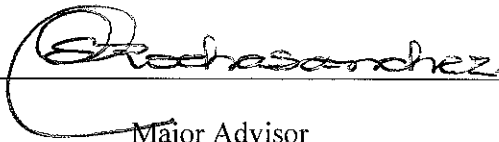


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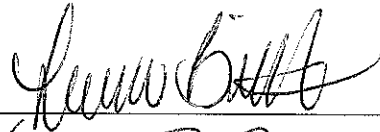
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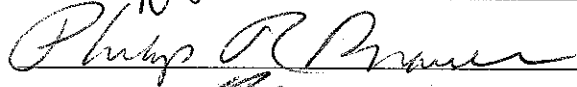
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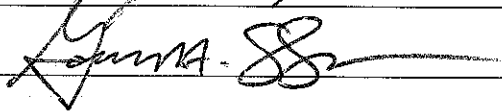
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The Role of MicroRNAs in Craniofacial Development

By

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A THESIS

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Abstract

Cleft palate and orofacial clefts are common congenital anomalies whose etiology results from deficient fusion of the bilateral processes of the first pharyngeal arch and/or the frontonasal process. Craniofacial clefting can result from genetic and teratogenic disruptions affecting the growth and development of the first pharyngeal arch. Many of the molecular pathways regulating normal and abnormal growth and development of the first arch have been studied; however, the role of microRNA (miRNA) in mammalian palatal development has not been elucidated. miRNA are a class of short 19-21 nt non-coding RNA whose function is in the repression and attenuation of translation of their target mRNA. The function of specific miRNA is known in many developmental events and disease processes, and they are estimated to regulate up to one third of human genes. We hypothesized that miRNA are essential to mammalian orofacial development, and as such, a disruption of miRNA biogenesis will disrupt established orofacial and palatal developmental processes. To test this hypothesis a conditional *Dicer* knockout mouse model was used. In this model, floxed *Dicer* alleles were knocked out in the first pharyngeal arch precursor tissues of the palate. Embryos lacking *Dicer* in their palatal precursors experienced craniofacial developmental defects that included several missing viscerocranial and chondrocranial bones and a complete cleft of the secondary palate. The developmental defects of the conditional *Dicer* knockout were similar to defects seen

as a result of aberrant cranial neural crest cell migration and differentiation. *Sox9* is a molecular marker of cranial neural crest cells and participates in their maintenance and ability to respond to chemotactic signals during migration. Investigation of *Sox9* expression, between E9.5 and E12.5, showed ectopic *Sox9* expression in the *Dicer* knockout embryos between E9.5 and E11.5, in the rhombencephalon and midbrain; and decreased *Sox9* expression in the maxillary process at E12.5, indicating that improper development or migration of cranial neural crest cells may be responsible for the viscerocranial malformations. These results demonstrate the importance of miRNA in mammalian palatogenesis and viscerocranial morphogenesis.

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Table of Contents

I.	INTRODUCTION	
A.	Hypothesis	1
B.	Introduction to microRNA	2
C.	Biogenesis of miRNA	3
D.	Mechanisms of miRNA function:	3
E.	Effects of inhibition of miRNA function	6
F.	Rate of miRNA turnover, and its effects on investigation of conditional <i>Dicer</i>	7
G.	miRNA in orofacial development	7
H.	Palatogenesis	9
I.	Early Development	10
J.	Induction of neural crest cells	10
K.	Rhombomere segmentation	11
L.	Hox gene patterning in facial development	12
M.	Directional cues during neural crest migration	13
N.	Molecular markers of first arch CNC	15

O.	Segmentation of CNC into mandible or maxillary process of first pharyngeal arch	16
P.	FGF signaling in palatogenesis	18
Q.	BMP signaling in palatogenesis	18
R.	Development of the palatal shelves	19
S.	miRNA in palatogenesis	20
T.	Epidemiology of craniofacial clefting	21
U.	The present study	23
V.	Summary	24
II.	MATERIALS AND METHODS	
A.	Animal care and handling	25
B.	Generation of conditional dicer knockout, timed pregnancy, and C-sections	25
C.	Genotyping	26
D.	Bone and cartilage staining	27
E.	Paraffin embedding and sectioning	28
F.	Hematoxylin and Eosin staining	28
G.	RNA extraction	29
H.	Riboprobe generation	30
I.	Quantitative PCR	31
J.	miRNA Quantitative PCR	32
K.	Whole mount ISH	33

III.	RESULTS	
A.	Palatal development in control and <i>Pax2Cre/Dicer^{LoxP}</i> littermates	35
B.	Bone and cartilage staining of E17.5 control and <i>Pax2Cre/Dicer^{LoxP}</i> littermates	40
C.	<i>Pax2</i> and <i>Cre</i> expression profile at E12.5	45
D.	Reciprocal expression patterns of <i>Cre</i> and <i>Dicer</i>	48
E.	Relative expression levels of <i>Bmp4</i> , <i>Fgf8</i> and <i>Fgf10</i> in E17.5 control and <i>Pax2Cre/Dicer^{LoxP}</i> palatal tissue	52
F.	Relative Quantification of select miRNAs	54
G.	Spatiotemporal expression of <i>Sox9</i> in control and <i>Pax2Cre/Dicer^{LoxP}</i> littermates.	57
III.	DISCUSSION	
A.	Overview	64
B.	Morphological consequence of <i>Dicer</i> deletion in developing orofacial tissue	64
C.	<i>Pax2Cre</i> expression in the viscerocranium	67
E.	Loss of miRNA mediated regulation	68
F.	Cre – mediated <i>Dicer</i> deletion	70
G.	The role of <i>Bmp4</i> , <i>Fgf8</i> , and <i>Fgf10</i> in <i>Pax2Cre/Dicer^{LoxP}</i> palatogenesis	72
H.	The role of CNC in <i>Pax2Cre/Dicer^{LoxP}</i> palatogenesis	73

IV.	CONCLUSION	78
VI.	CITATIONS	80

LIST OF FIGURES AND LEGENDS

Figure 1.	Biogenesis pathway of miRNA in WT and <i>Pax2Cre/Dicer^{LoxP}</i> embryos	4
Figure 2.	Relative sizes of PCR products of <i>Cre</i> and floxed <i>Dicer</i>	36
Figure 3.	Onset of palatal development in E11.5 control and <i>Pax2Cre/Dicer^{LoxP}</i> mice	37
Figure 4.	Coronal sections through palatal shelves of E12.5 control and <i>Pax2Cre/Dicer^{LoxP}</i>	38
Figure 5.	Coronal sections through the palate of E16.5 WT and <i>Pax2Cre/Dicer^{LoxP}</i> embryos	39

Figure 6.	Facial skeleton of E17.5 <i>Pax2Cre/Dicer^{LoxP}</i> and control embryos (lateral view)	42
Figure 7.	Hypoplastic growth of the mandible in <i>Pax2Cre/Dicer^{LoxP}</i>	43
Figure 8.	View of the maxillary process in E17.5 control and <i>Pax2Cre/dicer^{LoxP}</i> embryos.	44
Figure 9.	Expression pattern of <i>Pax2Cre</i> -driven LacZ	46
Figure 10.	Exogenous <i>Cre</i> expression overlaps with endogenous <i>Pax2</i> Expression in E12.5 palatal shelves	47
Figure 11.	<i>Cre</i> expression in <i>Pax2Cre/Dicer^{LoxP}</i> and <i>Pax2Cre/Dicer^{WT}</i> E12.5 embryo brains	49
Figure 12.	ISH using a probe against <i>Dicer</i> in E12.5 control and <i>Pax2Cre/Dicer^{LoxP}</i> brains.	50
Figure 13.	Decreased <i>Dicer</i> mRNA in E12.5 <i>Pax2Cre/Dicer^{LoxP}</i> palates	51
Figure 14.	Q-PCR comparing relative expression of <i>Bmp4</i> , <i>Fgf8</i> , <i>Fgf10</i> in control and <i>Pax2Cre/Dicer^{LoxP}</i> E17.5 embryos	53

Figure 15.	Relative quantification indicates a significant decrease in miRNA 101b, and 200b	55
Figure 16.	<i>Sox9</i> detection at E9.5 is similar in the first pharyngeal arch in Control and <i>Pax2Cre/Dicer^{LoxP}</i> littermates	58
Figure 17.	<i>Sox9</i> detection at E10.5 is similar between control and <i>Pax2Cre/Dicer^{LoxP}</i> littermates	59
Figure 18.	<i>Sox9</i> expression at E11.5.	60
Figure 19.	<i>Sox9</i> staining patterns in the brains and palates of <i>Pax2Cre/Dicer^{LoxP}</i> and control littermates	62

LIST OF ABBREVIATIONS

AGO2	argonaute
BMP	bone morphogenic protein
bp	base pair
BrdU	Bromodeoxyuridine
cDNA	complementary DNA
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsRNA	double stranded RNA
EF	elongation factor
Exp5	exportin 5
FGF	fibroblast growth factor
HIV	human immunodeficiency virus
ISH	In Situ Hybridization
KO	knockout
miRNA	MicroRNA

mRNA	messenger RNA
nt	nucleotide
OP	otic pit
P-Body	processing-body
PCR	polymerase chain reaction
Pre-miRNA	precursor microRNA
Pri-miRNA	primary microRNA
Pdgfr α A	platelet derived growth factor alpha A
Pdgf	platelet derived growth factor
PdgfA	platelet derived growth factor A
Q-PCR	quantitative polymerase chain reaction
RISC	RNA induced silencing complex
RNase	ribonuclease
RT-PCR	reverse transcription polymerase chain reaction
RNA	ribonucleic acid
Sdf1b	stromal cell derived factor 1b
UTR	untranslated region
WT	wild type

I. Introduction

A. Hypothesis

MicroRNA (miRNA) represents one component of the cellular machinery that regulates gene expression (Zamore and Haley 2005). They function by fine tuning the translation of their mRNA targets through RNA interference (RNAi) pathways (Hannon 2002). Many miRNA have been shown to play important roles in many developmental and disease processes, and are currently being investigated for potential therapeutic uses (Kim 2005; Wang and Wu 2009; Stenrang and Kauppinen 2008). The role of miRNA in orofacial development has scarcely been studied, and the studies are limited to zebrafish, whose orofacial development differs from mammalian development (Eberhart et al. 2008). My hypothesis was: “Conditional mutagenesis of *Dicer* in cranial neural crest cells will disrupt mammalian orofacial and palatal development through disruption of miRNA mediated regulation of gene expression.” To test this hypothesis *Pax2Cre/Dicer^{LoxP}* transgenic mice, in which floxed *Dicer* alleles are knocked out following *Pax2* directed *Cre* expression, were analyzed. This study was organized into the following aims: I. Characterization of the orofacial development of the conditional *Pax2Cre/Dicer^{LoxP}* knockout mouse during palatogenesis II. Initial assessment of the effects of microRNA depletion on skeletogenic cranial neural crest cells through analysis of *Sox9* – a molecular marker of a subset of differentiating cranial neural crest cells that populate the face.

B. Introduction to microRNA

MicroRNA (miRNA) belongs to a class of RNA molecules called non-coding RNA. miRNA have diverse functions in cells ranging from conservation of chromosomal architecture to maintenance of the stem cell undifferentiated state to roles in cancer biology (Zhang et al. 2009; Livingston and Rajewsky et al. 2005). They are short, ~ 21 nucleotide RNA transcripts that function within the RNA interference (RNAi) pathway to attenuate gene expression (Hannon 2002).

