deciduous tooth set contains six teeth in each quadrant, two molars, one premolar, one canine and two incisors, while the mouse dentition is more simplified with only two types of teeth, one incisor and three molars on each quadrant of the jaw (Figure 1.4).

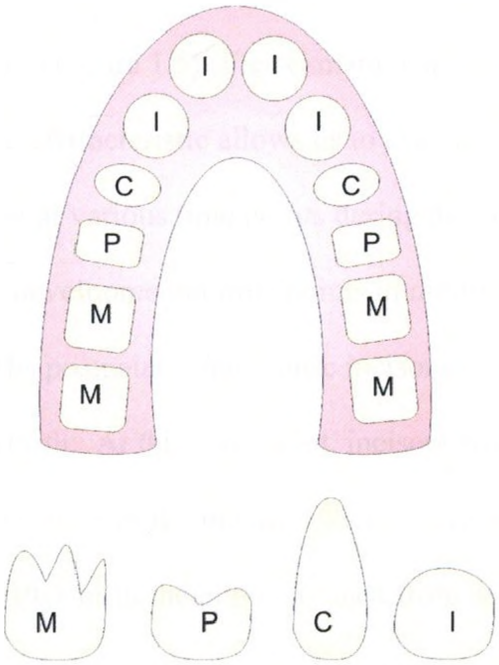
Initiation of tooth development involves determination of the dental mesenchyme and formation of the epithelial dental lamina. The dental lamina of mammalian teeth is derived from the ectoderm, whereas the mesenchymal component of the teeth derives from cranial neural crest in the midbrain region (Imai et al., 1996; Kontges and Lumsden, 1996).

During the morphogenesis phase of tooth development, the shape of each tooth is established. Morphogenesis of the tooth depends on epithelium-mesenchymal interactions (Thesleff and Hurmerinta, 1981), much like the formation of other epithelial appendages, such as hair and glands. Epithelial-mesenchyme interactions involve bone morphogenetic proteins, fibroblast growth factors, and sonic hedgehog signalling molecules (Maas and Bei, 1997). It is now evident that the mesenchymal tissue directs the development of the enamel organ. Transplants of dental mesechyme were able to induce formation of enamel epithelium in non-dental epithelia including the foot pad. Further, these epithelial cells differentiated into ameloblasts to secrete enamel matrix (Caton and Tucker, 2009; Harada and Ohshima, 2004).

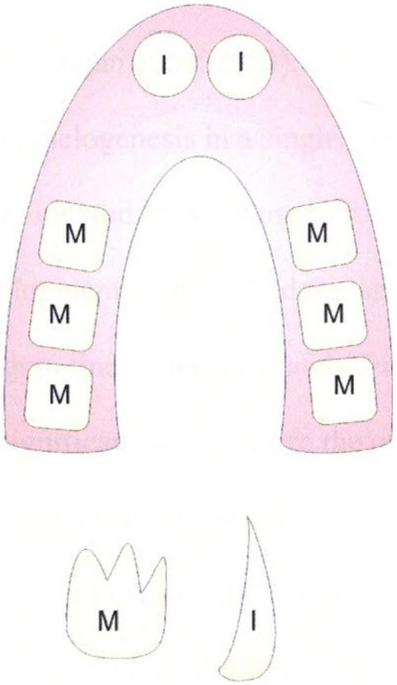
Morphogenesis is accompanied by differentiation of the tooth-specific cells, the odontoblasts from mesenchyme and ameloblasts from inner enamel epithelium. These differentiation processes result in the production of the dentin and enamel matrices,

Figure 1.4 Mouse and human mandibular dentition. The human deciduous tooth layout is shown with six teeth is each quadrant: two incisors (I), one canine tooth (C), one premolar (P) and two molars (M). The mouse tooth pattern is less complex with one incisor and three molars per quadrant, separated by a toothless gap. Tooth shape also varies between human and mouse. Modified from Tucker and Sharpe, 2004.

Human Dentition



Mouse Dentition



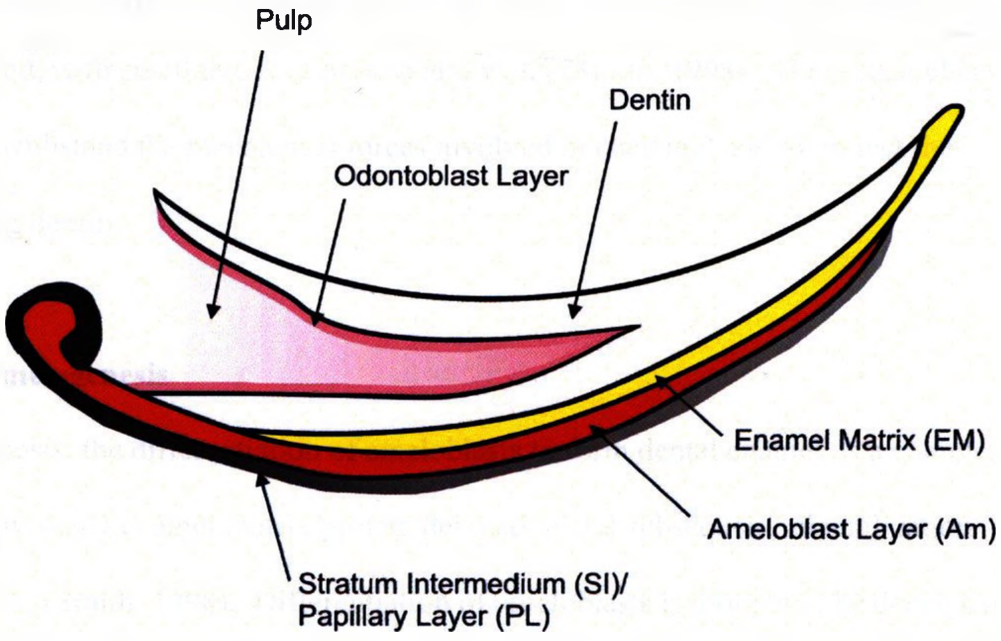
respectively. Differentiation of the mesenchymal tissue precedes the epithelial tissue. Mesenchymal odontoblasts polarize and begin secreting predentin, the organic matrix of dentin. The ameloblasts of the epithelium differentiate shortly after, occurring at the time that initial mineralization is seen in the dentin layer.

Unlike those of other mammals, rodent incisors are continuously growing throughout adult life, with a highly active ameloblast layer positioned at the labial incisor surface (Figure 1.5) (Fleischmannova et al., 2008; Thyagarajan et al., 2003). This unique rodent characteristic allows us to evaluate all stages of amelogenesis in a single mouse incisor at various time points during development. For our studies we chose to focus on three developmental time points in mouse incisor development, 7 days, 21 days, and 2 months postnatal. The mouse incisor erupts from the gum line at approximately 7 days after birth. At this time point, incisors have not been significantly exposed to the oral environment of the mouth, which includes digestive enzymes and occlusive forces. At 21 days after birth the pup is weaned from its mother and begins eating solid foods. At this time point the tooth has not been significantly exposed to the additional forces involved in chewing. Finally at two months after birth, the tooth has been exposed to the oral environment and the effect of this exposure on the enamel layer over time can be analyzed.

1.11 Enamel Structure and Function

Dental enamel is the external covering of the tooth crown which overlays and protects the dentin and pulp layers (Figure 1.5). Enamel is the hardest tissue in the body, composed mostly of calcium salts. Enamel is composed of microscopic rods formed by

Figure 1.5 The mouse incisor anatomy. When sectioned longitudinally through the incisor, all anatomical regions of the tooth can be viewed. The main compartments of the tooth are the central pulp cavity, dentin comprising the bulk of the tooth, and the outer enamel layer. In the mouse incisor, enamel is found only on the facial side of the tooth. External to the developing enamel are the ameloblast and the stratum intermedium and papillary cell layers.

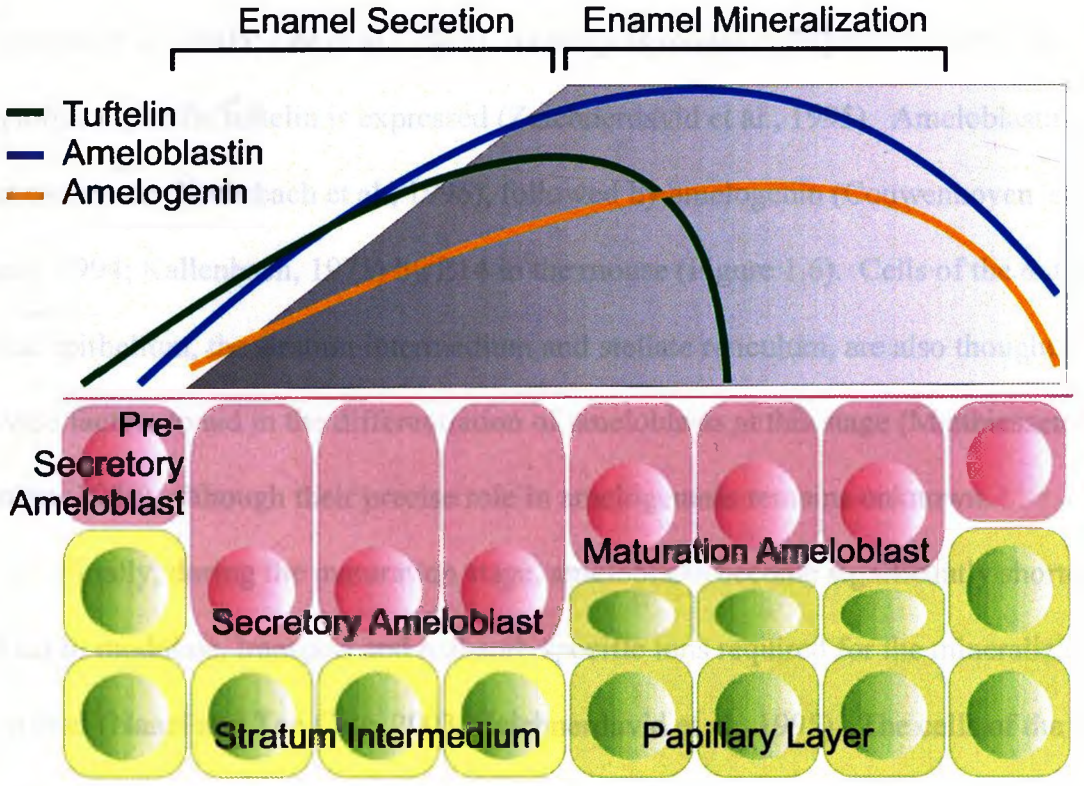

Pulp
Dentin
Odontoblast Layer
Enamel Matrix (EM)
Ameloblast Layer (Am)
Stratum Intermedium (SI)/
Papillary Layer (PL)

ameloblasts. In cross sections, enamel rods are roughly key-hole in shape and run parallel to each other and perpendicularly through the enamel matrix. Individual rods are composed of organized hydroxyapatite crystals, which require enamel matrix proteins to influence their orientation within a lattice. The proteins in enamel are unique to the tissue, including amelogenin and enamelin (Hu et al., 2001). As the crystals grow longer and wider, the organic protein is digested by enzymes and removed from the developing matrix. The resulting fully formed enamel is acellular and almost completely (95%) mineralized, with small traces of protein and water (Smith, 1998). Thus, enamel is well suited to withstand the mechanical forces involved in chewing, and to protect the underlying dentin.