The first known miRNA, called *lin-4*, was discovered in 1993, by Ambros *et al.* They found that expression of a very short RNA transcript (*lin-4*) correlated with down regulation of Lin-14, a protein whose mRNA transcript had several antisense complementary sequences to *lin-4*. The presence of antisense complementary sequences suggested that *lin-4* might regulate translation of Lin-14 through RNA-RNA interactions (Ambros et al. 1993). With their initial discovery and using intuitive reasoning, Ambros *et al.* predicted the now known miRNA biogenesis pathway. For years their discovery remained relatively obscure and prior to the year 2000 no miRNA function had been found in mammalian biology (Harfe et al. 2006).

In the 21st century, the field of non-coding RNA research has rapidly progressed. miRNA are now believed to play a role in virtually all physiologic processes and in humans, Bartel et al. (2007) have estimated that at least 5,000 genes are likely targets of miRNA, based on bioinformatics.

C. Biogenesis of miRNA

Biogenesis of miRNA occurs in several steps, and takes place in both the nucleus and the cytoplasm (Figure 1). First, in the nucleus, primary transcripts known as pri-miRNA, are predominately transcribed by RNA Polymerase II, but can also be transcribed by RNA polymerase III (Davidson et al. 2006; Shomron and Levy 2009). These pri-miRNA transcripts are recognized by an RNase III like enzyme called Drosha, which, along with its partner DCGR8, cleave pri-miRNA into 60 – 80 bp precursor miRNA, known as pre-miRNA (Kim et al. 2006; Soifer et al. 2008). Pre-miRNA transcripts contain a hairpin, and a two nucleotide 3' single stranded RNA (ssRNA) overhang. The two nucleotide 3' ssRNA overhang is recognized by the nuclear protein Exportin5 (Exp5) which translocates the pre-miRNA out of the nucleus and into the cytoplasm. Once in the cytoplasm, another RNase III like enzyme, Dicer, cleaves the pre-miRNA hairpin into a 19-21 bp double stranded miRNA duplex. The miRNA duplex is loaded onto the Argonaute (AGO2) protein while the antisense strand of the miRNA duplex is removed, leaving an AGO2-ssRNA complex known as the RNA Induced Silencing Complex (RISC) which functions to guide translational repression.

D. Mechanisms of miRNA function

The basis of miRNA function is homologous binding of a region of the miRNA, known as the “seed sequence,” with the antisense complement of a target mRNA within its 3' untranslated region (UTR) (Bartel et al. 2003; Bartel et al. 2007). Within each 21 nt miRNA there is a 6 to 8 base pair seed sequence located at the 5' end of the miRNA, which is critical for miRNA mediated mRNA targeting (Bartel et al. 2003). The seed

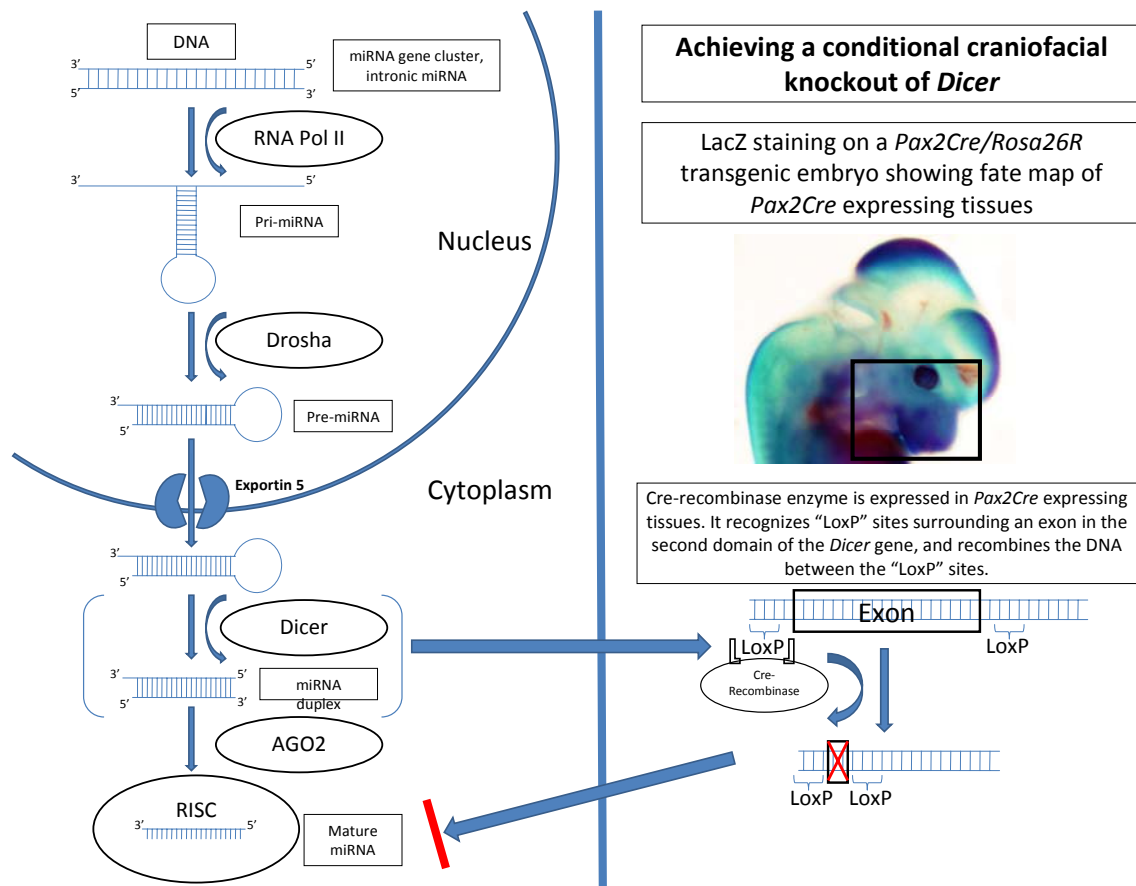


Figure 1. Biogenesis pathway of miRNA in control littermate and conditional *Dicer*

knock out. In normal miRNA biogenesis the RNaseIII enzyme Dicer participates in the final step of miRNA biogenesis pathway by cleaving the pre-miRNA hairpin to produce a miRNA/miRNA* duplex of 19-21 bp. In the conditional knockout mice, floxed *Dicer* alleles are permanently knocked out in all *Pax2Cre* expressing tissues, inhibiting their production of mature miRNA once residual *Dicer* mRNA and Dicer enzyme are depleted.

sequence comprises nucleotides 2-8 when counting from the 5' end (Joshua-Tor et al. 2004; Pillai et al. 2007). The context of the miRNA – 3'UTR mRNA interaction site, and the number of complementary base pairs binding between them, helps to determine the downstream effect their complexation has (Bartel et al. 2007).

miRNA function is accomplished through four similar, but distinct mechanisms; co-translational repression, degradation of mRNA transcripts, repression of translation initiation, and localization of target mRNA to processing-bodies (P-bodies), which are localized sites of mRNA degradation (Parker et al. 2005; Izaurralde et al. 2008). In the literature, there exists some debate over the exact functionality of P-Bodies and whether they can be used to store mRNA for later use, or if mRNA is immediately degraded once localized to a P-body (Parker et al. 2005; Pillai et al. 2007).

Studies in mammalian cell cultures have provided evidence for co-translational repression (Izaurralde et al. 2008), wherein actively translating mRNA targets of miRNA are found in the presence of the miRNA, but no completed protein product is detected (Izaurralde et al. 2008). In co-translational repression, the mRNA begins to be translated, but gets interrupted by the miRNA/RISC complex and the functional protein is never completed or detected. In this function of miRNA, the expression of a given miRNA has an inverse correlation with the expression of its mRNA target (Izaurralde et al. 2006; Izaurralde et al. 2008). Direct cleavage of an mRNA target can be directed by miRNA when they are loaded into a RISC complex; miRNA can direct a cleavage of their mRNA target at a site on the mRNA strand, located between nucleotides 10 and 11 of the miRNA, when counted from the 5' end of the miRNA (Zamore and Haley 2005). This

cleavage is accomplished by an RNase domain on the Argonaut2 protein (Joshua-Tor et al. 2004).

E. Effects of inhibition of miRNA function

In genetic and molecular based pharmaco-therapeutics, miRNA are being investigated both as mediators and targets for treatment of disease. Targeting of miRNA to attenuate their function can be accomplished through anti-sense complementary binding using anti-miRNA oligonucleotides. Targeting of miRNA-oncogenes, such as miRNA-24, could lead to new treatments for cancers, or cancer drug resistance (Zhang et al. 2009; Mishra 2009). When miRNA function is attenuated, such as with a mutation within the seed sequence of the miRNA, or in the target sequence on the mRNA 3' UTR, there can be multiple consequences relating to the function of that target within the cell or tissues' regulatory networks. The primary effect of attenuation of miRNA function may be small, resulting in small changes in its target mRNA concentration and protein concentration, but secondary effects may result in a large phenotypic response if the miRNA target is involved in a sensitive regulatory or genetic pathway (Soukup and Weston 2009). This has been illustrated by a single nucleotide mutation in the seed region of miR-96 and the resulting inner ear phenotype and progressive hearing loss in mice (Soukup and Weston 2009; Moreno-Pelayo et al. 2009). Mutation of a single nucleotide in the seed region of miR-96 causes an alteration of the seed sequence which directs miR-96 to its targets; resulting in a primary loss of its ability to regulate its targets, and a secondary disruption of downstream signaling molecules associated with the target of miR-96 (Moreno-Pelayo et al. 2009).

F. Rate of miRNA turnover and its effects on investigation of conditional *Dicer* deletion

Just like other RNA molecules, miRNA experience a rate of turnover related to the current functions of the cell or tissue in which they are expressed. Active degradation through exoribonuclease activity may be one pathway for modification of miRNA accumulation and activity (Grosshans and Chatterjee 2009). In conditional *Dicer* knockout mice, the extent of their phenotypic response is known to be a function of the residual level of miRNAs already present in those tissues (Soukup et al. 2009). The residual level of miRNA following conditional *Dicer* deletion may be a result of many factors including the rate of miRNA degradation, the rate of *Dicer* mRNA degradation, and the rate of Dicer protein turnover. Variability in the rate of degradation of residual miRNA, *Dicer* mRNA, and Dicer protein has been seen in other transgenic models using the *Cre/LoxP* system for *Dicer* deletion (Wang et al. 2008). This variability can make the outcome of conditional *Dicer* deletion difficult to interpret without quantifying the residual products. This can be further complicated when conditional deletion occurs during development which has a variable effect on protein, mRNA, and miRNA metabolism.

G. miRNA in orofacial development

Currently in the literature there is a single study suggesting that miRNA have a role in palatogenesis (Eberhart et al. 2008). The field of miRNA biology within orofacial and palatal development is in its infancy, and outside the single article, there are many

questions still to be answered. Given the broad role of miRNA in other systems, it is easy to imagine that many, if not all, the developmental processes involved in palatogenesis have some influence resulting from miRNA mediated regulation of protein expression.

In 2008, in zebrafish, miRNA miR-140 was shown to regulate and attenuate the expression of *Pdgfra* α , which is a receptor located on cranial neural crest (CNC) cells, for the chemoattractant *Pdgfaa* ligand, released from the optic stalk during CNC migration. Over expression of miR-140 in CNC resulted in a cleft primary palate, attributed to failure of down regulation of the receptor *Pdgfra* α , and the resulting accumulation of CNC at the optic stalk. Although this study was groundbreaking, the results are not readily extended to human development since zebrafish do not have a secondary palate (Eberhart et al. 2008).

To investigate the role of miRNA in mammalian palatogenesis and orofacial development, we analyzed a conditional *Dicer* knockout mouse, where *Dicer* is conditionally knocked out using the Cre-LoxP system. In our transgenic model, floxed *Dicer* alleles are knocked out through Cre – mediated recombination, following *Pax2Cre* expression (Harfe et al. 2005; Ohyama and Groves 2004). Cre is a type I topoisomerase that recognizes and recombines DNA between *LoxP* sites, which is a form of site directed mutagenesis (Hoess 1986). In the *Pax2Cre/Dicer*^{LoxP} mouse model, *LoxP* sites are flanking an exon within the *Dicer* gene, so that when *Cre* is expressed, *Dicer* is knocked out.

The *Pax2Cre/Dicer*^{LoxP} mouse model shows that *Dicer* is necessary for mammalian palatogenesis. Considering the important role that miRNA play in many

developmental processes, it will be vital to understand the role that miRNA play in palatogenesis and orofacial development. As medicine becomes more personalized to the specific genetics of individuals, treatments of disorders of the first pharyngeal arch, such as a cleft lip or cleft palate, will depend on intimate knowledge of the genesis of those tissues, of which, miRNA may play a vital role.

H. Palatogenesis

Palatogenesis is a highly regulated and coordinated process that occurs within a dynamic framework during development. Many developmental events, signaling centers, and signaling molecules have been elucidated both in normal and dimorphic developmental patterns and to date over 300 syndromic disorders have been described in which a cleft of the palate is a clinical feature (Von den Hoff et al. 2009). Mutations in many different genes such as *Tgf β 's*, *Bmp's*, and *Fgf's* have been associated with orofacial dimorphisms (Ferguson et al. 1999; Neubuster et al. 2000; Chen et al. 2002; Chai et al. 2003; Nie et al.2006); however, in mammalian models, discussion or implication of miRNA's role in palatogenesis is absent from the literature. Considering the precision of the developmental sequence of events during palatogenesis, it is easy to imagine that miRNA play an important role in regulation of the gene expression patterns and timing. To assess what potential roles miRNA may play in palatogenesis, it is necessary to understand the details of the process, which spans from gastrulation to late embryogenesis. The following is a summary of palatogenesis and the role of CNC, as well as the role miRNA may play in mediating the CNC contribution.

I. Early development

The mammalian palate develops from contributions from both the frontonasal process and the first pharyngeal arch. Mammals have two distinct regions of the palate, known as the primary and secondary palate (Ferguson 1988). The primary palate develops from the frontonasal prominence, while the secondary palate develops from a portion of the first pharyngeal arch (Ferguson 1988). One of the early developmental events that is crucial for palatogenesis and orofacial development may begin as early as gastrulation, as shown in chick, with the induction of neural crest cells (Mayor et al. 2009). In mice, specific palatogenic events do not finish until approximately E16.5, with the degradation of the midline seam following palatal shelf fusion, indicating a wide range of developmental time over which palatogenesis occurs (Trainor et al. 2003; Ferguson 1988).

J. Induction of neural crest cells

The first critical event during palatogenesis is the induction, specification, and differentiation of CNC cells (Krumlauf and Trainor 2002) which occurs during gastrulation in chick. CNC are specified at the border of the epidermal ectoderm and the neural ectoderm (Trainor et al. 2003). Cytokine signals, including the Wnt and BMP families, converge at the border of the epidermal ectoderm and the neural ectoderm. The unique combination of Wnt and BMP signals helps to specify the cells at the border to take on a neural crest lineage (Mayor et al. 2009; Krumlauf and Trainor 2002).

Current evidence suggests that during CNC induction, BMP signals from the mesoderm provide competency to the prospective CNC, so they may receive and respond

to Wnt signals for specification (Raible 2006). In the literature there is ongoing debate as to the exact timing of these signals, and whether it is a multistep or a temporally dynamic process (Basch et al. 2006; Mayor et al. 2009). Recent evidence suggests that there are two temporally and molecularly different steps in which the prospective neural crest cells are induced during the gastrula stage; first through Wnt activation and BMP inhibition, then during the neurula stage, cells are specified and maintained through activation of both pathways (Mayor et al. 2009). Specification of CNC involves transition of the cell's phenotype and delamination and onset of migration is characterized by morphological changes as they transition from an epidermal ectoderm to a mesenchymal phenotype.

K. Rhombomere segmentation

Immediately prior to CNC migration, the rhombencephalon becomes transiently segmented into eight rhombomeres which are formed by condensation of mesenchymal cells through differential cell adhesion molecule expression, which may be orchestrated by differential nested expression patterns of *Hox* genes (Tanyhill 2008; Tumpel et al 2009). In mice, palatogenic CNC migrate in two separate subectodermal streams, originating laterally to the first, second and fourth rhombomeres from the area of the prospective midbrain/hindbrain boundary (Serbedzija 1992; Trainor 2003). These CNC migrate into, and populate the mesenchyme of the first two pharyngeal arches, which contribute to the mesenchymal condensations that form the skeletal elements of the face and palate.

DiI is a lipophilic, hydrophobic fluorescent dye that can be focally injected and allowed to incorporate into the plasma membrane of cells, where it remains until it is

diluted following cell divisions. Injections of DiI into the rhombencephalon indicate that neural crest cells that originate laterally to odd-numbered rhombomeres may encounter inhibitory signals that prevent them from migrating into the adjacent mesenchyme (Serbedzija et al. 1992). They instead must migrate ventrally or dorsally into the closest even numbered rhombomere to join other streams (Krumlauf et al. 2002).

L. Hox gene patterning in facial development

Rhombomeres provide the first segmental organization and fate specification of cranial neural crest cells. A part of their contribution to CNC segmental organization is through their *Hox* gene expression pattern, which correlates with the axial level of the CNC origin and provides a unique identity to the neural crest at that axial level.

Segmentation of the CNC represents the first step in the patterning of facial structures (Couly 2002). The CNC that migrate into the first pharyngeal arch are *Hox* negative, which may permit them to uniquely respond to regionally specific patterning cues after they have finished their migration (Couly 2002; Kurihara 2008).

In chick, forced, exogenous expression of *Hox* genes in the CNC of the first pharyngeal arch results in complete absence of a facial skeleton (Creuzet 2005). The CNC that migrate to the second pharyngeal arch all express *HoxA2*. Transgenic knockouts of *HoxA2* results in *Hox* negative CNC in the second pharyngeal arch. These ectopic *Hox* negative CNC fail to differentiate or develop properly, and follow the same differentiation program as their *Hox* null first arch counterparts, forming an ectopic lower jaw (Couly et al. 2002). These studies on the role of *Hox* gene identity of palatogenic CNC reveals how important *Hox* identity is for downstream developmental events.

Experiments by Rijli et al. (2005) have suggested the importance of maintenance of the *Hox* code for CNC determination, and just as importantly, they revealed a high degree of plasticity in post-migratory CNC. In mice, CNC retain their plasticity until they have finished their migration, which was demonstrated when Rijli et al. deleted *HoxA2* in post-migratory CNC, and found that they changed their cell fate (Rijli et al. 2005). Post-migratory CNC rapidly proliferate and differentiate into their fated structures, but loss of *Hox* identity can alter their differentiation pathway, possibly by allowing them to respond to different sets of differentiation cues (Kurihara et al. 2008). Proliferation and differentiation occurs under the influence of both intrinsic properties such as *Hox* gene identity, and through local influences and differentiation cues such as regionally specific signals from the ectoderm or mesenchyme (Brugman *et al.* 2006).

M. Directional cues during neural crest migration

During their migration, CNC cells must receive directional cues. A common scheme in development is for migrating cells to follow a chemotactic gradient created by a localized release of chemotactic factors that diffuse and establish a concentration gradient (Tannyhill 2008). In zebrafish, at least two such chemotactic factors are known to help to regulate the migration of CNC into the first pharyngeal arch.

Platelet derived growth factor alpha A ($\text{Pdgf } \alpha \text{ A}$) and stromal cell derived factor 1b (Sdf1b) are chemotactic proteins that are released from the optic stalk and the first pharyngeal arch endoderm respectively (Eberhart et al 2008; Olesnicky et al. 2009). They have been shown to provide directional cues during migration of CNC (Eberhart et al. 2008; Killian et al. 2009).

CNC express the receptor *Pdgfra*, and the expression of this receptor is *Sox9* dependent (Wegner et al. 2008). Eberhart et al. (2008) found that in zebrafish, microRNA miR-140, temporally regulates the CNC expression of the receptor *Pdgf α A*. During migration, the *Pdgf α A* ligand, *Pdgfa*, is released from the optic stalk to guide CNC during migration. Eberhart et al. (2008) demonstrated that expression of miR-140 in CNC was able to knock down expression of *Pdgf α A*, as the CNC reached the optic stalk, attenuating their receptivity to the *Pdgfa* ligand. They demonstrated that miR-140 could mediate the fine tuning of a cell's response to chemoattractant gradients. Eberhart's discovery was the first example of the regulation of CNC migration and palatogenesis being dependent on miRNA expression (Eberhart et al. 2008).

As CNC migrate past the optic stalk they express the receptor, *Cxcr4a*, whose ligand is *Sdf1b*. *Sdf1b* is released from the pharyngeal pouch endoderm during CNC migration, and it assists in directing condensation of CNC into the first arch mesenchyme. Loss of *Cxcr4a* results in aberrant CNC migration and defects in neurocranium morphogenesis (Olesnicki et al. 2009).

Early segmentation of the CNC cells results in the first regional patterning cues for downstream developmental processes. Evidence suggests that this early molecular segmentation of neural crest cells may specify, or limit the structures that they are able to form (Hunt et al. 1991; Iseki et al. 2008). The CNC that migrate into the first pharyngeal arch proliferate and differentiate according to local cues that may be provided by the ectoderm, mesoderm, and the transient endoderm (Trainor et al. 2001; Gritli-Linde 2007). As CNC cells receive cues from their neighboring tissues, they are induced to express a diverse set of transcription factors, the specific combination of which gives an identity to

the cells (Jeong 2008). Once inside the first arch, the CNC receive signals identifying their location along all three axis, guiding their differentiation.

N. Molecular markers of first arch CNC

During their migration CNC can express molecular markers that indicate their future downstream differentiation pathways. Wegner et al. (2008) have demonstrated that *Sox9* and the closely related *Sox10* are responsible for transcriptional regulation of *Pdgfra* which encodes a receptor for the platelet derived growth factor (PDGF) ligand. *Sox9* influences chondrogenesis, male sex determination, and neural crest development (Brugmann et al. 2006; Crombrughe et al. 2009; Bi et al. 2001). *Sox9* is expressed in chondrogenic precursor neural crest cells, and *Sox10* is a survival factor for neural crest cells (Bi et al. 2001; Mori-Akiyama et al. 2003; Brugmann et al. 2006).