1.12 Amelogenesis

Amelogenesis, the differentiation of ameloblasts to form dental enamel, is a complex process involving enamel matrix protein deposition and subsequent mineralization (Figure 1.6) (Smith, 1998). Differentiation of ameloblasts is thought to be dependent on interactions between odontoblasts and ameloblasts. The process of amelogenesis is remarkably constant among mammalian species (Aoba and Moreno, 1987; Gibson et al., 2005). In mammals, the ameloblast exhibits a unique life cycle characterized by progressive phenotype changes that reflect its primary activity during the various stages of enamel formation (Zeichnerdavid et al., 1995). The ameloblast can be described as sequentially differentiating from the inner enamel epithelium through three main and exclusive functional stages: pre-secretory, secretory and maturation (Smith, 1998). During the pre-secretory stage, differentiating ameloblasts acquire their

Figure 1.6 The process of amelogenesis. Ameloblasts form an epithelial cell layer external to the developing enamel. Ameloblasts transition from a pre-secretory stage to secretory, and finally to a maturation stage of differentiation. At the secretory stage ameloblasts secrete enamel matrix proteins to form the full thickness of the enamel. Cells of the stratum intermedium and stellate reticulum surround the secretory ameloblasts. Ameloblasts at the maturation stage resorb much of the broken down organic constituent of the matrix to favour enamel mineralization. Cells of the papillary layer surround and support the maturation stage ameloblasts.


Enamel Secretion
Enamel Mineralization
Tuftelin
Ameloblastin
Amelogenin
Pre-Secretory Ameloblast
Secretory Ameloblast
Maturation Ameloblast
Stratum Intermedium
Papillary Layer

phenotype, develop an extensive protein synthesis apparatus and prepare to secrete the organic matrix of enamel (Zeichnerdavid et al., 1995).

For the duration of the secretory stage, tall columnar ameloblasts secrete enamel-specific proteins into the enamel matrix. The majority of these proteins consist of amelogenins, as well as ameloblastin, enamelin and tuftelin (Begue-Kirn et al., 1998; Fukumoto et al., 2005; Lee et al., 2003). As early as mouse embryonic day (E) 13, ameloblast specific tuftelin is expressed (Zeichnerdavid et al., 1995). Ameloblastin is next expressed (Krebsbach et al., 1996), followed by amelogenin (Couwenhoven and Snead, 1994; Kallenbach, 1971) by E14 in the mouse (Figure 1.6). Cells of the outer dental epithelium, the stratum intermedium and stellate reticulum, are also thought to provide factors to aid in the differentiation of ameloblasts at this stage (Matthiessen and Romert, 1980), although their precise role in amelogenesis remains unknown.

Finally, during the maturation stage, ameloblasts become significantly shortened and act to modulate, transport and reabsorb specific ions required for the mineralization of enamel (Nanci and Ten Cate, 2003; Zeichnerdavid et al., 1995). The cells of the outer dental epithelium undergo hypertrophy and morphological changes to reorganize into a papillary layer during this maturation stage of amelogenesis (Matthiessen and Romert, 1980; Smith, 1998). As protein is broken down and reabsorbed from the enamel matrix, enamel crystals grow in width and achieve enamel's final hardened form (Smith, 1998). When the tooth erupts into the oral cavity, around post natal day 7 in the mouse or 6 months in the human, the ameloblast layer is lost and enamel is exposed to the environment of the mouth (Nanci and Ten Cate, 2003).

1.13 Enamel developmental defects

All enamel defects result from disturbances in the process of amelogenesis. This is not surprising given that amelogenesis involves strict regulation of the enamel matrix constituents. Amelogenesis imperfecta represents a group of developmental defects in enamel which affect the structure and clinical appearance of all teeth of the affected individual in an equal manner (Aldred et al., 2003; Crawford et al., 2007). The enamel may be hypoplastic (thinner), hypomineralized, or both and the affected teeth may be discoloured, sensitive, or prone to disintegration. These two distinct types of amelogenesis imperfecta are related to the two phases of amelogenesis (Aldred et al., 2003; Crawford et al., 2007). Some of the genes encoding specific enamel proteins important in amelogenesis have been implicated in amelogenesis imperfecta. Mutations in the amelogenin gene (AMELX) cause X-linked amelogenesis imperfecta, while mutations in the enamelin gene (ENAM) cause autosomal-inheritance forms of amelogenesis imperfecta (Stephanopoulos et al., 2005).

Enamel hypoplasia has long been known to be due to an ameloblast disturbance during matrix secretion. In general, enamel hypoplasia refers to a deficiency in the amount or thickness of enamel (Suckling, 1989). These defects may range from single or multiple pits to deep and wide troughs of decreased enamel thickness, and ultimately to complete lack of the tissue. However, in distinctive enamel hypoplasia, even the thin enamel is completely mineralized during enamel maturation. Similarly, enamel hypocalcification is known to be due to a disturbance in enamel maturation. Hypocalcification involves the change of colour and opacity of enamel, indicating differences in hardness or opacity of enamel (Clarkson and Omullane, 1989).

1.14 Connexins in the tooth

Gap junction plaques are present between all cells of the enamel organ, including the inner enamel epithelium and ameloblasts (Inai et al., 1997; Sasaki et al., 1983), suggesting that intercellular communication may in fact be necessary during all phases of enamel development. Information transferred across gap junctions could potentially control ameloblast cell proliferation or cell death and coordinate the activation and subsequent regulation of protein matrix deposition and mineralization through ameloblast differentiation.

Cx43 is the most ubiquitously expressed and has been found in the cells forming the human tooth during development, including the ameloblast, stratum intermedium, stellate reticulum and differentiating odontoblasts (About et al., 2002; Sasaki et al., 1985). Cx43 appears to be downregulated in the mature adult tooth, but can be seen in carious teeth localized to odontoblast processes (About et al., 2002; Ibuki et al., 2002). Likewise, the presence of Cx43 has been demonstrated in ameloblasts and stratum intermedium of the enamel organ in rats (Fried et al., 1996; Joao and Arana-Chavez, 2003) as well as the rat preodontoblast and odontoblast (Joao and Arana-Chavez, 2003; Murakami et al., 2001). Given its broad distribution in the enamel organ, Cx43 and GJIC may play an important role in the coordinated events that lead to the differentiation of ameloblasts during enamel development.

Cx32 has also been observed in the preameloblast, ameloblast and stratum intermedium of the embryonic rat, while the presence of Cx26 was not noted in these cells (Fried et al., 1996). Connexin expression in the mouse enamel organ has not been investigated in the literature to date and is one of the topics investigated in this thesis.

1.15 Hypothesis

We hypothesize that mice harboring an autosomal-dominant mutation in the gene encoding Cx43 will have fewer Cx43 gap junctions in the developing tooth resulting in a dysfunctional enamel organ, thin enamel layer, and undersized incisors.

1.16 Objectives

1. Localize connexins and the frequency of Cx43 plaques in the enamel organ of wild-type and *Gja1*^{Jrt}/+ mice harboring an autosomal-dominant Cx43 mutant.
2. Assess enamel organ morphology, development and differentiation in *Gja1*^{Jrt}/+ mice.
3. Characterize incisor dentin and enamel development in *Gja1*^{Jrt}/+ mice.

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CHAPTER 2

Decreased levels of Cx43 gap junctions result in ameloblast dysregulation and enamel hypoplasia in *Gja1*^{Jrt}/+ mice

2.0 Rationale

In this chapter, we wanted to examine the role of Cx43 on enamel organ differentiation and disease. We hypothesized that amelogenesis is governed by Cx43-based gap junctional intercellular communication as oculodentodigital dysplasia (ODDD) patients harboring autosomal-dominant mutations in Cx43 exhibit enamel defects typically resulting in early adulthood tooth loss. To assess the role of Cx43 in tooth development we employed a mouse model of ODDD that harbors a G60S Cx43 mutant, *Gja1*^{Jrt}/+, and appears to exhibit tooth abnormalities that mimic the human disease. Using the *Gja1*^{Jrt}/+ mouse as a knock-down model of Cx43, we tested the hypothesis that Cx43 mutation results in enamel organ dysfunction and that this may account for the enamel hypoplasia displayed by ODDD patients.

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2.1 Introduction

Tooth development follows patterning, morphogenesis, and cell differentiation pathways programmed into odontoblasts and ameloblasts that are responsible for the formation of dentin and enamel, respectively. It is predicted that gap junctions play a critical role in the differentiation of these cells leading to their ability to deposit the mineralized components of the developing tooth.

Gap junctional intercellular communication (GJIC) has multiple and diverse roles regulating aspects of signaling, communication, differentiation, proliferation, polarity and development (Neijssen et al., 2007). A gap junction is formed when two contacting cells each contribute a connexon (hemichannel) which dock to form a pore connecting their cytoplasms. These cell-cell channels allow for ionic and metabolic coupling (Evans and Martin, 2002). Each connexon consists of six connexin (Cx) proteins of the same or different type. There are 21 connexins expressed in humans and 20 in mouse, all of which share similar transmembrane topologies (Evans and Martin, 2002). Cx43 is the most ubiquitously expressed and has been found in the cells forming the human tooth during development, including the ameloblast, stratum intermedium, stellate reticulum, papillary layer and differentiating odontoblasts (About et al., 2002; Sasaki et al., 1985). Cx43 appears to be downregulated in the mature adult tooth, but can be seen in carious teeth localized to odontoblast processes (About et al., 2002; Ibuki et al., 2002). In rats, Cx43 immunoreactivity has been found in the ameloblasts of the enamel organ (Fried et al., 1996; Joao and Arana-Chavez, 2003) as well as the preodontoblast and odontoblast (Joao and Arana-Chavez, 2003; Murakami et al., 2001). In addition to Cx43, Cx32 and Cx26 have been found localized to the developing enamel organ and mature odontoblasts

in rats, respectively (Fried et al., 1996; Inai et al., 1997). Given the broad distribution of Cx43 in the developing enamel organ, we postulate that GJIC is required to coordinate the differentiation of ameloblasts during enamel development.

Humans with mutations in the Cx43-encoding gene, *GJA1*, exhibit oculodentodigital dysplasia (ODDD), an autosomal-dominant disorder defined by skeletal, ocular and tooth abnormalities (Paznekas et al., 2003). Patients with this disease display enamel hypoplasia and multiple cavities resulting in early tooth loss (Paznekas et al., 2003). To date, 62 Cx43 mutations have been linked to the pleiotropic symptoms of ODDD. Many of these mutant proteins have been shown *in vitro* to act as dominant-negatives to co-expressed wild-type Cx43 and reduce GJIC far below 50% and in some cases to as little as 15% of normal levels (Flenniken et al., 2005; McLachlan et al., 2005; Shibayama et al., 2005). A mouse harboring a G60S mutation in the Cx43 protein ($Gja1^{Jrt}/+$) mimics the human symptoms of ODDD including craniofacial abnormalities and syndactyly (Flenniken et al., 2005). Preliminary characterization of this mouse also revealed defects in the enamel of mutant mouse incisors (Flenniken et al., 2005), indicating that this mouse mimics the enamel defect found in human ODDD patients.