Sox9 is a critical factor in endochondral bone formation, which is a process by which mesenchymal cells differentiate into chondrocytes that lay down a cartilaginous extracellular matrix. This cartilage matrix forms the model over which differentiated osteoblasts calcify and replace the cartilage with bone matrix. Mutation of *sox9* in CNC results in CNC cells that migrate properly, but fail to express characteristics of chondrocytes, such as the molecular markers *Col2a1*, *Col11a2*, or *Aggrecan* (Bi et al. 2001).

In humans, a mutation in a *Sox9* allele results in campomelic dysplasia, a syndrome characterized by sex reversal and malformation of endochondrally derived skeletal elements (Bi et al. 2001; Gordon et al. 2009). Facial features of people with campomelic dysplasia include micrognathia and cleft palate (Gordon et al. 2009).

In 2003, Mori-Akiyama et al. conditionally inactivated *Sox9* in cranial neural crest cells, using the Cre/LoxP system, promoted by *Wnt1-Cre*. Using a Rosa26r reporter to make a fate map of *Wnt1-Cre* expressing cells, they found that migration and localization of the mutant (*Sox9^{fl/fl}*) neural crest cells was normal, but the CNC failed to contribute to the mesenchymal condensations and form the chondrogenic precursors of the endochondrally derived bones of the face. Embryos in which *Sox9* has been conditionally knocked out in *Wnt1-Cre* expressing CNC were missing the basisphenoid, presphenoid, malleus, incus, and nasal capsule; but the bones derived from intramembranous ossification were all present. These embryos develop a cleft of the secondary palate as a secondary effect of their missing basisphenoid and presphenoid bones, demonstrating that the endochondrally derived bone structures of the cranial base are necessary support palatal development. This is similarly seen in people with campomelic dysplasia who have a cleft of the secondary palate.

O. Segmentation of CNC into mandible or maxillary process of first pharyngeal arch

In mice, at approximately E10.0 the first pharyngeal arch divides into the maxillary and mandibular processes. Upon reaching the first arch, one of the first local patterning cues for the CNC is whether to become part of the maxillary or mandibular jaw structures. This patterning information is directed by the Endothelin-1(End1)/Endothelin receptor (Ednra) pathway and the *Dlx* gene family. All CNC cells express the receptor, Ednra, but only those in the first pharyngeal arch, which are *Hox* negative, receive the Ednra ligand, Edn1 (Kurihara et al. 2008). Edn1 is released from

the mandibular portion of the first pharyngeal arch endoderm and directs the CNC to differentiate into lower jaw structures. Artificial activation of *Ednra* throughout the first arch results in a homeotic transformation of the upper jaw into lower jaw structures (Kurihara et al. 2008). Likewise, exogenous activation of the downstream effectors of the *Ednra* pathway, e.g., through *Hand2* over expression, also results in transformation of the upper jaw into lower jaw structures. Kurinara et al. (2008) show that the potential to respond to endothelin-1/*Ednra* signaling may be restricted to the *Hox* negative CNC, since the only affected structures were those originating from *Hox* negative CNC.

In addition to the rostral – caudal patterning within the arch mediated by *Edn1* expression, dorsal-ventral patterning is mediated through differential expression of the *Dlx* gene family. *Dlx* genes have specific, nested, local expression patterns within the first arch that provide cues for dorsal-ventral patterning. *Dlx* genes provide specific local regulation in the dorsal-ventral axis for morphogenesis of larger structures such as the mandible or maxilla. This patterning information has been illustrated through the inactivation of specific *Dlx* family members. Inactivation of *Dlx1* and *Dlx2* causes defects in the maxilla. Inactivation of *Dlx5* and *6* results in a homeotic transformation of the lower jaw into the upper jaw (Jeong et al. 2008). Prior to the work of Jeong et al. (2008) the fate of the upper jaw was thought to be the default state for the first arch, upon which the lower jaw differentiation pathway was imposed, however, Jeong et al. (2008) found that there is a gene specific program that guides the development of the upper jaw.

P. FGF signaling in palatogenesis

At E10.5, the first arch is split into bilateral maxillary and mandibular processes that form the upper and lower jaws, respectively (Ferguson 1988). Around this time, the maxillary process fuses with the frontonasal process and fuses at the midline to form the upper lip and nasal process. Local factors and reciprocal signaling between the facial ectoderm and mesenchyme help to regulate the patterning of the upper and lower jaws. Between E9.5 and E11.5 many of the *Fgf* family of growth factors are broadly expressed in the facial ectoderm, specifically, *Fgf3*, 8, 9, 10, and 17 at E9.5; and *Fgf3*, 8, 9, 10, 15, 17, and 18 at E10.5 (Neubuser and Bachler 2001). The Fgf receptors are expressed predominately in the facial mesenchyme. The broad expression pattern of the Fgf receptors and the more localized expression patterns of their ligands indicate a wide range of competency of the midfacial mesoderm to receive and respond to Fgf signaling (Neubuser and Bachler 2001).

Q. BMP signaling in palatogenesis

Bone Morphogenic Proteins (BMP's) are a subset of the *TGF- β* family. They are present throughout embryogenesis and are known to be essential for many developmental processes. There are over 20 members of the *BMP* sub-family of *TGF- β* s (Nie et al. 2006). During facial development, *BMP* genes are expressed in the ectoderm and signal to the developing mesenchyme (Chen et al. 2002). They have been shown to be essential for the fusion of the palate at the midline (Chen et al. 2002).

R. Development of the palatal shelves

At E11.5, after the maxillary and mandibular processes have each fused at the midline, the maxillary process develops bilateral, medial outgrowths which constitute the palatal buds. This is equivalent to week six in human embryogenesis. During palatal growth, *Shh* signaling helps to regulate reciprocal interactions between the palatal oral ectoderm and the underlying CNC derived mesenchyme. *Shh* signaling has been shown to directly regulate palatal mesenchymal cell proliferation through activation of *CyclinD1* and *CyclinD2* signaling (Jiang and Lan 2009). During this initial stage of palatal shelf development, *Shh* and *Fgf10* function in a positive feedback loop to regulate palatal outgrowth; *Shh* is expressed in the palatal mesenchyme, and induces it to proliferate via the cyclins, and to release *Fgf10* onto the palatal epithelium (Jiang and Lan 2009). The release of *Fgf10* onto the palatal epithelium helps induce proliferation and signals the mesenchyme to release more *Shh* in a positive feedback loop. The palatal shelves continue to grow and proliferate initially in a vertical direction, until E13.5.

Between E13.5 and E14.5 in mice, and week nine in humans, the palatal shelves elevate into a horizontal position. To accomplish the elevation, the developing tongue must move ventro-caudally as the oral cavity grows and creates more room. Hyaluronic acid accumulates in the base of the palatal shelf mesenchyme and the resulting fluid accumulation causes a mechanical elevation (Ferguson 1988). At E15.5, or week 12 in humans, the medial edges of the growing palate meet and fuse at the midline. TGF- β signaling helps to regulate the fate of medial edge epithelium during fusion of the palatal shelves. Studies examining the temporal and spatial expression of TGF- β in mice during embryogenesis indicate that they play a key role during the elongation and palatal

fusion stages of palatogenesis (Ferguson 1999). In the developing palatal shelves, *Tgf β 1* and *Tgf β 3* are expressed along the medial edge epithelia (Ferguson, 1995). *Tgf β 3* knockout mice have a cleft of the secondary palate due to the failure of adhesion of the shelves at the midline seam (Ferguson 1995; Chai et al. 2003). *Tgf β 2* is expressed in the CNC derived underlying mesenchyme, while the receptor, *Tgf β 2r*, is expressed in both the medial edge epithelium and the mesenchyme (Chai et al. 2003). In 2003, Chai et al. examined the effects of a conditional knock out of the receptor *Tgf β 2r* in CNC cells. They found that the mice exhibited not only a cleft secondary palate, but they also had severe calvaria defects. They found that the *Tgf β 2r* knockout resulted in inhibition of expression of CyclinD1 causing a decreased rate of proliferation in the palatal shelf mesenchyme and a failure of palatal shelf fusion (Chai et al. 2003).

Palatal shelf fusion is accompanied by removal of the medial edge epithelium from each palatal shelf through a combination of epithelial-to-mesenchymal transition, migration of the epithelial cells both nasally and orally, and apoptosis (Ferguson 1995; Svoboda and Kang, 2005). By E16.5 the midline seam is degenerated and the palate is fully fused. Complete fusion accompanies the fusion of the nasal septum with the palatal shelves.

S. miRNA in palatogenesis

Within palatogenesis, whose developmental sequence spans gastrulation to palatal shelf fusion, it is easy to imagine that part of the regulatory scheme might involve miRNAs. Currently, there is evidence that miRNA may play a role in the CNC contribution to palatogenesis. In zebrafish, Eberhart et al. found that miR-140 helped to

temporally regulate the expression of the CNC's receptor for the Pdgfr α A ligand, which provides a directional cue during their migration. In the literature there is currently no evidence for a role of miRNA in mammalian palatogenesis.

T. Epidemiology of craniofacial clefting

Defects of palatogenesis and orofacial development are common, with an incidence of greater than 1:700 live births worldwide (Bian 2009; Murray 2002). Clefts of the lip and palate occur early in development as a result of insufficient growth and/or fusion during the genesis of the derivatives of the first pharyngeal arch and the frontonasal prominence. Orofacial clefts can be attributed to teratogenic interference, genetic factors, or a combination of a genetic predisposition and a teratogenic trigger (Wilke and Morris-Kay 2001; Murray 2002; Murray and Schutte 2004; Helms 2006). There are over 300 syndromes in which an orofacial cleft is an accompanying feature, and current estimates are that over 70% of non-syndromic orofacial clefts are a result of genetics (Wilke and Morris-Kay 2001).

Asians, Native Americans, and Northern Europeans have the highest incidence, while Africans have the lowest at ~ 1/2500 (Murray 2002). Twin and inheritance studies suggest a strong genetic component to isolated clefts, with 30-50% of the incidences attributed to genetics, and the remainder attributed to a combination of genetic and environmental factors (Grittle-Linde et al. 2006). As an example, maternal smoking is thought to contribute to approximately 4% of cleft lip and palate cases, and approximately 12% of bilateral clefts of the lip and palate (Honein 2007).

Cleft palates can be repaired surgically, but the repair involves multiple surgeries over the lifetime of the affected individual. Despite surgical repair in individuals with facial clefting, long term morbidity studies suggest an increased risk of death of all major causes including cancer and suicide (Druschel 1996, Murray et al. 2004). During the first year of life, there is an approximately 3.7 fold increase in mortality in individuals with a non-syndromic cleft (Druschel 1996).

In addition to physical health issues, there is a higher incidence of psychiatric illness in individuals with cleft lips or palates. Some researchers believe the correlation between facial clefting and psychiatric illness is a result of the intimacy in development between the face and the brain (DeMeyer 1964; Veira 2008). Individuals with an orofacial cleft have been found to have a significant reduction in the size of their cerebrum and cerebellum, and this is found to be associated with mild cognitive impairments (Murray et al. 2004).

Given the large impact that orofacial clefts have on health and well being of the afflicted, as well as the financial impact on our health care system, it is vital to understand as much as possible about the molecular mechanisms of facial clefting. Several genes and genetic pathways are known to be involved in the development of cleft lip or palate, including the Fgfs, Bmps, Tgf β s, Shh, and other families of growth factors, as well as their receptors and downstream mediators, such as the SMAD family (Nie et al. 2006; Marcucio et al. 2009; Dijke et al. 1997; Lagna et al. 1996; Helms et al. 2006). Over the past twenty years there have been many discoveries about the molecular mechanisms of craniofacial clefting, but to date, no mammalian studies have been performed on the role of the enzyme Dicer, or its product, miRNA, in orofacial

development and palatogenesis. Investigation into the role of miRNA in palatogenesis and orofacial dimorphisms could possibly lead to novel therapeutic targets and agents for molecular based therapies.

U. The present study

The present study analyzed the role of *Dicer* in craniofacial development and its role in mediating the CNC contribution to the orofacial and palatal region. The tested hypothesis was “*Dicer* is essential to mammalian orofacial development, conditional mutagenesis of the *Dicer* in palatal precursors will disrupt established orofacial and palatal developmental processes.” Mouse embryos were analyzed, in which a conditional *Dicer* knockout is driven by *Pax2Cre* expression, using the LoxP/Cre system. *Pax2Cre* has previously been shown to be expressed in the ear and midbrain/hindbrain and was briefly described in the literature as being expressed in the first pharyngeal arch (Ohyama and Groves 2004). In addition, conditional *Dicer* knockouts driven by *Pax2Cre* have been described in the literature showing miRNAs have a role in inner ear development (Soukup et al. 2009).

V. Summary

Palatogenesis is a complex developmental process that occurs within a dynamic spacio-temporal framework within the first pharyngeal arch and the embryonic orofacial region. Correct development of the palate is dependent on the correct sequence of events from CNC specification and migration, to the final closure and elimination of the medial edge epithelial seam. The process is assisted by a multitude of regulatory molecules,

transcription factors, chemotactic molecules, cell-cell adhesion molecules, and growth factors. One piece of the palatogenesis regulatory puzzle that is currently missing is what role, if any, miRNA have in mediating the process in mammals. Current evidence in the literature suggests that in zebrafish miRNA may play an important role in mediating the migration of CNC into the first pharyngeal arch from which the palate develops.

My specific aims were:

- I. Characterization of the orofacial development of the conditional *Pax2Cre/DicerLoxP/LoxP* knockout during palatogenesis
- II. Initial assessment of the effects of microRNA depletion on skeletogenic cranial neural crest cells through analysis of *Sox9* – a molecular marker of a subset of differentiating cranial neural crest cells that populate the face.

In this study, I investigated whether *Dicer* plays a role in palatogenesis, and how loss of *Dicer* affects *Sox9* expression patterns within the first pharyngeal arch. Further studies will be necessary to elucidate how loss of *Dicer* results in loss of miRNA and siRNA contributions to the regulation of palatogenesis; and proteomics studies will be necessary for a complete picture of how the loss of miRNA in the palatal precursors affects gene expression. My thesis lays the necessary foundation for these further investigations.

II. Materials and Methods:

A. Animal care and handling

All animal care and use was approved by the Creighton University Institutional Animal Care and Use Committee (CU –IACUC) under the listed protocol 0852 for all breeding, and 0853 for all experimental animals. All measures possible were taken to reduce pain and suffering for experimental animals.

B. Generation of conditional *Dicer* knockout, timed pregnancy, and c-sections

Conditional *Dicer* knockout mice were obtained from Dr. Garrett Soukup of the Creighton University School of Medicine. These mice were created by Harfe et al (2005) through the insertion of *LoxP* sites around an exon in the coding sequence of the second domain of the *Dicer* allele. Cre – mediated recombination between the *LoxP* sites results in the deletion of ~ 90 amino acids, rendering the enzyme non-functional (Harfe et al. 2005).

Dicer^{LoxP/Wt} mice were mated with transgenic *Pax2Cre*, or *Pax2Cre/Rosa26R* mice (Ohyama and Groves 2004) to generate *Dicer*^{LoxP/Wt}/*Pax2Cre* and *Pax2Cre/Rosa26R* mice. Males containing these genotypes were then used as studs for timed pregnancy mating with *Dicer*^{LoxP/LoxP} females.

Timed pregnancies were set up by first visually checking for estrus in the *Dicer*^{LoxP/LoxP} females, and then placing the female into the *Pax2Cre*, or *Pax2Cre/Rosa26R* male's cage overnight, followed by confirmation of a sperm plug, and removal from the male's cage in the morning. Noon the next day was considered embryonic day 0.5, and timed pregnancies were counted forward from that time point.

All c-sections were performed between 11:30 AM and 12:00 PM to ensure maximum consistency in development of the embryos. All embryos used in these experiments were between E9.5 and E17.5.

C-sections were carried out on clean, RNase free surfaces (RNase Zap, Ambion, Austin TX, Cat No. AM9780), using sterile, autoclaved instruments. Embryos were placed into diethyl pyrocarbonate (DEPC) (Cat No. D5758-25ML, Sigma-Aldrich, St. Louis, MO) treated 0.1M Phosphate Buffered Saline (PBS) or DEPC treated 4% Para-formaldehyde (PFA) (Cat No. T353-500, Fischer-Scientific, Fair Lawn N.J) following snipping of their tail for genotyping. Embryos older than E15.5 were cardiac perfused with ice cold DEPC treated PBS followed by ice cold DEPC treated 4% PFA.

C. Genotyping

5-10 day post-natal mice and C-sectioned embryos were tail clipped for genotyping and tattooed for record keeping. DNA was extracted from tails using the Phenix Genomic DNA Purification Kit (Phenix, Candler NC, Cat No. PKG-120L). Purified tail DNA was used for genotyping using the Fast Start Taq DNA Polymerase PCR kit (ROCHE, Cat No. 04738420001) using forward and reverse primers for *Cre* (5'-GCCTGCATTACCGGTCGATGCAACGA and 5'-GTGGCAGATGGCGCGGCAACACCATT primer sequence) generating a 726 bp sequence, for floxed *Dicer* (5'-CCTGACAGTGACGGTCCAAAG and 5'-CATGACTCTTCAACTCAAACACT) generating a 420 bp product for *Dicer*^{LoxP/LoxP}, and 351 bp product for *Dicer*^{wt}, and for *LacZ* (5'- TACCACAGCGGATGGTTTCGG and 5'-GTGGTGGTTATGGCGATCGC) generating a 350 bp product.

The PCR product was separated on 2 % agarose (Gene Pure LE Quick Dissolve Agarose, Cat. No. E-3119-500, ISC BioExpress, Kaysville UT) using a horizontal separation system (Owl Separation Systems, Portsmouth NH, Model A1) run at 115V (BioRad, Hercules CA, PowerPac HC 250V) made with 1X TAE. DNA was intercalated with Gel Green (Cat No. 41004, Biotium Inc. Hayward CA), and visualized using the Kodak Gel Logic 2200 Imaging System and Kodak MI imaging software.

Embryos were considered knockouts when they were *Pax2Cre* + and homozygous for the *LoxP* insertion in the *Dicer* allele (*Dicer*^{LoxP/LoxP}) which increases the PCR product for *Dicer* by 80bp.

D. Bone and cartilage Staining

To stain the bone and cartilage we adapted a protocol from Lufkin et al. (1992). Embryos were eviscerated, their skin removed, and placed into 95% ethanol for 48hr rotating at RT. 95% ethanol was changed every 12 hours. After 48 hours, ethanol was changed with Alcian Blue staining solution (0.1% Alcian blue 8 GX (Cat No. 720, Allied Chemical Corp., New York, NY), 80% ethanol, 20% acetic acid) and was stained at RT, rocking, for 72 hours. Alcian blue solution was changed with 95% ethanol for 6 hours, followed by 2% potassium hydroxide (KOH) (Cat No. P250 – 77501, Fisher Scientific) for 24 hours, rocking at RT. Alizerin red solution (0.06% Alizerin Red (Cat No. A5533-25G, Sigma, St. Louis, MO), 1% KOH, Water) replaced the KOH and stained the bone for 36 hours, rocking at RT. Embryos were cleared in 1% KOH/20% glycerol until completely cleared. Embryos were then transferred to 1:1 Glycerol/95% ethanol for 12

hours, and then passed through a series of increasing glycerol concentrations until they were in 100% glycerol (Cat No. G5516-1L, Sigma) for long term storage.

E. Paraffin embedding and sectioning

To embed E11.5, E12.5 and E16.5 embryo heads in paraffin they were first dehydrated in 80% ethanol (Cat No. S93232, Fisher Scientific, Fair Lawn NJ) for 15 minutes, followed by 95% ethanol for 45 minutes, 100% ethanol for 45 minutes, 1:1 100% ethanol / 100% xylenes (Cat No. 3175-L-12734, Fisher Scientific) for 15 minutes, and 100% xylene for 30 minutes. After 100% xylene wash, embryo heads were transferred to paraffin (FisherBrand Paraplast Tissue Embedding Medium, Cat No. 23-021-399, Fisher Scientific) in a vacuum oven (Model No. 3608, Lab-Line, Dubuque Iowa) for 15 minutes, and transferred four times. Heads were embedded to allow coronal sections.

10 μ m coronal sections were made through face and secondary palate using a Leica microtome (Leica Microsystems, Bannockburn IL, Model No. RM2125). Sections were melted onto Superfrost Plus Slides (Cat No. 12-550-15, Fisher Scientific) using a slide warmer (Cat No. 66632, GCA/Precision Scientific, Chicago IL), warmed to 55 ° C. Control and *Pax2Cre/Dicer^{LoxP}* knockout sections were location matched using morphological markers such as the eye and the tongue.

F. Hematoxylin and Eosin Staining

Slides were dewaxed and rehydrated prior to Hematoxylin (Surgipath Hematoxylin - Harris Formula, Richmond IL, Cat No. 01562) and Eosin (Surgipath

Eosin, Cat No. 01602) staining. Slides were placed into xylene for 5 minutes, followed by 1:1 Xylene:Ethanol for 2 minutes, 100% ethanol for 3 minutes, 95% ethanol for 2 minutes, 80% ethanol for 2 minutes, 70% ethanol for 2 minutes, 50% ethanol for 2 minutes, and DI water for 5 minutes.

Hematoxylin was diluted 1:4 in DI water, and applied to slides for 2 minutes. Slides were rinsed with gently running water for 2 minutes, rinsed with acid alcohol (230mL Ethanol, 70mL Water, 1mL 12N HCl), rinsed with running water for 5 minutes, rinsed with ammonia solution (1L H₂O, 3mL 28% W/V NH₄OH Stock) for 1 minute, rinsed with running water for 5 minutes, rinsed with 80% ethanol briefly, rinsed with diluted eosin (diluted 1:4 with 100% ethanol), rinsed with 95% ethanol, and 100% ethanol. Slides were mounted in glycerol and a cover slip was applied.

G. RNA extraction

Tissues containing the RNA of interest were harvested in *RNAlater* (Ambion Inc Austin TX, Cat No. AM7021), homogenized (OmniTip Homogenizing Kit, Omni International, Kennesaw GA), and RNA was extracted for Quantitative-PCR (Q-PCR) and riboprobe synthesis using the RNeasy Mini Kit (Qiagen Inc. Valencia CA, Cat No. 74104). The quantity of RNA was determined using the NanoDrop ND – 1000 spectrophotometer. The quality of RNA used for Q-PCR was analyzed using the Model 2100 Agilent Bioanalyzer (Agilent Inc. Santa Clara, CA)

H. Riboprobe generation

Riboprobes for in situ hybridization (ISH) were generated from both plasmid vectors containing the cDNA sequence and from cDNA generated from a reverse transcription using tissue extracted RNA as a template.