Ameloblasts form an epithelial cell layer external to the enamel of the developing tooth (Bei, 2009). These cells differentiate from the inner enamel epithelium sequentially through stages which reflect their primary functions during enamel development which include inductive, secretory and maturation phases (Bei, 2009; Smith, 1998). At the cervical loop, the pre-ameloblasts are induced to differentiate into ameloblasts (Bei, 2009). The tall, columnar secretory ameloblasts actively produce and secrete enamel proteins into the matrix to form the thickness of the enamel layer (Bei, 2009). The

majority of these proteins consist of amelogenin, as well as ameloblastin, and enamelin (Bei, 2009; Fukumoto and Yamada, 2005; Lee et al., 2003). Ameloblasts then transition into a maturation state to secrete proteases which break down enamel matrix proteins and then resorb much of the water and organic matrix to favor mineralization of the tissue (Lu et al., 2008). Ameloblasts finally commit to a quiescent state external to the developed enamel until the tooth erupts from the gum line whereupon this cell layer is lost (Nanci and Ten Cate, 2007). External to the differentiating ameloblasts are supporting cells consisting of the stratum intermedium, stellate reticulum and outer enamel epithelium at the secretory stage of differentiation, and the papillary layer at the maturation stage of differentiation (Harada et al., 2006). The enamel organ supporting cell layers have been proposed to provide factors critical for ameloblast differentiation and survival (Kawano et al., 2004; Satokata et al., 2000; Tompkins, 2006). The process of amelogenesis is remarkably constant among mammalian species (Gibson et al., 2005). However, unlike those of other mammals, rodent incisors are continuously growing throughout adult life, with a highly active ameloblast layer positioned at the labial incisor surface (Boran et al., 2009; Fleischmannova et al., 2008; Thyagarajan et al., 2003). This unique rodent developmental characteristic allows us to evaluate all stages of amelogenesis in a single longitudinal section of the mouse incisor.

To date, the function of Cx43 in the enamel organ is still unknown. In other mineralized tissues such as bone, Cx43 has been shown to play a role in cell differentiation as well as respond to physiologic signals and mechanical stresses (Castro et al., 2002; Donahue, 2000; Jiang et al., 2007; McLachlan et al., 2008; Watkins et al.,

2006). To investigate the biological importance of Cx43 in the tooth, we used the *Gja1*^{Jrt}/+ mouse as a Cx43 knock-down model that leads to disease.

2.2 Materials and Methods

2.2.1 Animals

Gja1^{Jrt}/+ mice were bred with a mixed genetic background of C3 and C57BL/6J to carry a heterozygous mutation in the *Gja1* gene (Flenniken et al., 2005). All mice used in this study were from the second, third, or fourth generation of back-crossing to C57BL/6J mice. Mice were maintained ad libitum on a high fat diet of chow and ground meal (Harlan Laboratories). Mice were sacrificed using CO_2 and O_2 at 7 days, 21 days, or 2 months postnatal. Mouse genotype was determined by polymerase chain reaction (PCR) as previously described (Flenniken et al., 2005). Experimental procedures used were approved by the Animal Care Committee at the University of Western Ontario and followed the guidelines of the Canadian Council on Animal Care.

2.2.2 Tissue collection and treatment

Upper and lower incisors were collected from *Gja1*^{Jrt}/+ and wild-type littermate mice at 7 days, 21 days, or 2 months postnatal. Teeth and control liver sections were fixed overnight in 10% neutral buffered formalin (NFB) (BDH, West Chester, PA, USA). Teeth were additionally demineralized for up to 7 days in 10% formic acid (Fluka, Steinheim, Germany), and embedded in paraffin. Paraffin-embedded tissues were sectioned longitudinally at 5μm intervals and used for immunofluorescent labeling or staining with hematoxylin and eosin.

2.2.3 Radiography

Upper and lower incisors were collected from *Gja1*^{Jrt}/+ and wild-type littermate mice 2 months postnatal, fixed, and radiographed using a Belmont 096 Belray Dental X-ray machine (70kVp, 10mA, 0.02s exposure; Takara Belmont Corporation, Osaka, Japan).

2.2.4 Immunohistochemistry and confocal microscopy

Demineralized paraffin sections cut longitudinally through the incisors were stored in 10% NFB before immunolabelling. Sections were deparaffinized with xylene then subjected to rehydration in descending grades of ethanol baths followed by microwave antigen retrieval using citric acid solution. Detection of Cx43 was performed using a monoclonal mouse antibody (1:500; Chemicon International). Detection of Cx32 was performed by using a polyclonal rabbit antibody (1:2000; Sigma, Saint Louis, MI, USA). Detection of Cx26 required the use of a polyclonal rabbit antibody (1:100; Zymed, Carlsbad, CA, USA). Amelogenin expression was assessed by using a rabbit polyclonal antibody (1:500; Santa Cruz Biotecnologies, Santa Cruz, CA, USA). Secondary Alexa Fluor 488 or 555-conjugated anti-mouse or anti-rabbit antibodies were used at a 1:500 dilution (Molecular Probes, Eugene, OR, USA). Nuclei were stained with Hoechst 33342 (1:10,000; Molecular Probes, Eugene, OR, USA). Tissue sections were imaged on a Zeiss LSM 510 inverted confocal microscope as previously described (Thomas et al., 2005). In cases where protein expression levels and patterns were

compared between wild-type and *Gja1*^{Jrt}/+ tissue sections, imaging conditions were kept constant between matched samples.

2.2.5 Quantification

Enamel organ images used in our assessments were delineated as supporting cells (SC), including stratum intermedium, stellate reticulum or papillary cell layers and ameloblast (Am), based on their well-described morphological appearances (Nanci and Ten Cate, 2003). The number of gap junction plaques was counted under conditions where the investigator was blinded to whether the sample was from wild-type or mutant mouse. The resulting gap junction counts per field of view were normalized to the number of cells in the area as defined by Hoechst-stained nuclei. A gap junction plaque was defined as a discernable Cx43-labelled bright fluorescent structure of at least 0.5μm in length located at sites of cell-cell apposition. Supporting cell and ameloblast areas were counted separately, and combined for a total enamel organ assessment. Statistical analysis included the calculation of standard error and unpaired Student's t-test where data from wild-type and mutant mice were compared.

2.2.6 Histology

To assess the histology of the mouse incisor, 5μm paraffin-embedded sections of wild-type and *Gja1*^{Jrt}/+ incisors were stained with hematoxylin and eosin. Briefly, paraffin-embedded incisor sections were deparaffinised in xylene, rehydrated in descending grades of ethanol baths, and stained with 1% hematoxylin (5 min) and 1% eosin (5 min). Sections were dehydrated in ascending grades of ethanol and xylene baths

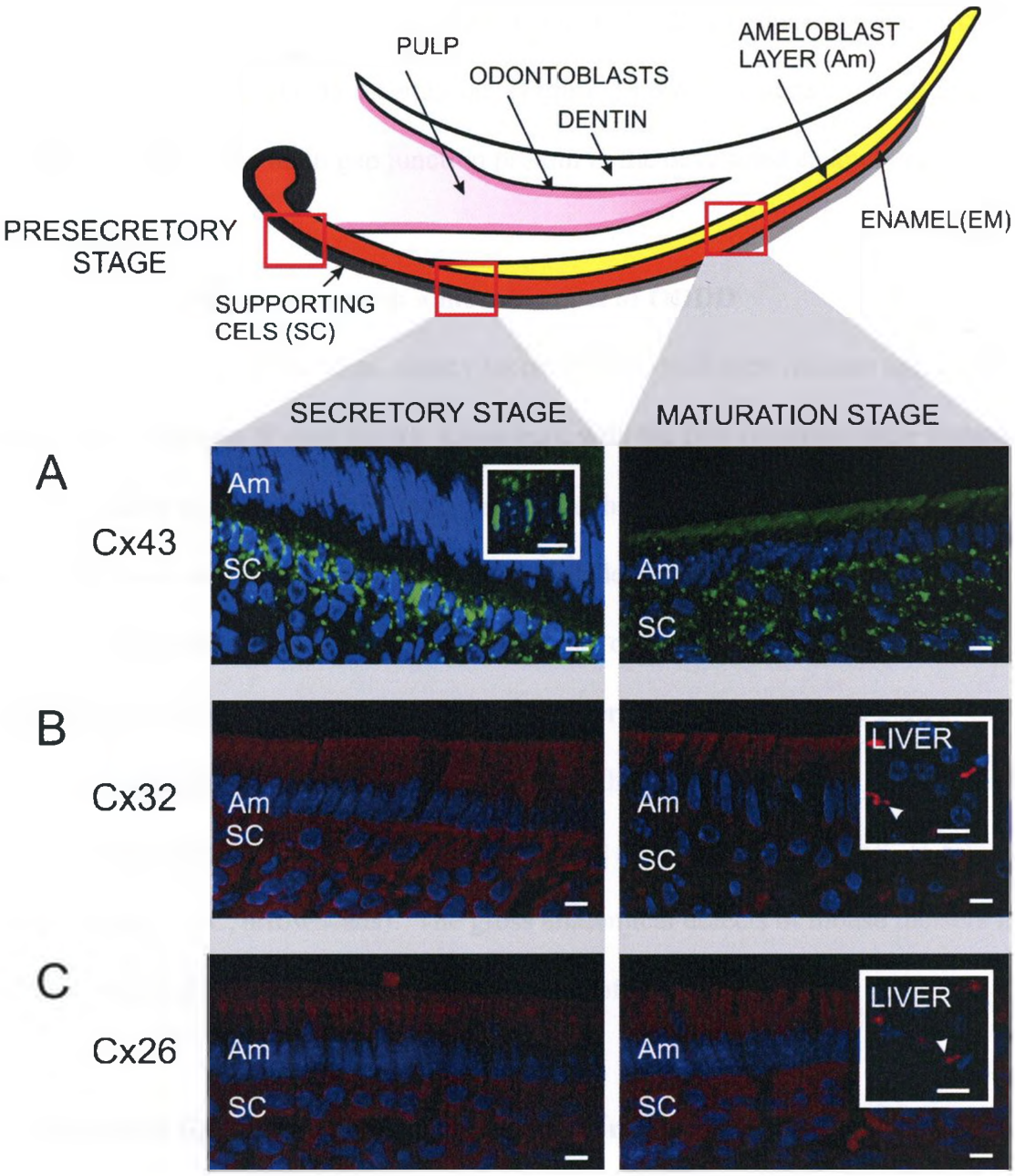
and mounted with Cytoseal. The enamel organ and overall tooth were imaged under 63X, 40X, or 5X objective lenses mounted on a Zeiss Axioscope microscope workstation equipped with a Sony PowerHAD camera and Axiovision LE imaging software (Carl Zeiss Vision). Images of secretory and maturation stages of enamel organ were selected based on their well-described anatomical region within the tooth (Nanci and Ten Cate, 2003). Linear measurements were made either perpendicularly through the mature enamel layer (enamel thickness), mature dentin layer (dentin thickness), or from the enamel organ cervical loop to the enamel tip (incisor length) using imaging software. Statistical analysis included standard error calculations and comparisons between wild-type and mutant measures with a Student's t-test.

2.3 Results

2.3.1 Connexin expression in the enamel organ

Cx43 is present in many tissues and organs in the body, and it has been reported to be localized to the developing tooth (About et al., 2002). However, little information has been published on its cellular and subcellular localization during the differentiation of the mouse enamel organ. To that end, we first evaluated fully developed murine incisors for the presence of Cx43. Cx43 staining was noted primarily in the cells of the stratum intermedium and stellate reticulum at presecretory and secretory stages and in the papillary cell layer at maturation stages of differentiation as punctate fluorescence between apposing cell borders (Figure 2.1). Cx43 was found in low levels in the ameloblast cell layer at these stages, but appeared to be transiently upregulated in the ameloblasts transitioning from secretory to maturation stages of differentiation (Figure

Figure 2.1 Cx43 expression is predominant in the murine enamel organ. Cx43 (A), Cx32 (B), and Cx26 (C) immunofluorescence were imaged in the 7d lower incisor at secretory and maturation stages of ameloblast differentiation. Nuclei were stained with Hoechst (blue). (A) Punctate Cx43 structures at cell-cell interfaces were visible in the enamel organ at both stages of ameloblast differentiation, particularly in the supporting cell layers. At the transition point between secretory and maturation stages of differentiation, Cx43 became more evident between ameloblasts (insert). (B&C) Cx32 and Cx26 were not observed in the enamel organ of the murine incisor but were detected in liver sections prepared under standard conditions (inserts, arrowheads). Similar results were found for 21 day and 2 month sections (data not shown). Am, ameloblast layer; SC, supporting cells (stratum intermedium and stellate reticulum at the secretory stage and papillary layer at the maturation stage). Bars = 20μm.


PULP
ODONTOBLASTS
DENTIN
AMELOBLAST LAYER (Am)
ENAMEL(EM)
PRESECRETORY STAGE
SUPPORTING CELS (SC)
SECRETORY STAGE
MATURATION STAGE
A
Cx43
Am
SC
B
Cx32
LIVER
C
Cx26

2.1A, insert). Cx26 and Cx32 have been reported in cells of ectodermal origin (Evans and Martin, 2002). However, we did not detect Cx26 or Cx32 in the murine differentiating enamel organ using antibodies that could identify these connexins in liver sections prepared under standard conditions (Figure 2.1B & C, inserts). These data suggest that Cx43 is the main gap junction protein in the developed enamel organ.

2.3.2 Altered dental phenotype in a mouse model of ODDD

Gja1^{Jrt}/+ mice have white, chalky incisors while wild-type incisors appear yellow due to enamel staining (Figure 2.2A). Consistent with the first report on these mutant mice (Flenniken et al., 2005), this finding suggests that there is a lack of enamel on the *Gja1*^{Jrt}/+ incisors resulting in the underlying white dentin layer being visible. The *Gja1*^{Jrt}/+ mice incisors appear smaller, although the overall size of the mutant mice is also smaller which is taken into consideration in later experiments (Figure 2.2B). Using x-ray imaging, a dense enamel layer was visible on the facial surface of 2 month incisors from wild- type mice while this layer appears absent in litter-matched *Gja1*^{Jrt}/+ mouse incisors (Figure 2.2C, arrowheads). The gross anatomical defects of mouse incisors from *Gja1*^{Jrt}/+ mice led us to examine further the nature of this defect.

2.3.3 Impaired *Gja1*^{Jrt}/+ mouse enamel organ morphology

Histological assessment of the enamel organ from wild-type mice revealed a tall, columnar ameloblast epithelial cell layer and overlying stratum intermedium and papillary cell layers in both the secretory and maturation components of the enamel organ, respectively (Figure 2.3). In contrast to the wild-type littermate control, the

Figure 2.2 The *Gja1*^{Jrt}/+ mouse displays an altered dental phenotype. Upper and lower *Gja1*^{Jrt}/+ and wild-type mouse incisors were analyzed at 2 months of age. (A) The *Gja1*^{Jrt}/+ incisors appear white and chalky compared to the yellow, enamel stained, wild-type incisors. (B) Mutant incisors appear smaller in size than wild-type when not corrected for body weight. (C) X-ray images reveal the mineralized content of incisors from wild-type and *Gja1*^{Jrt}/+ mice, including the enamel layer (arrowheads). Bars = 1mm.

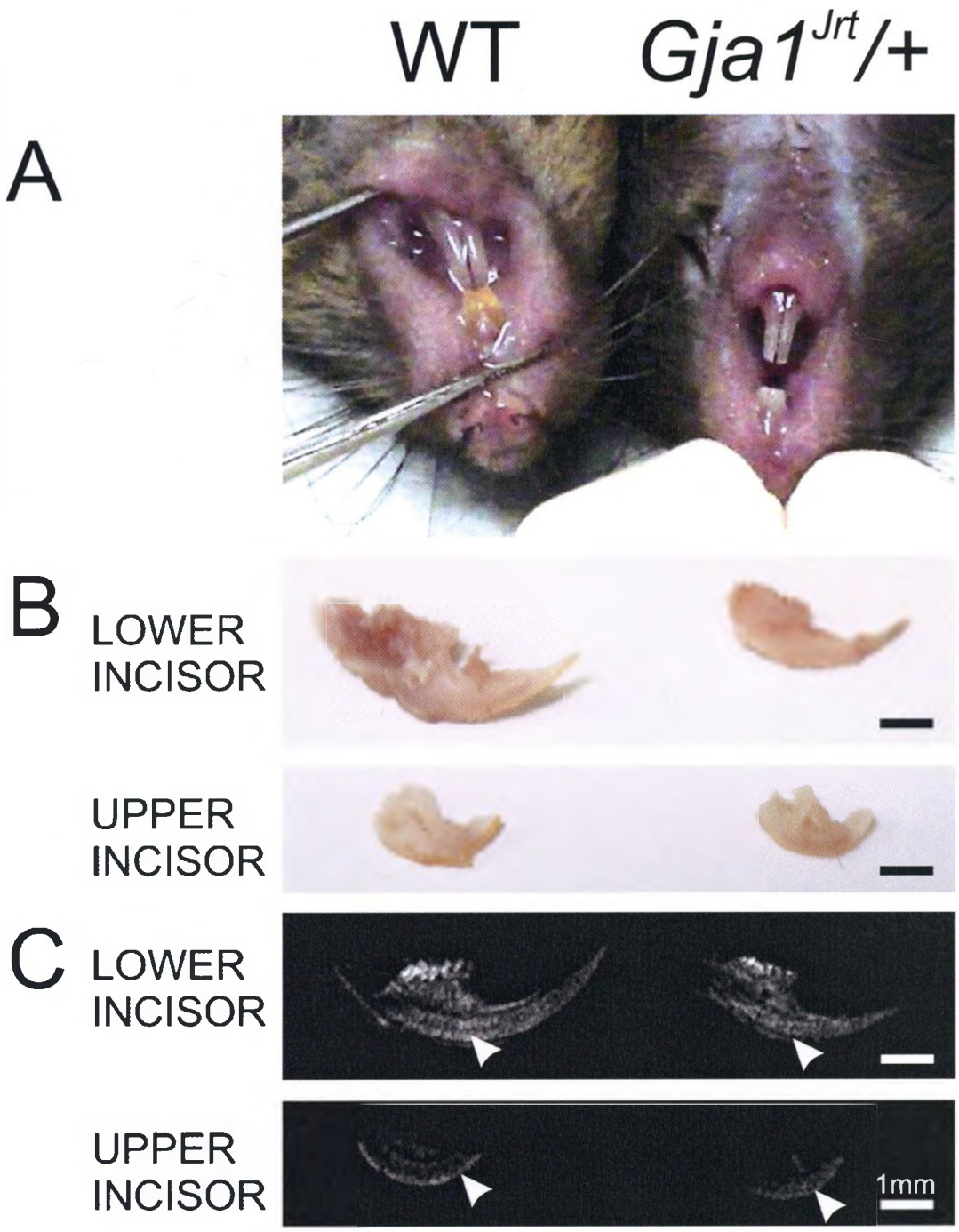
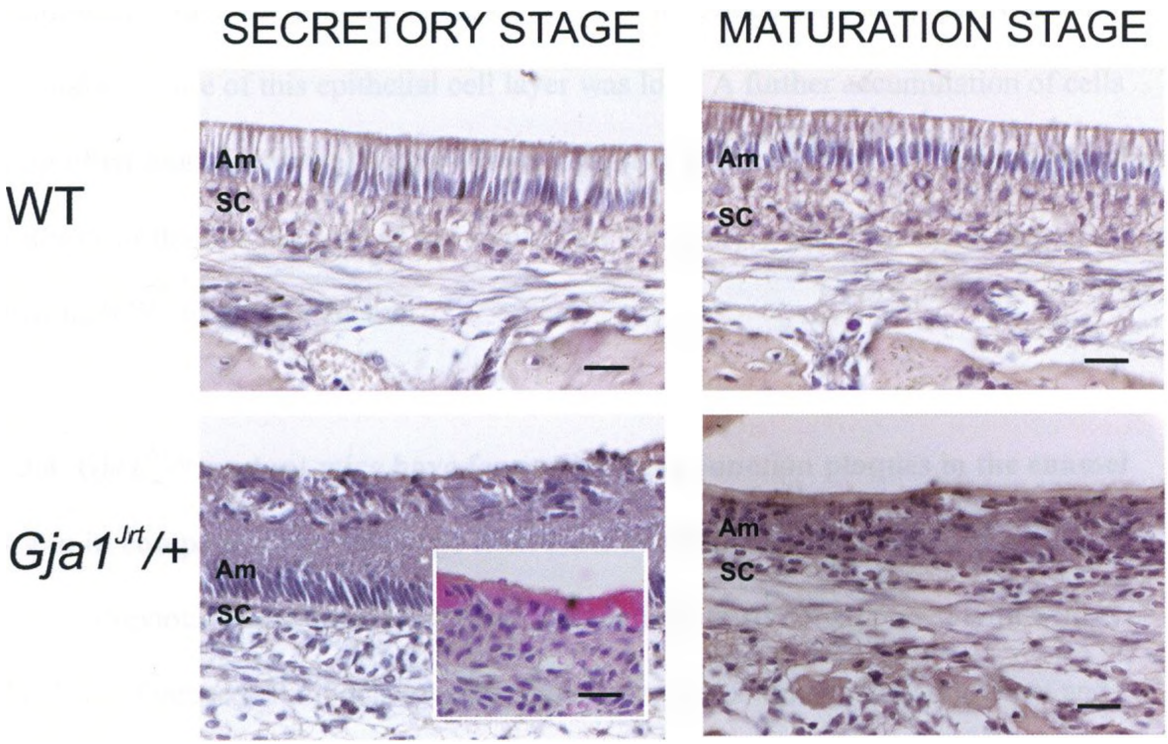

WT
Gja1Jrt/+
A
B
LOWER INCISOR
UPPER INCISOR
C
LOWER INCISOR
UPPER INCISOR
1mm

Figure 2.3 Enamel organ morphology was found to be severely altered in *Gja1*^{Jrt}/+ mice. Representative demineralized paraffin sections of the enamel organ are shown of 7d wild-type (upper panels) and *Gja1*^{Jrt}/+ mice (lower panels). Sections were stained with hematoxylin and eosin to denote the columnar ameloblasts (Am) between the supporting cells (SC) and the enamel at secretory and maturation stages of differentiation. Ameloblasts from *Gja1*^{Jrt}/+ mutant mice revealed an accumulation of eosinophilic-stained amorphous protein within the epithelial cell layer (insert) as well as disorganized cell shape and polarity, and additional misplaced cells between the ameloblasts and enamel matrix (~70% prevalence). Similar results were found for 21 day and 2 month sections (data not shown). Bars = 50μm.