In the RT-PCR method, total RNA was extracted and purified from tissue known to express the target gene, using the method discussed above. The total RNA was then reverse transcribed into cDNA using random hexamers, using the Superscript III First-Strand Synthesis kit from Invitrogen (Invitrogen Inc. Carlsbad CA, Cat no. 16080-051). PCR using gene specific primers amplified the cDNA of interest. Another PCR using gene specific primers, with one containing the T7 recognition site (TAATACGACTCACTATAGGG) re-amplified the cDNA into the template DNA. This cDNA was purified on a 1% agarose gel run for 2 hours at 115V, extracted with phenol/chloroform, and sequenced in the Creighton University Molecular Biology Core Laboratory. The sequence analysis confirmed that the riboprobe product would be complementary to the mRNA target. Template cDNA containing the T7 site was transcribed and DIG labeled (Roche DIG RNA Labeling Kit SP6/T7, Cat no 11175025910).

In the plasmid method for riboprobe generation, single colonies of *E. coli* containing the plasmid vector were grown overnight in LB media. Plasmid cDNA containing the clone of interest was extracted (QIAprep Spin MiniPrep Kit, Cat 27106, Qiagen), and the clone (*Pax2*, *Cre*, *Dicer*) was amplified by PCR using gene specific primers (see Table 1. for primer sequences). The PCR product was re-amplified using gene specific primers with the T7 sequence inserted into the reverse primer. Template

cDNA was then run on a 1% agarose gel at 115V to confirm its size and purity, and was extracted for sequencing at the Creighton Molecular Core Lab using . The output of the sequencing reaction was BLASTed against the gene using the reverse complement of the sequencing output to confirm it was targeting correctly. The template cDNA containing the T7 sequence was transcribed and DIG labeled as described above.

DIG labeled RNA probes were purified on 6% acrylamide gels (American Bioanalytical Sequel NE Reagent, Natick MA, Cat No. AB13021-01000), extracted overnight in “Crush-Soak Buffer” (0.2M Tris HCL, 3.8M NaCl, 0.02M EDTA) ethanol precipitated, resuspended in 25 μ l of nuclease free water (Ambion, Cat. No. AM9937), and quantified using the NanoDrop ND 1000.

I. Quantitative PCR

RNA was extracted and the quantity and quality were determined as described above. Total RNA was reverse transcribed and amplified with random hexamers (Applied Biosystems, High Capacity cDNA Reverse Transcription Kit, Cat No. 4368814). Q-PCR was performed at the Creighton University Molecular Core Facility on the PRISM 7000 Sequence Detection System (Applied Biosystems) using Applied Biosystem Taq Man Gene Expression Assays custom made primers for *Bmp4*, *Fgf8*, and *Fgf10*, and β -*Actin* as the endogenous control, using the Applied Biosystems one step RT-PCR master mix reagent (Cat No. 4309169).

Prior to Q-PCR, several house keeping genes, including *Gapdh*, β -*Actin*, *Tbp*, and *Jag1*, were tested in logarithmic dilutions from 1:1 to 1:10,000 using E17.5 WT cDNA to determine amplification efficiency and to optimize the endogenous control. β -

Actin was determined to have the optimum amplification efficiency and thus was used as an internal control.

J. miRNA Quantitative PCR

RNA used for Q-PCR with *Bmp4*, *Fgf8*, and *Fgf10* was also used for Q-PCR with miRNA specific probes. RNA was treated with DNase (Turbo DNA Free Kit, Ambion, Cat No. AM1907), and its integrity was assessed on the Agilent Bioanalyzer using the Aligent RNA 6000 Pico Kit (Cat No. 5067-1513, Aligent Technologies, Santa Clara, CA). E17.5 control littermate miRNA was reverse transcribed (TaqMan MicroRNA Reverse Transcription Kit, ABI, Cat No. 4366596), using 5 μ l DNase treated RNA [10ng/ μ l], in a 15 μ l reaction (3 μ l 5X TaqMan MicroRNA RT Primer, 4.2 μ l Nuclease Free H₂O, 1.5 μ l ul 10X RT Buffer, 0.2 μ l 100mM dNTPs, 1 μ l MultiScribe Reverse Transcriptase (50U/ μ l), 0.2 μ l RNase Inhibitor). Amplification efficiency of each miRNA probe was tested using logarithmic dilutions of control cDNA (1:1 to 1:10,000) which were run on the Applied Biosystems StepOne Plus RT-PCR System (Cat No 4376598, Applied Biosystems, Foster City, CA) using 20 μ l reactions (7.7 μ l Nuclease Free H₂O, 10 μ l TaqMan 2X Universal PCR Master Mix (No AmpErase UNG), 1 μ l, TaqMan Assays 20X TaqMan Assay, 1.3 μ l cDNA) amplified with an initial holding stage of 50° C for 2 min, 95° C for 10 min, followed by 50 cycles of 95° C for 15s and 60° C for 1 min. Once amplification efficiency of each miRNA probe was determined, RNA from both control and *Pax2Cre/Dicer^{LoxP}* palatal tissue was again reverse transcribed, and Q-PCR was performed in quadruplicate for each of three biological replicates, using both the control and *Pax2Cre/Dicer^{LoxP}* cDNA with miRNA specific

probes for miR-101a, miR-101b, miR-140, miR-145, and miR-200b (Applied Biosystems custom made TaqMan Assays). A *Sno-202* probe was used as the endogenous control. Results were made into a box plot comparing the relative CT value of each probe between the control and *Pax2Cre/Dicer^{LoxP}* littermates with the control *Sno-202*. Statistical significance was determined using a Students T-test between the relative CT value of the control and the *Pax2Cre/Dicer^{LoxP}*.

K. Whole mount ISH:

Timed-pregnancy embryos were harvested in ice cold PBS and then transferred to ice cold 4% PFA, and left at 4C for at least three days before use. Embryos E12.5 and older were dissected to separate the lower jaw from the upper, and their brain, and brain stem were removed and kept intact. During whole mount ISH, one embryo of each time point would not receive a probe, and was used as a negative control. Embryos were rinsed in PBS / 0.1% Tween – 20 and then digested in PBS containing 2.5ug/ml proteinase K. Digestion occurred at room temperature, and length of time varied based on the age of the embryo. Proteinase K digestion was stopped by rinsing tissue in 4% PFA, and then tissues were rinsed 3 x 10 minutes in PBS/0.1% Tween-20. Tissues were then prehybridized at 60C for 1hr in prehybridization solution (50% formamide, 2X SSC, 0.1% Tween-20, and 7.5% denatured salmon sperm DNA) before the addition of 100ng of DIG labeled riboprobe. Tissue was hybridized overnight at 60C, in a rotating incubator, for ~ 14 to 16 hours. Following hybridization, tissue was washed in 2X SSC 2 x 10 minutes at 60C, and then for 1 hour at 70C. Tissue was then washed in RNase A buffer (500mMol NaCl, 100mMol Tris, 100ul Tween-20, in DEPC treated water, pH 7.5)

for 10 minutes, followed by digestion in RNase A at 37C for 1hr. Digestion was terminated through two washes in PBS/0.1% Tween 20. Tissues were then incubated in DIG Wash and Block Buffers (DIG Wash and Block Buffer Set, cat no 11585762001, Roche) for 1 hour, followed by the addition of sheep anti-DIG FAB antibody (Roche) at 1:1000 dilution, tissues were incubated overnight at room temperature. Following incubation with anti-DIG antibody, tissues were subjected to 48 hours of washes in Levamisole wash buffer (0.1g levamisole in 90ml H2O and 10ml of Roche 10X wash buffer). Following 48 hours of washing, tissues were rinsed for 30 minutes with detection buffer (DIG wash and Block Buffer Set, Cat No 11585792001) and detected with BM purple precipitating solution (Cat No 11442074001, Roche) at room temperature.

III. Results

A. Palatal development in control and *Pax2Cre/Dicer^{LoxP}* littermates.

Embryos were considered control littermates when they lacked the *Cre* positive band (Fig. 2). Conversely, embryos were considered to be a conditional knockout when they showed the *Cre* positive band, and were homozygous for the floxed *Dicer* allele, *Pax2Cre/Dicer^{LoxP}* (Fig. 2). Control littermate embryos could be either heterozygous or homozygous for the floxed *Dicer* allele, but lacking the *Cre* gene. Floxed *Dicer* Heterozygous, *Cre* positive embryos were not used in our analysis.

Morphological differences in palatal development in control littermates and *Pax2Cre/Dicer^{LoxP}* embryos were compared at three time points; E11.5, E12.5 and E16.5. Contrasting to the control littermates littermates, E11.5 *Pax2Cre/Dicer^{LoxP}* failed to show the characteristic swellings in the medial maxillary process which later develops into the palatal shelves (Fig. 3). At E12.5, *Pax2Cre/Dicer^{LoxP}* embryos have small bilateral bulges in their medial maxillary process whereas the control littermate embryo has a definitive palatal shelf (Fig. 4). At E16.5, when palatal development is normally complete, *Pax2Cre/Dicer^{LoxP}* show a cleft of the secondary palate and the overall extent of their palatal development is equivalent to the morphology normally seen in WT E12.5 (Fig. 5).

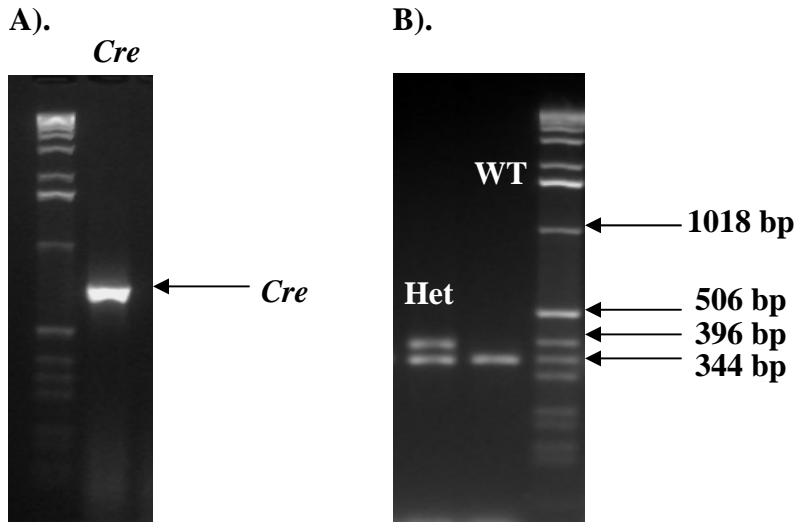


Figure 2. Relative sizes of PCR products of *Cre* and floxed *Dicer*. A) PCR product amplified from tail DNA showing the *Cre* allele. B) PCR products amplified from tail extracted DNA showing the relative sizes of endogenous *Dicer* (WT), and floxed *Dicer* alleles. The PCR product of floxed the *Dicer* allele is approximately 80bp larger, and is represented by the top band in the Het PCR product.