SECRETORY STAGE
MATURATION STAGE
WT
Gja1Jrt/+
Am
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Am
SC
Am
SC
Am
SC

ameloblast layer from both the secretory and maturation regions of the enamel organ from *Gja1*^{Jrt}/+ mutant mice exhibited a massive accumulation of eosinophilic-stained amorphous protein material within the ameloblast cell layer (Figure 2.3, insert). In addition, ameloblasts from mutant mice revealed no evidence of cell polarity and the columnar profile of this epithelial cell layer was lost. A further accumulation of cells were often found between the ameloblast layer and enamel matrix. Thus, in mutant mice, it was clear that the integrity of the ameloblast was grossly disturbed which may result in their inability to deposit enamel.

2.3.4 *Gja1*^{Jrt}/+ mutant mice have fewer Cx43 gap junction plaques in the enamel organ in comparison to wild-type littermate controls

Previous studies where the G60S Cx43 mutant was co-expressed with wild-type Cx43 in reference cell models revealed that the mutant is functionally dominant and reduces GJIC well below 50% (McLachlan et al., 2005). Moreover, cardiomyocytes, and osteoblasts obtained from *Gja1*^{Jrt}/+ mice exhibited greatly reduced gap junction coupling, suggesting that the G60S mutant is also dominant to the co-expressed Cx43 in *Gja1*^{Jrt}/+ mice (Manias et al., 2008; McLachlan et al., 2008). In the enamel organ of mutant mice, the Cx43 localization pattern did not appear significantly different from wild-type mice (Figure 2.4A). However the total level of Cx43 protein in the enamel organ, as assessed by the prevalence of gap junction plaques, was decreased at all stages of ameloblast differentiation (Figure 2.4). Thus, the *Gja1*^{Jrt}/+ mouse is essentially a knock-down model of Cx43 in the enamel organ. However, while the level of total Cx43 gap junction plaque knock-down in the enamel organ was evident at all stages of

Figure 2.4 Cx43 plaques were less frequent in the *Gja1*^{Jrt}/+ mouse enamel organ compared to wild-type mice. (A-C) Cx43 gap junctions were frequently found in the enamel organ obtained from the lower incisors of 7d wild-type mice. Similar results were found for 21 day and 2 month sections (data not shown). Cx43 was most evident in the supporting cells including the stratum intermedium and stellate reticulum at the secretory stage and papillary cell layer at the maturation stage of differentiation. The frequency of total Cx43 gap junctions in the enamel organ of mutant mice was halved. Bars = 20μm. Quantification of Cx43 gap junctions per cell was delineated in both ameloblast and supporting cell layers at inductive (A), secretory (B), and maturation (C) stages of differentiation. Total Cx43 gap junction plaques were significantly reduced in incisors obtained from *Gja1*^{Jrt}/+ mice at all stages of differentiation compared to wild-type littermates. ($^{*}p < 0.05$; $^{**}p < 0.005$; $n \geq 6$ animals).

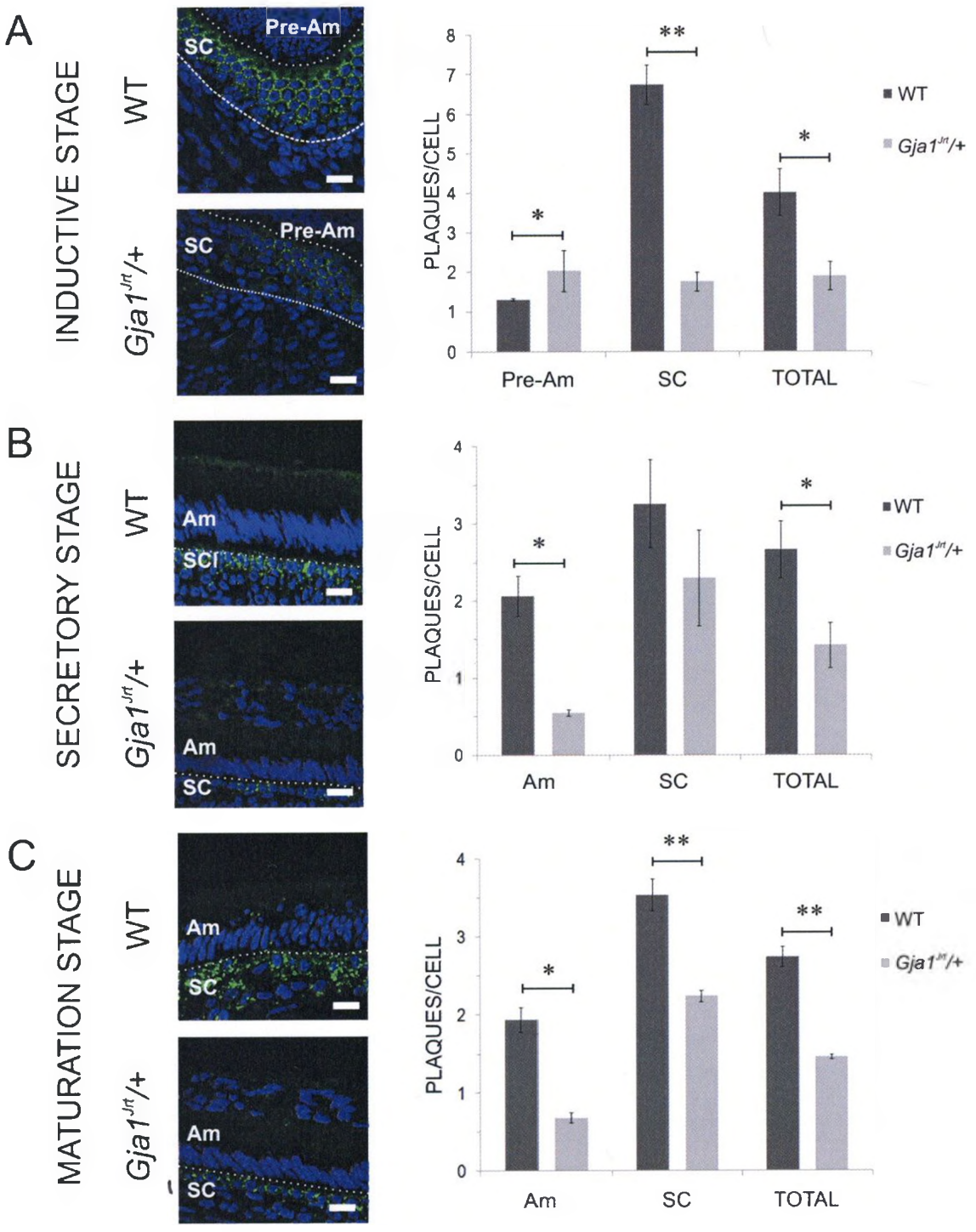
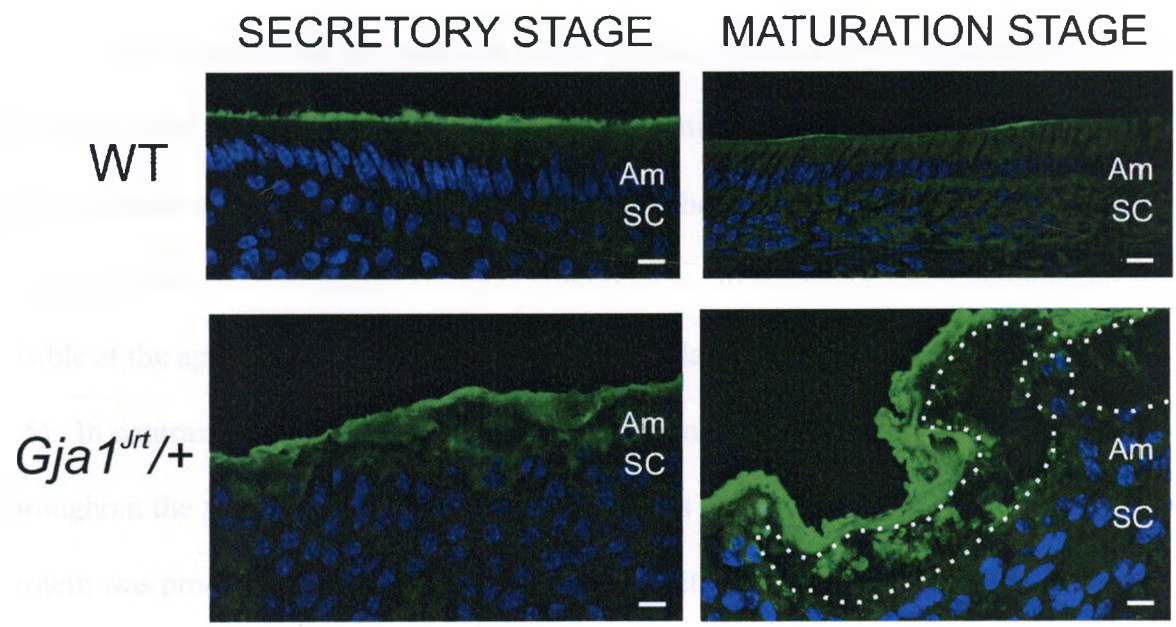

A
INDUCTIVE STAGE
WT
Gja1Jrt/+
Pre-Am
SC
PLAQUES/CELL
Pre-Am
SC
TOTAL
WT
Gja1Jrt/+
B
SECRETORY STAGE
WT
Gja1Jrt/+
Am
SC
PLAQUES/CELL
Am
SC
TOTAL
WT
Gja1Jrt/+
C
MATURATION STAGE
WT
Gja1Jrt/+
Am
SC
PLAQUES/CELL
Am
SC
TOTAL
WT
Gja1Jrt/+

Figure 2.5 Amelogenin production and localization is altered in *Gja1*^{Jrt}/+ mice. Seven day lower incisor demineralised sections were immunofluorescently labelled for the secretory protein amelogenin. Wild-type ameloblasts displayed low levels of amelogenin at the apical region of the ameloblast layer at the secretory stage and maturation stage. Amelogenin was found throughout the ameloblasts at the secretory phase and accumulated within and near ameoloblasts at the maturation stage in *Gja1*^{Jrt}/+ mice. Frequently, cysts were found between the ameloblast layer and maturing enamel (between dotted lines). Similar results were found for 21 day and 2 month sections (data not shown). Bars = 20μm.


SECRETORY STAGE
MATURATION STAGE
WT
Gja1Jrt/+
Am
SC
Am
SC
Am
SC
Am
SC

differentiation, this was not equally evident in all regions of the enamel organ (Figure 2.4).

2.3.5 Impaired cell differentiation in the enamel organ of *Gja1*^{Jrt}/+ mice

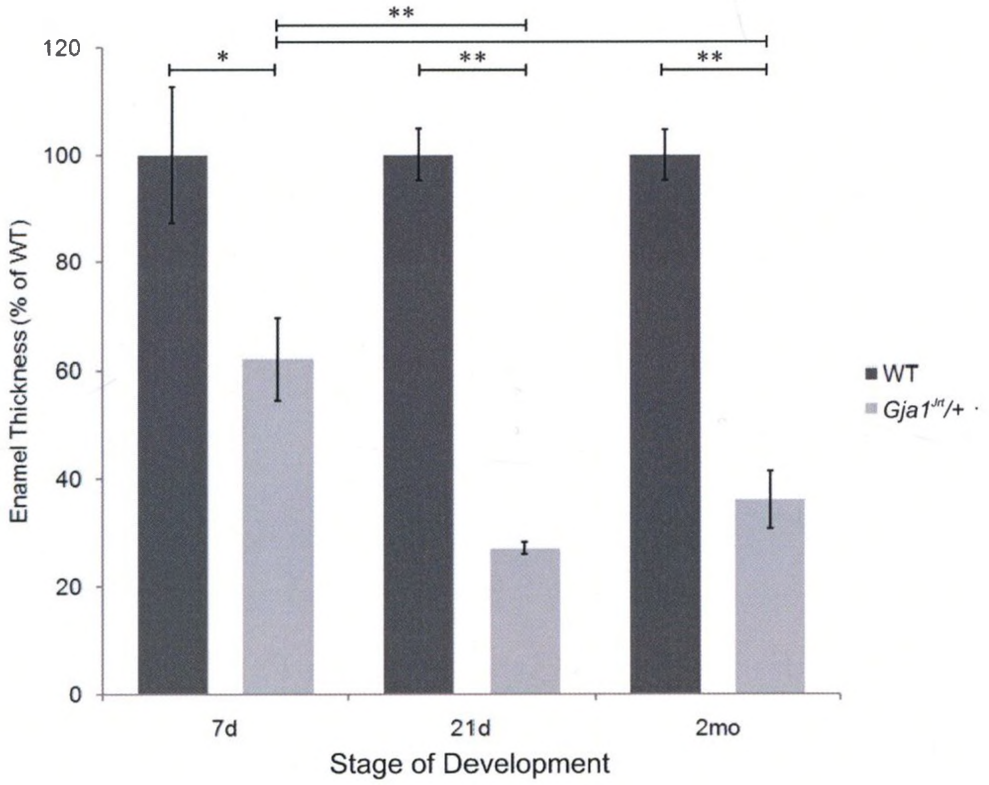
Amelogenin is an extracellular matrix protein produced by ameloblasts and thus, it is often used as an index of ameloblast differentiation (Hu et al., 2001). Under normal circumstances this protein is typically localized to the most apical regions of ameloblasts as it is secreted to become part of the enamel matrix. In wild-type mice, amelogenin was visible at the apical secreting surface of the ameloblast during enamel secretion (Figure 2.5). In contrast, in *Gja1*^{Jrt}/+ mice excessive amounts of amelogenin were localized throughout the ameloblast layer in secretory regions of differentiation suggesting that this protein was produced but may not be properly secreted. In addition, visible cysts between the ameloblast layer and developing enamel were observed in 80% of mutant incisors (Figure 2.5, between dotted lines) suggesting that contact with the enamel surface is lost and ameloblasts exhibit a defect in the mineralization of enamel.

2.3.6 Reduction of the enamel layer in *Gja1*^{Jrt}/+ mice

We sought to quantify the difference in enamel thickness through histological measures of the enamel layer at different stages of tooth maturation and aging. At 7 days postnatal, *Gja1*^{Jrt}/+ enamel thickness was less than wild-type incisors (Figure 2.6A). When enamel thickness was normalized to mouse body weight this trend remained the same (Figure 2.6B). This difference was amplified at 21 days and two months postnatal, after the teeth had erupted from the gum line. These data suggest that upon exposure to

Figure 2.6 Reduced lower incisor enamel thickness in the *Gja1*^{Jrt}/+ mouse compared to wild-type. (A) Enamel thickness was less in 7d, 21d and 2 month mutant mice compared to littermate controls. Enamel measures were taken longitudinally through the fully developed enamel layer of *Gja1*^{Jrt}/+ and wild-type mouse incisors and wild-type values were set to 100%. (B) When measures were normalized to the body weight of the mouse, enamel thickness of the *Gja1*^{Jrt}/+ mouse was less than wild-type at all stages of development. Bars represent average relative enamel thickness ±SEM. ($^{*}p < 0.05$; $^{**}p < 0.005$; $n \geq 7$ animals).

A


**
*
**
**
120
100
80
60
40
20
0
Enamel Thickness (% of WT)
7d
21d
2mo
Stage of Development
WT
Gja1Jrt/+

B

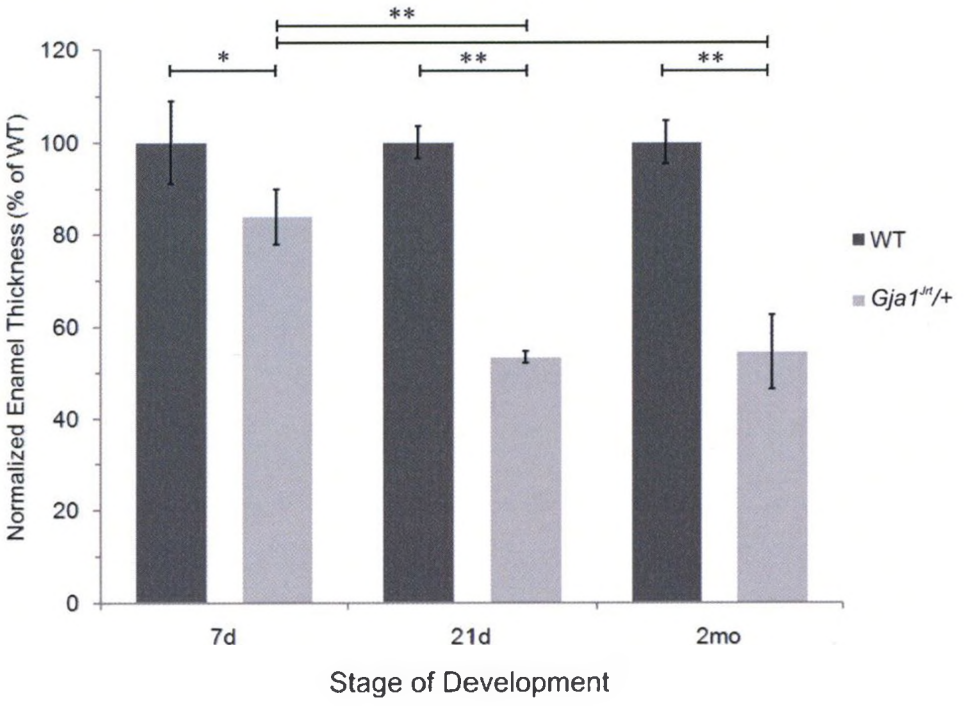
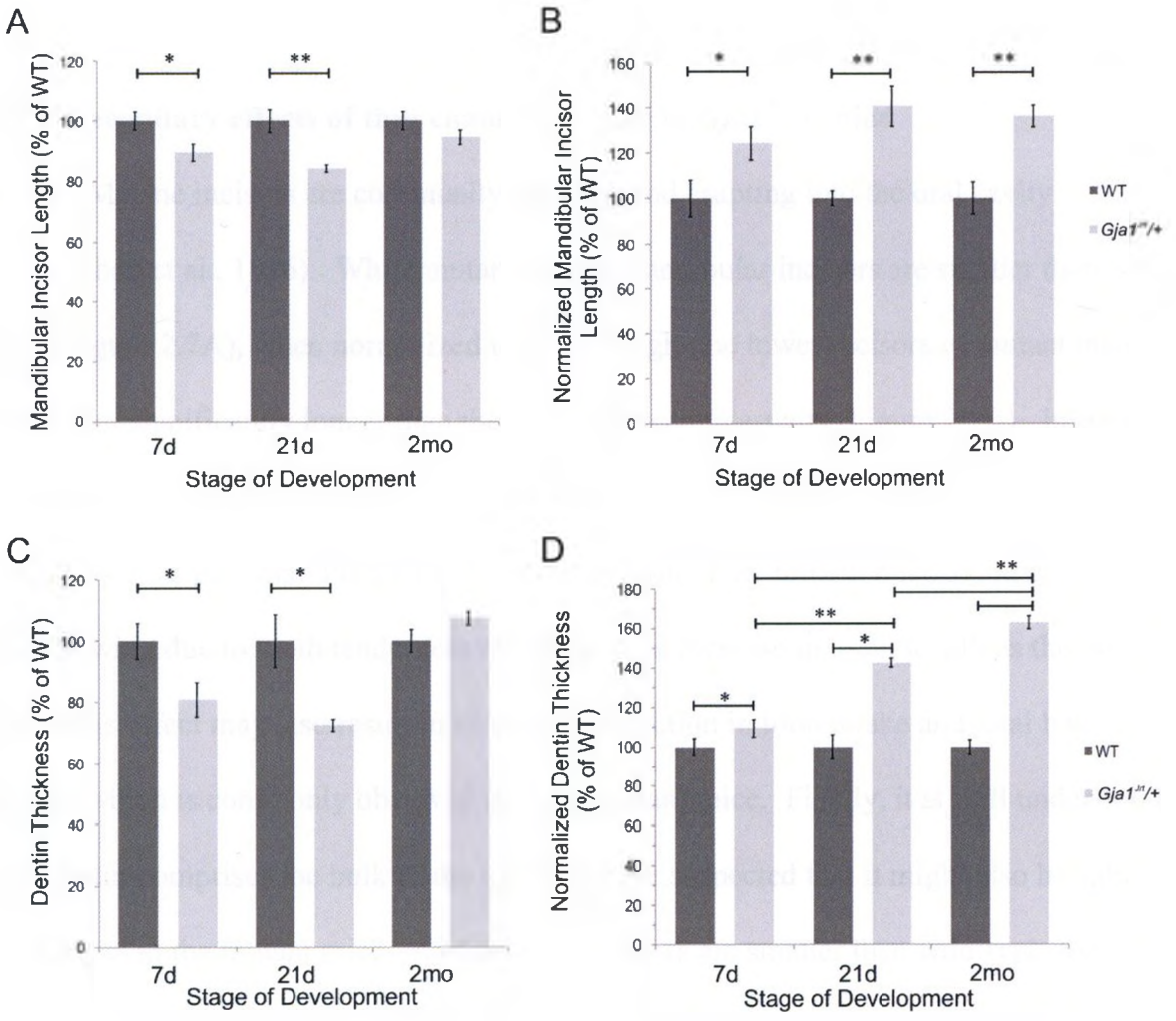

**
*
**
**
120
100
80
60
40
20
0
Normalized Enamel Thickness (% of WT)
7d
21d
2mo
Stage of Development
WT
Gja1Jrt/+

Figure 2.7 Secondary effects in the *Gja1*^{Jrt}/+ mouse lower incisors. (A) *Gja1*^{Jrt}/+ mouse mandibular incisor length was less than that found in wild-type mice. Incisor length measures were taken linearly from the cervical loop to the enamel tip of *Gja1*^{Jrt}/+ mice and wild-type incisors and wild-type values were set to 100%. (B) When normalized to the body weight of the mouse, the *Gja1*^{Jrt}/+ mouse mandibular incisor length was greater than that found in wild-type mice. Bars represent average relative lower incisor length ±SEM. ($^{*}p < 0.05$; $^{**}p < 0.005$; $n \geq 7$). (C) *Gja1*^{Jrt}/+ mouse lower incisor dentin thickness was less than wild-type mice. Dentin thickness measures were taken longitudinally through the dentin layer of *Gja1*^{Jrt}/+ and wild-type mouse incisors and wild-type values set to 100%. (D) When corrected for body weight, the *Gja1*^{Jrt}/+ mouse lower incisor dentin thickness was greater than wild-type mice. Bars represent average relative lower incisor dentin thickness ±SEM. ($^{*}p < 0.05$; $^{**}p < 0.005$; $n \geq 7$ animals).