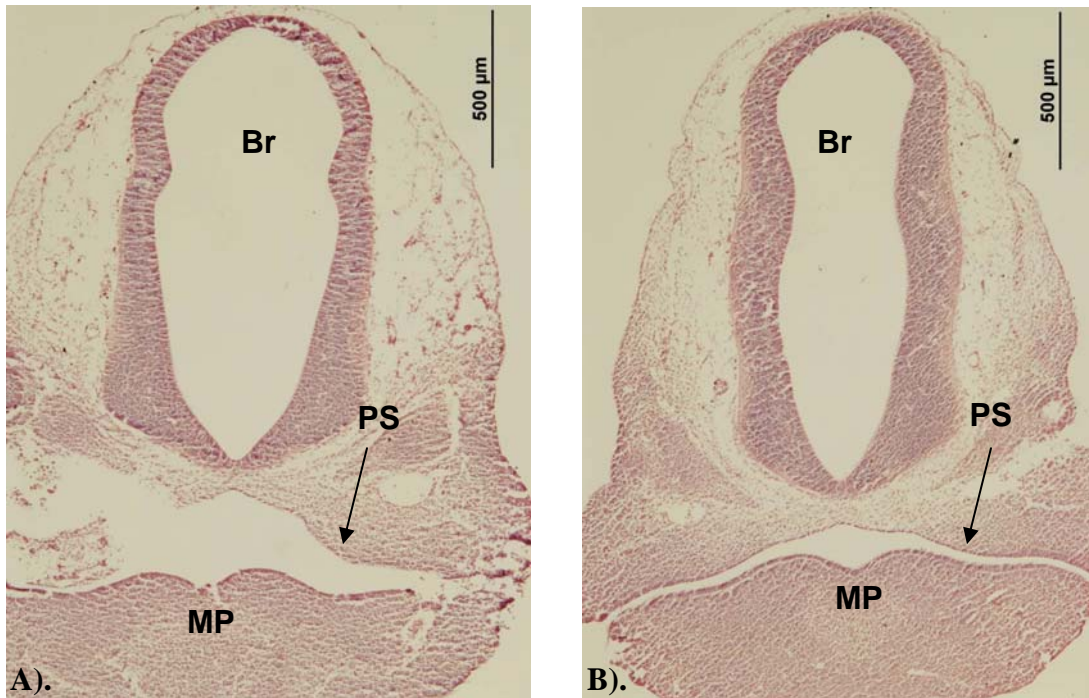


Figure 3. Onset of palatal development in E11.5 control and *Pax2Cre/Dicer^{LoxP}* mice.

Coronal sections through the developing embryonic face show at the level of the palatal shelves (PS) (arrows). A). The palatal bulge is visible in the control littermate's medial maxillary process. B). In the *Pax2Cre/Dicer^{LoxP}*, the medial maxillary process is relatively flat. Sections were location matched based on the midbrain (Br) and mandibular process (MP) morphology to facilitate comparison. Scale bar = 500 μ m.

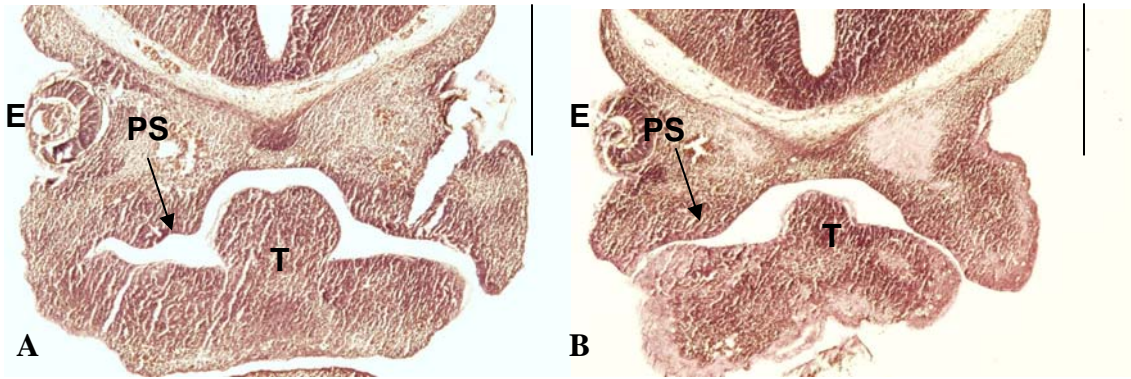


Figure 4. Coronal sections through palatal shelves of E12.5 control and *Pax2Cre/Dicer^{LoxP}* A). At E12.5, control littermate embryos have bilateral vertical outgrowths of palatal shelves (PS) (arrow) descending from the medial edges of the maxillary process. B). *Pax2Cre/Dicer^{LoxP}* embryos exhibit delayed outgrowth of the palatal shelves (arrow). Sections were site matched based on the location and morphology of the brain and the eye. Eye (E), Tongue (T), Palatal Shelf (PS). Scale Bar = 1mm.

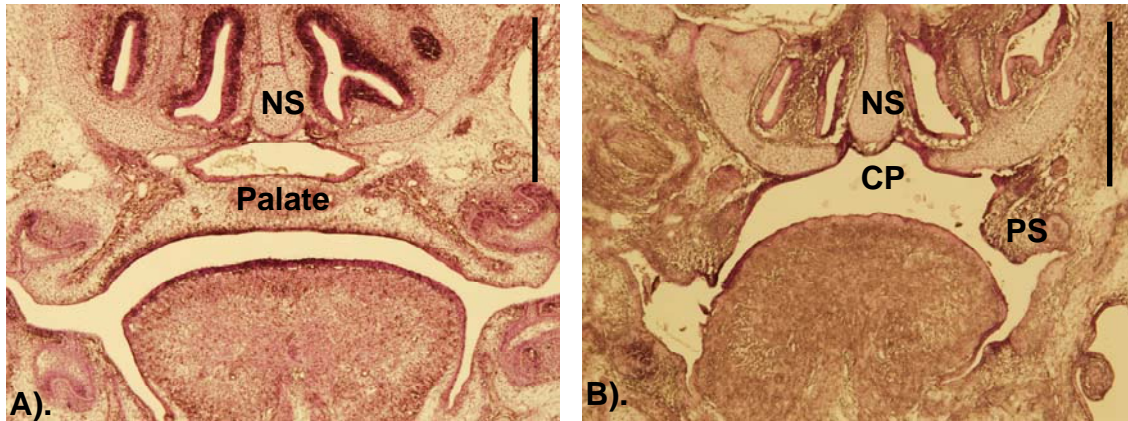


Figure 5. Coronal sections through the palate of E16.5 control and *Pax2Cre/Dicer^{LoxP}* embryos. A). At E16.5, control littermate embryos have a completely fused secondary palate. B). *Pax2Cre/Dicer^{LoxP}* palatal shelves (PS) have failed to develop past the morphology normally seen in E12.5 WT embryos resulting in a complete cleft of the secondary palate (CP) and exposure of the nasal septum (NS) to the oral cavity. Sections were site matched based on the morphology of their eyes and brains which are not visible in the images. Tongue (T), Cleft Palate (CP), Palatal Shelves (PS), Nasal Septum (NS). Scale bar = 2mm.

B. Bone and cartilage staining of E17.5 control and *Pax2Cre/Dicer^{LoxP}* littermates

Comparison of gross skeletal morphology between *Pax2Cre/Dicer^{LoxP}* embryos and control littermates revealed either a total absence or hypoplastic growth of several bones in *Pax2Cre/Dicer^{LoxP}* embryos. A sagittal view (Fig. 6) comparing the control and *Pax2Cre/Dicer^{LoxP}* embryos shows the complete absence of the tympanic ring, the temporal bones, and zygomatic bones in the *Pax2Cre/Dicer^{LoxP}* mice. Removal of the jaw allowed for direct comparison of the lower jaw of the *Pax2Cre/Dicer^{LoxP}* and control embryos. *Pax2Cre/Dicer^{LoxP}* embryos exhibit hypoplastic growth of the mandible and the overall smaller size of the *Pax2Cre/Dicer^{LoxP}* embryos (Fig 7). Moreover, with the jaw removed, a view of the maxilla shows that *Pax2Cre/Dicer^{LoxP}* embryos are missing their palantine bones, and have hypoplastic pre-sphenoid, and basisphenoid bones (Fig. 8).

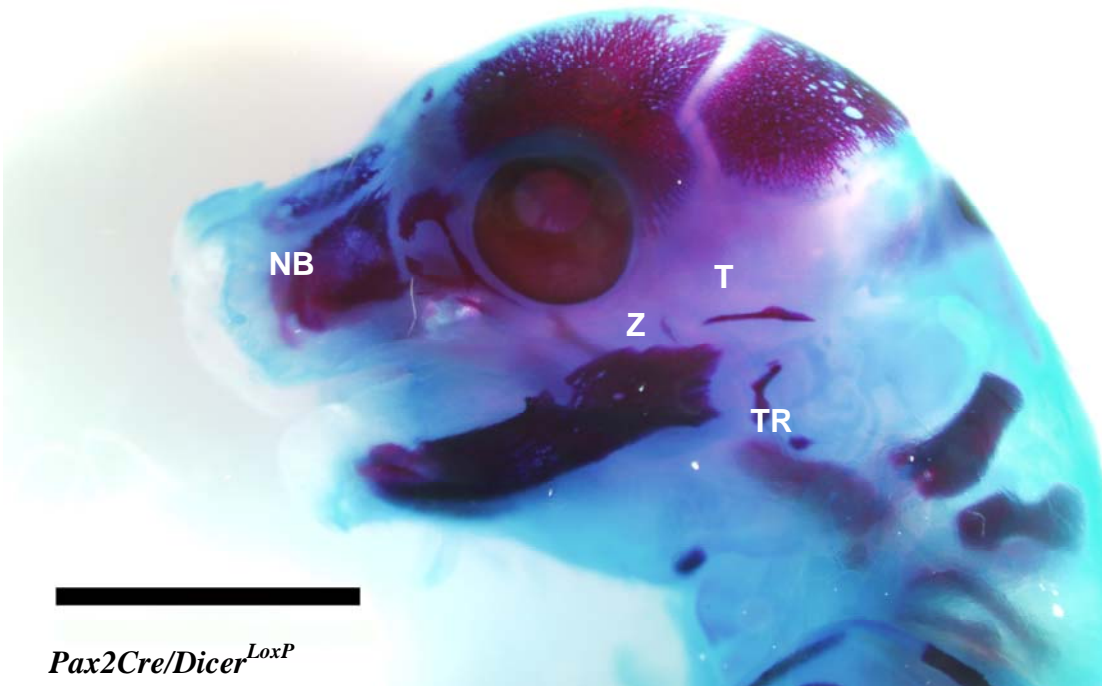
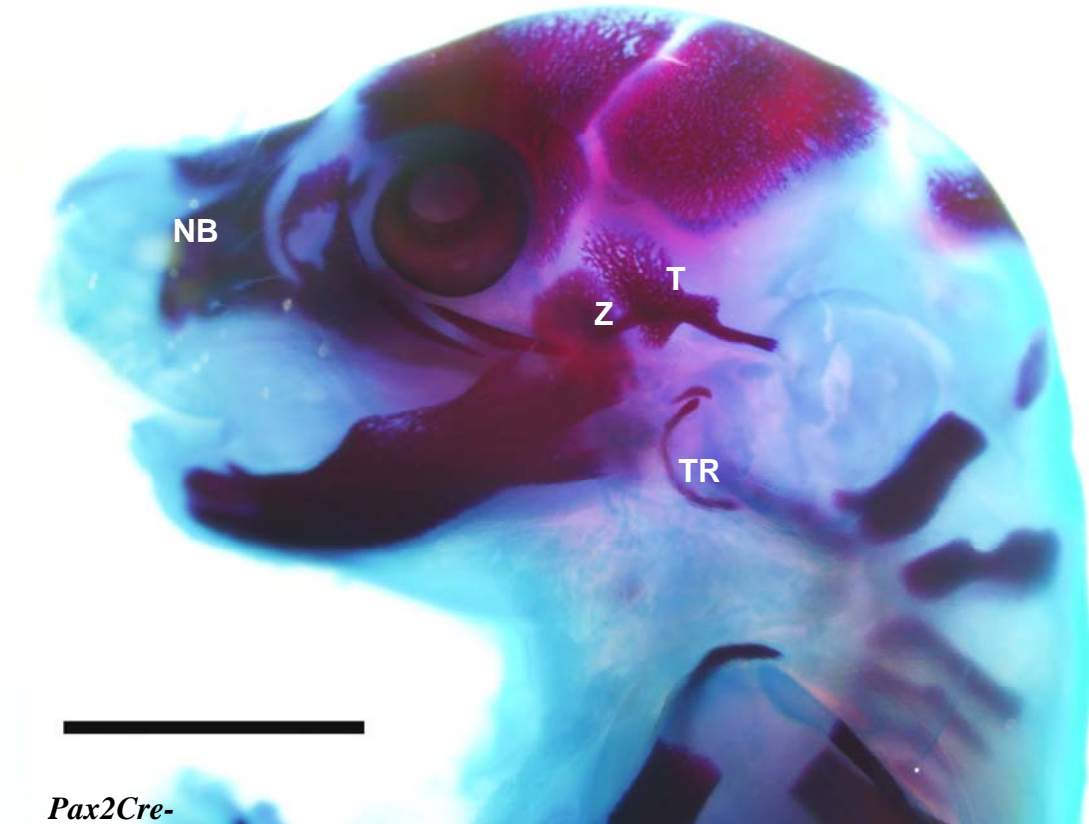