A
Mandibular Incisor Length (% of WT)
120
100
80
60
40
20
0
*
**
7d
21d
2mo
Stage of Development
B
Normalized Mandibular Incisor Length (% of WT)
160
140
120
100
80
60
40
20
0
*
**
**
7d
21d
2mo
Stage of Development
WT
Gja1tm1/+
C
Dentin Thickness (% of WT)
120
100
80
60
40
20
0
*
*
7d
21d
2mo
Stage of Development
D
Normalized Dentin Thickness (% of WT)
180
160
140
120
100
80
60
40
20
0
*
**
**
*
**
7d
21d
2mo
Stage of Development
WT
Gja1tm1/+

the oral cavity, the enamel layer of the *Gja1*^{Jrt}/+ mouse incisors rapidly and prematurely degrades. Thus it seems likely that the integrity of the matrix secreted by the ameloblast has altered organic constituent and/or inferior mineralization.

2.3.7 Secondary effects of thin enamel are seen in *Gja1*^{Jrt}/+ mice

Murine incisors are continually growing and erupting into the oral cavity (Moinichen et al., 1996). While mutant mouse mandibular incisors are smaller than wild-type (Figure 2.7A), when normalized to body weight the lower incisors of mutant mice are in fact significantly longer than their wild-type counterparts (Figure 2.7B). In weaned littermate control mice, characteristic gnawing wears teeth down to maintain a consistent length. We suspect that due to the reduction in enamel the mutant mice avoid gnawing and chewing due to tooth tenderness resulting in an increase in tooth length as the mice age. This effect may also result in an overall reduction in food intake and total body weight which is commonly observed in these mutant mice. Finally, it is well understood that dentin comprises the bulk of the tooth, and we suspected that it might also be subject to changes in the mutant mice. As *Gja1*^{Jrt}/+ incisors are smaller than wild-type, the thickness of the dentin layer is less in these mice (Figure 2.7C). At all stages of development, the lower incisor dentin of the *Gja1*^{Jrt}/+ mice was thicker than wild-type when normalized to mouse body weight (Figure 2.7D).

2.4 Discussion

Normal tooth development requires highly coordinated events between the mesenchyme-derived odontoblasts and the ectoderm-derived ameloblasts (Bei, 2009).

The tooth phenotype of ODDD is apparent in both the primary and permanent dentition in almost all documented patients (Paznekas et al., 2009), suggesting that there is a developmental defect in the enamel layer. We hypothesized that this defect may be a result of abnormal protein deposition, poor mineralization and/or loss of enamel architecture as a consequence of loss-of-function mutations in Cx43. We chose to use a *Gja1*^{Jrt}/+ mutant mouse that harbors a G60S Cx43 mutant as a model of human ODDD. We compared enamel organ tissue from *Gja1*^{Jrt}/+ and wild-type littermates for connexin expression, enamel organ architecture, dentin thickness and state of differentiation. The enamel organ from wild-type mice displayed high levels of Cx43 in the supporting cells with more limited expression in ameloblasts. In the developed enamel organ of *Gja1*^{Jrt}/+ mice we observed considerably lower levels of Cx43 gap junction plaques. The morphology and architecture of the enamel organ from *Gja1*^{Jrt}/+ mice revealed a loss of cell polarity and organization with abnormal accumulation of amelogenin. Surprisingly, the teeth from mutant mice are not actually smaller when normalized to body weight but do have considerably less enamel and more dentin. Collectively, these studies reveal that the reduction of Cx43 gap junctions in the enamel organ has both direct consequences on enamel formation and indirect effects on dentin deposition and overall tooth constitution.

2.4.1 *Gja1*^{Jrt}/+ mice as a model of human ODDD dental problems

ODDD is an autosomal-dominant disease of high penetrance resulting in ocular, craniofacial and dental abnormalities (Paznekas et al., 2003; Paznekas et al., 2009). Genetic studies have revealed 62 mutations in the Cx43 gene which result in ODDD with varying degrees of severity. Most ODDD patients incur tooth abnormalities including

enamel hypoplasia, leading to cavities and early tooth loss, and microdontia (Paznekas et al., 2003). In order to examine the cells and mechanisms associated with these clinical symptoms we employed the *Gja1*^{Jrt}/+ mouse model which exhibits many of the symptoms of ODDD including tooth abnormalities (Flenniken et al., 2005). During the initial characterization of this mouse model, it was noted that the tooth defects of this mouse included an apparent enamel hypoplasia, and a smaller size of dentition (Flenniken et al., 2005). We sought to expand on the mechanisms of these initial findings here.

Importantly, the *Gja1*^{Jrt}/+ mouse shares genotype similarities with ODDD patients, with a 1:1 ratio of mutant to wild-type Cx43. The mouse has a single set of dentition where humans have a primary and permanent set, both of which are affected by ODDD (Debeer et al., 2005; Paznekas et al., 2009). Yet, amelogenesis is identical for both human and mouse dentition (Gibson et al., 2005). Thus, we believe that the *Gja1*^{Jrt}/+ mouse is a useful model to study the dental abnormalities of ODDD and also to evaluate the consequence of Cx43 knock-down on enamel organ development and function. In cell types from other *Gja1*^{Jrt}/+ mutant tissues, greatly reduced GJIC has been documented leading to various tissue abnormalities (Langlois et al., 2007; Manias et al., 2008; McLachlan et al., 2008). For example, previous studies investigating the consequence of mice harboring the G60S Cx43 mutant revealed impaired late-stage osteoblast differentiation (McLachlan et al., 2008) and impaired keratinocyte differentiation (Langlois et al., 2007) which is perhaps not surprising given the fact that Cx43 is expressed in over 35 distinct cell and tissue types (Laird, 2006). Here, we sought

to determine whether similar differentiation defects would be evident in the enamel organ of the *Gja1*^{Jrt}/+ mouse.

2.4.2 Connexin expression during amelogenesis

Previous studies have localized Cx43 to the embryonic rat enamel organ (Fried et al., 1996; Inai et al., 1997; Joao and Arana-Chavez, 2003). Specifically, immunolabeling studies localized Cx43 to the rat molar ameloblasts and stratum intermedium at a late stage of embryonic tooth development, with an increase in expression as differentiation proceeded (Joao and Arana-Chavez, 2003). Similar expression patterns were observed in rat upper incisor ameloblasts (Inai et al., 1997) while connexins in ameloblasts from mouse incisors have not been significantly studied (Yamada et al., 2007). It is rare for any cell type to express a single member of the connexin family as most tissues express two or more connexins (Laird, 2006). In the case of the enamel organ, a weak Cx32 signal was previously detected at the apical surface of embryonic rat molar preameloblasts (Fried et al., 1996), however a role for connexins at the enamel interface was not proposed and is not probable given the well documented role of connexins as gap junction proteins that function at intercellular interfaces. Cx32 has also been observed in the stratum intermedium during embryonic rat molar development, while other connexins such as Cx26 have not been detected in the enamel organ (Fried et al., 1996). In our studies, staining for the most likely candidates to be found in the ectoderm-derived enamel organ, Cx26, Cx32 and Cx43, revealed only Cx43 which may in fact be the only, or primary, connexin expressed in the differentiating mouse enamel organ. In particular, Cx43 was enriched in the supporting cells including the stratum intermedium, stellate

reticulum and papillary cell layers. The role of the supporting cells within the enamel organ remains poorly understood, but they have been proposed as regulators of ameloblast differentiation (Harada et al., 2006; Kawano et al., 2004; Satokata et al., 2000). Our data would support this position as reduced supporting cell levels of Cx43 were correlated with architectural and functional defects in ameloblast layers.

2.4.3 Decreased levels of Cx43 disrupt enamel organ differentiation and enamel development

ODDD enamel abnormalities could also be classified in a similar manner as amelogenesis imperfecta, which is defined as an enamel hypoplastic or hypomineralized disease caused by protein malfunctions in the enamel layer (Crawford et al., 2007). This condition causes teeth to be abnormally small, discolored, and prone to rapid wear and breakage (Crawford et al., 2007). Amelogenesis imperfecta has been linked to a variety of mutations in ameloblast genes encoding proteins that are critical for different aspects of enamel formation (Gibson et al., 2001; Hu et al., 2008; Lezot et al., 2008). Based on knock-out mouse studies, amelogenins play an important role in enamel mineralization (Gibson et al., 2001). In the $AMELX^{-/-}$ mouse, the enamel layer was thinner, while ameloblast differentiation remained normal (Gibson et al., 2005; Gibson et al., 2001; Hatakeyama et al., 2009). Ameloblastin (AMBN) serves as a cell adhesion molecule for ameloblasts (Fukumoto et al., 2004). In AMBN null mice, ameloblasts differentiate properly, but at the secretory stage the cells detach from the matrix surface and lose polarity (Fukumoto et al., 2004). In our studies, we looked at the regulation of the predominant protein of the enamel layer, amelogenin. Our results revealed dysregulated

amelogenin secretion and localization within the enamel layer in *Gja1*^{Jrt}/+ mice. Enamel hypoplasia also arises when there are defects in the secretion of the enamel layer while enamel hypomineralization occurs when there is a deficiency in the maturation stage of enamel development (Wright et al., 2009). Amelogenin secretion in the *Gja1*^{Jrt}/+ mouse incisor appeared dysregulated, with an abundance of amelogenin trapped in the ameloblast cells with lower than wild-type levels at the enamel interface. This situation could lead to an enamel hypoplasia, where the secreted enamel layer is thinner than normal as confirmed in mutant mice at 7 days postnatal, before tooth eruption.

During maturation of the enamel layer ameloblasts move calcium, phosphate, and bicarbonate ions into the matrix and remove water and cleaved proteins (Brookes et al., 2001). It appears as though this maturation phase is altered in *Gja1*^{Jrt}/+ mice and that full mineralization of the enamel layer is impaired. Thus, the architecture of the fully formed enamel layer would be expected to be softer with possibly more organic constituent than normal, and may be more prone to abrasion in the oral cavity. This is supported by the increased loss of enamel in older 21 day and 2 month mice when the teeth have been exposed to the oral cavity as compared to young animals. In the most extreme of cases involving an ENAM knock-out mouse the tooth is coated by a calcified material that cannot be classified as enamel, and is prone to severe abrasion and occlusal wear (Hu et al., 2008). Therefore, *Gja1*^{Jrt}/+ mice exhibit symptoms of both enamel hypoplasia and hypomineralization, which are ultimately linked to the dysregulation of amelogenesis at all stages of ameloblast differentiation.

Gja1^{Jrt}/+ mice are up to 50% smaller than their wild-type littermates. It remains unknown as to whether this size deficiency is due to a smaller bone stature of the mutant

mouse, or a lack of caloric intake. This deficiency seems unique to the mouse model, as such a severe defect has not been noted in the human ODDD population. In humans and primates, tooth size shows correlation to body weight, as found useful in anthropological studies (Anderson et al., 1977; Gingerich, 1977). In addition, enamel thickness has been correlated to the size of the tooth (Grine et al., 2005). As such, in order to account for the size difference of the mutant mouse compared to wild-type, we chose to normalize our measured parameters to the body weight of the mouse.

2.4.4 Secondary effects of enamel hypoplasia observed in the *Gja1*^{Jrt}/+ mouse

Several reports suggest that the loss of enamel leads to secondary effects in the surrounding tissue and in animal feeding behavior. Consistently, we determined that the lower incisors of *Gja1*^{Jrt}/+ mice were longer than their wild-type littermates when accounting for the body weight of the mouse. This finding was not reported in the original article describing the teeth of these mutant mice as no adjustment was made to account for the much smaller body size of the mutant mice (Paznekas et al., 2003). Mice have incisors which are normally continuously erupting at a constant rate and are regulated by characteristic gnawing behavior (Risnes et al., 1995). Due to a thin enamel layer in mutant mouse incisors, the mice may experience tooth sensitivity and fail to actively gnaw to regulate their incisor size. Interestingly, the *Gja1*^{Jrt}/+ mice had an aversion to chewing and were put on a powdered diet to stimulate caloric intake. Thus, tooth sensitivity may also be one of the contributing factors to the smaller size of the *Gja1*^{Jrt}/+ mice.

ODDD patients often display microdontia or a smaller tooth size (Paznekas et al., 2003). Since dentin is a porous mineralized tissue that underlies the enamel (Pashley and Carvalho, 1997) and comprises the bulk of the tooth we investigated its thickness in our mutant mice. Intriguingly, the thickness of dentin was greater in *Gja1*^{Jrt}/+ mice compared to wild-type and normalized to mouse body weight. This may be due to the well-characterized secondary dentin deposition that occurs in sensitive teeth (Pashley and Carvalho, 1997). It is documented that when the overlying enamel layer is lost, dentinal tubules establish direct contact between the nerve endings of the pulp and stresses presented in the oral cavity such as heat and pressure (Pashley and Carvalho, 1997). Upon odontoblast stress, a layer of dentin is deposited, the thickness of which is dependent on the intensity and duration of the stimulus (Pashley, 1990; Tziafas, 1994). Thus, in mutant mice where the enamel is thin we suspect that dentin deposition is activated leading to the increase in dentin thickness and overall increase in tooth size.

2.4.5 Significance

In the present study, we demonstrate the importance of a full complement of Cx43 in normal enamel organ differentiation and enamel development. Cx43 in the stratum intermedium and ameloblast layers allows the enamel organ to properly differentiate, secrete and mineralize enamel. Thus, through insights provided by the use of the *Gja1*^{Jrt}/+ mutant mouse, we show that decreased Cx43 gap junctions lead to enamel organ morphological defects and enamel hypoplasia. Understanding the role of gap junctions in normal enamel development is critical to elucidating their function in amelogenesis imperfecta and diseases such as ODDD.

2.5 Acknowledgements

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CHAPTER 3

Discussion

Cx43 is the most abundant gap junction protein in mammals. The expression of Cx43 is linked to the proper development and differentiation of many tissues (Cruciani and Mikalsen, 2006). Therefore, aberrant expression of Cx43 is implicated in morbidity and disease (Krutovskikh and Yamasaki, 2000; Laird, 2006). The overall goal of this thesis was to examine the role of Cx43 in enamel organ differentiation and disease. We chose to study the enamel organ as the human disease ODDD is linked to patients that harbor Cx43 mutations which ultimately lead to enamel dysfunction. In this section we will summarize some of our findings with respect to the role of Cx43 in tooth development as observed through the use of a mouse model of Cx43-linked disease and address possible future studies that could further assess the role of Cx43 in the tooth.

In the current study, we compared the enamel organ from *Gja1*^{Jrt}/+ and wild-type mice. The *Gja1*^{Jrt}/+ mice were used since they harbor an autosomal-dominant G60S mutant that rendered the total Cx43 functional level in these mice to between 15-40% as assessed in a number of tissues (Flenniken et al., 2005; McLachlan et al., 2005; Roscoe et al., 2005). Specifically, the connexin expression within the enamel organ, enamel organ architecture and state of differentiation, as well as dentin thickness and tooth size were compared. Characterization of the enamel organ connexin levels in wild-type mice revealed high levels of Cx43 in the stratum intermedium and papillary layer with more limited expression in ameloblasts. Cx43 appeared to be the predominant gap junction protein in the differentiating enamel organ, as other epithelial connexins were not detected in the enamel organ at the postnatal stages of development we used in our studies. In general, the enamel organ of *Gja1*^{Jrt}/+ mice displayed considerably lower

total levels of Cx43 gap junction plaques compared to wild-type. The enamel organ from *Gja1*^{Jrt}/+ mice revealed defects in cell morphology and architecture, with a loss of cell polarity and organization and an abnormal intracellular accumulation of amelogenin. The differentiation defects in the *Gja1*^{Jrt}/+ mouse led to a thin enamel layer, which was further depleted when the teeth were exposed to the oral cavity. While the mandibular incisors from mutant mice are smaller than their wild-type counterparts, when normalized to body weight these teeth were surprisingly longer than those found in wild-type mice, a finding attributed to a secondary effect of the thin enamel layer. The dentin layer of the *Gja1*^{Jrt}/+ mouse incisor is thicker than wild-type when normalized to body weight, revealing what may be a secondary dentin deposition in response to the stress of thin enamel. Collectively, these studies demonstrate that the reduction of Cx43 gap junctions in the *Gja1*^{Jrt}/+ enamel organ has both direct consequences on enamel organ differentiation and enamel formation and indirect effects on dentin and tooth structure.

3.1 Cell-cell contacts in the enamel organ

As gap junctions require cells to come in close contact to one another, junction formation requires proper adhesive interactions between cells. Thus, gap, tight, and adherens junctions exist in close relationship within epithelial cells (Cotrina et al., 2008; Laird, 2006). Cx43 is known to interact with several proteins, including cytoskeletal and anchoring proteins, such as cadherins, caveolin-1, and ZO-1 (Laird, 2006). Comparative microarray studies on wild-type and Cx43 ablated mice revealed the differential impact of Cx43 expression on other genes, with a portion of these dysregulated genes being related to cell junctions, adhesion, and extracellular matrix proteins (Iacobas et al., 2004).

Thus, looking at the effect of reduced levels of Cx43 gap junction plaques in *Gja1*^{Jrt}/+ mice on other junctional complexes may be of interest in future studies.

The structural proteins involved in cell-cell contact are of interest during tooth development. For example, N- P- and E-cadherins are expressed spaciotemporally in various parts of the enamel organ prior to amelogenesis (Fausser et al., 1998; Obara et al., 1998; Palacios et al., 1995). Cadherins are transmembrane glycoproteins which belong to the superfamily of calcium-dependent cell adhesion molecules of the adherens junction. Extracellular domains of cadherins are involved in homophilic cell-cell interactions, while the intracellular domain interacts with the cytoskeleton (Takeichi, 1995; Tsukita et al., 1992). The relationship between gap and adherens junctions has been well documented (Frenzel and Johnson, 1996; Jongen et al., 1991). Antibodies against E-cadherin and N-cadherin can alter the formation of gap junctions (Jongen et al., 1991; Meyer et al., 1992). Conversely, antibodies against Cx43 can also inhibit the assembly of adherens junctions (Meyer et al., 1992; Wei et al., 2005). In addition, Cx43 can complex with N-cadherin and the disruption of the expression of either protein of the complex affects the membrane localization of the other (Wei et al., 2005). It seems possible that reduced Cx43 gap junctions in the *Gja1*^{Jrt}/+ enamel organ could affect the localization and assembly of adherens junctions in this tissue, accounting for the loss of cell-cell adhesions and tissue integrity in the mutant enamel organ. Additionally, desmosomes and hemidesmosomes provide mechanical integrity to tissues, and might be involved in tissue remodelling during development (Garrod et al., 1996; Green and Jones, 1996). The desmosome protein desmoglein was detected at the basal pole of ameloblasts and between cells of the stratum intermedium during amelogenesis (Sasaki et al., 1984),

while the hemidesmosome protein BP-230 accumulates at the secretory pole of differentiating ameloblasts (Fausser et al., 1998). As we have shown a major loss of ameloblast cell morphology and integrity in the *Gja1*^{Jrt}/+ mouse enamel organ, perhaps cell adhesions are altered to account for the observed disruption in the tissue architecture. In particular, this may account for the abnormal accumulation of cells between the ameloblast cell layer and the enamel matrix in *Gja1*^{Jrt}/+ mouse enamel organ tissues.

Other epithelial junctional complexes known to be involved in amelogenesis are the tight junctions, which function as a barrier to regulate the paracellular transport of ions, small solutes, and water (Gumbiner, 1993). Tight junction proteins including claudins and occludins have been well documented in the enamel organ (Inai et al., 2008). It is thought that tight junctions in ameloblasts act to regulate paracellular permeability to create a microenvironment suitable for enamel deposition and matrix maturation (Inai et al., 2008). As there appears to be defects in both enamel matrix deposition and mineralization in the *Gja1*^{Jrt}/+ mouse, perhaps the integrity of the enamel microenvironment is affected by the reduced level of Cx43 and their association with tight junctions.

3.2 Murine models of tooth abnormalities

Human teeth are classified as incisor, canine, premolar, and molar, all varying in size and shape. All human deciduous teeth undergo replacement by the permanent dentition. The mouse has a comparatively reduced dentition, with three molars and one incisor in each quadrant (Tucker and Sharpe, 2004). In mice, replacement teeth are unnecessary in the incisors, as they are able to grow continuously due to a stem cell niche

located within the cervical loops at the apical part of the tooth (Harada and Ohshima, 2004). The unique feature of detecting all stages of enamel organ development in rodent incisors provides an excellent experimental model for examining amelogenesis in the Cx43 knock-down *Gja1*^{Jrt}/+ mouse model. However, the incisor may not be the best tooth to model the tooth size defect found in ODDD patients, as our data show that when normalized to size of mouse, the incisor of mutant mice are longer than their wild-type counterparts. However, the human phenotype of ODDD typically yields a smaller tooth size in patients with this disease (Paznekas et al., 2003; Paznekas et al., 2009).

The size of the tooth is a factor of the mesenchyme (Cai et al., 2007), which differentiates into odontoblasts to form the dentin of the tooth (Lisi et al., 2003). After development, the dentin layer will form the size and shape of the tooth. The shape of the three mouse molars are thought to be analogous to that of the back three molars of the human dentition. Each of the three mouse molars in all quadrants of the dentition develop similarly to the human tooth during a single developmental time frame. Further characterization of the *Gja1*^{Jrt}/+ mouse dentition should involve investigation into the development of the molars in this mutant mouse.

3.3 Conclusion

In summary, the results presented in this thesis suggest that Cx43 has an important role in enamel organ differentiation and formation of enamel. Cx43 likely plays a role in coordinating the differentiation of the ameloblasts involved in enamel matrix deposition and mineralization. Thus, the phenotype of ODDD patients with mutant forms of Cx43 results from enamel organ dysfunction and lack of appropriate

differentiation. Further studies need to be completed in order to determine the effect of Cx43 on junctional complexes resulting in enamel organ disorganization, as well as on other aspects of tooth development such as tooth size.

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