Figure 6. Facial skeleton of E17.5 *Pax2Cre/Dicer^{LoxP}* and control embryos (Lateral view). Comparing control littermate with *Pax2Cre/Dicer^{LoxP}* mice shows the temporal (T) and zygomatic bones (Z), and the tympanic rings (TR) are missing in the *Pax2Cre/Dicer^{LoxP}* mice, moreover, they also have hypoplastic nasal bones (NB). The *Pax2Cre/Dicer^{LoxP}* embryos are noticeably smaller than their control littermates. Bone and cartilage stained with alizarin red and alcian blue respectively. Scale bar = 2mm.

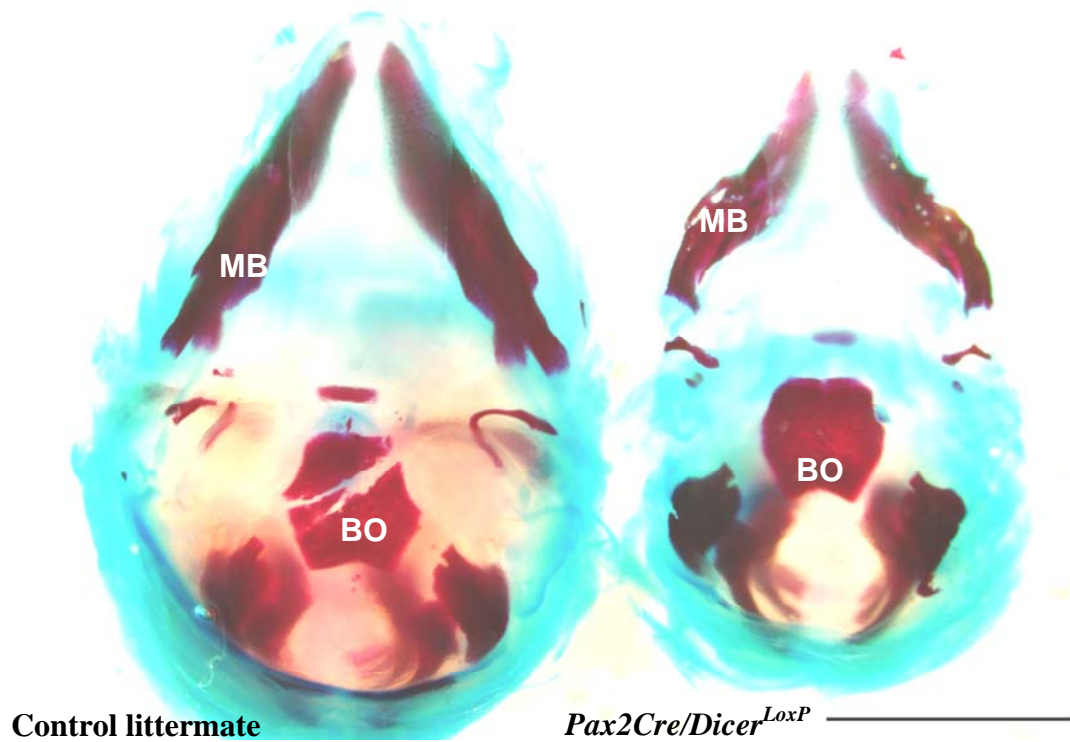


Figure 7. Hypoplastic growth of the mandible in *Pax2Cre/Dicer^{LoxP}*. A superior view of the mandible after it has been removed shows that control embryos have a fully formed mandible whereas *Pax2Cre/Dicer^{LoxP}* embryos have hypoplastic mandibles (MB) compared to their control littermates. The basoccipital bone (BO), which is not derived from the first pharyngeal arch, is similarly developed in both embryos. In addition, the *Pax2Cre/Dicer^{LoxP}* embryos are smaller than their littermates. Mandible (MB), Basoccipital bone (BO). Scale bar = 2mm.

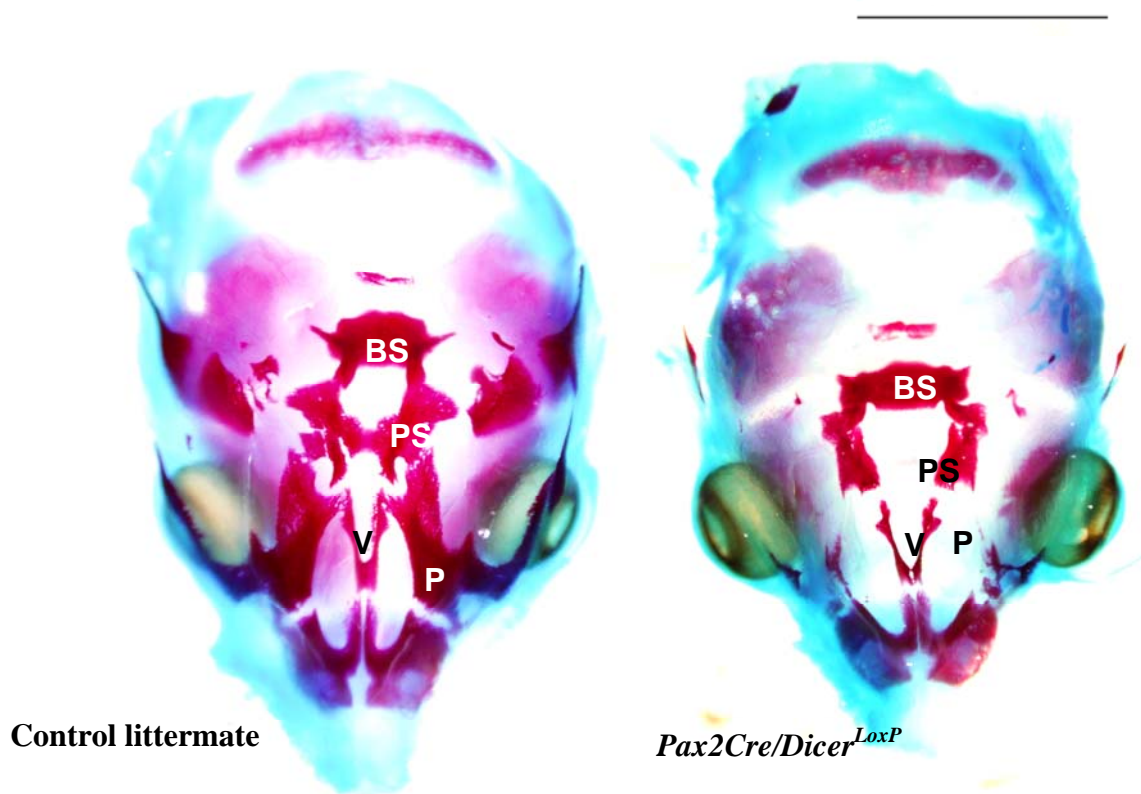


Figure 8. View of the maxillary process in E17.5 control and *Pax2Cre/Dicer^{LoxP}* embryos. With the mandible removed, an inferior view of the cranial base shows that at E17.5, *Pax2Cre/Dicer^{LoxP}* (B) embryos are missing the palatine bones (P), basisphenoid (BS), and have a malformed pre-sphenoid (PS) and hypoplastic vomer (V), compared with their littermate control. Scale Bar = 2mm. Palatine bones (P), Basisphenoid(BS), Pre-sphenoid (PS), Vomer (V).

C. *Pax2* and *Cre* expression profile at E12.5

To create a fate map of *Pax2Cre* expressing tissues in the embryonic face, *Pax2Cre*⁺ mice were mated with *Rosa26R*⁺ mice, generating offspring in which the enzyme LacZ is permanently expressed in tissues derived from *Pax2Cre* expressing precursors. Tissues of the *Pax2Cre/Dicer*^{LoxP} viscerocranium expressed LacZ which was actively detected histochemically, indicating these cells had at one time expressed *Pax2Cre* (Fig. 9).

To further confirm the LacZ data, riboprobes generated for *Pax2* and *Cre*, which were used to look at the expression patterns of *Pax2* and *Cre* mRNA in E12.5 WT embryos carrying the *Cre* transgene (*Pax2Cre/Dicer*^{WT}). Both *Pax2* and *Cre* overlap in expression with LacZ staining, showing expression in the maxillary and mandibular processes and throughout the viscerocranium (Fig. 10). Taken together, these data suggest craniofacial morphology differences are a primary effect due to the loss of *Dicer* in *Pax2* expressing cells of the first pharyngeal arch, rather than a secondary effect.

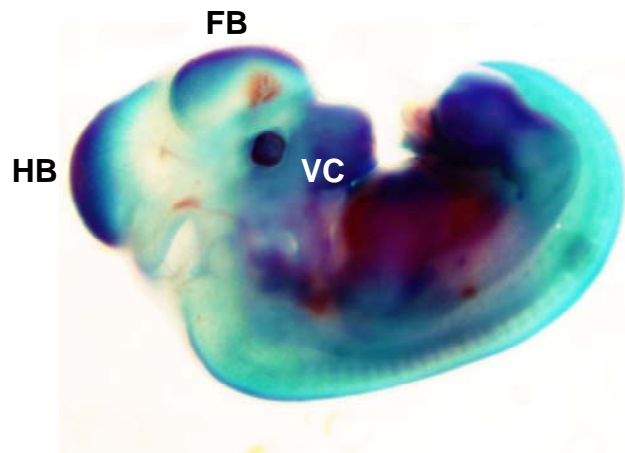


Figure 9. Expression pattern of *Pax2Cre*-driven *LacZ*. At E12.5 the hindbrain (HB), forebrain (FB), and throughout the viscerocranial (VC) regions are stained blue, indicating that they descended from tissues that once expressed *Pax2Cre*. Scale bar = 2mm.

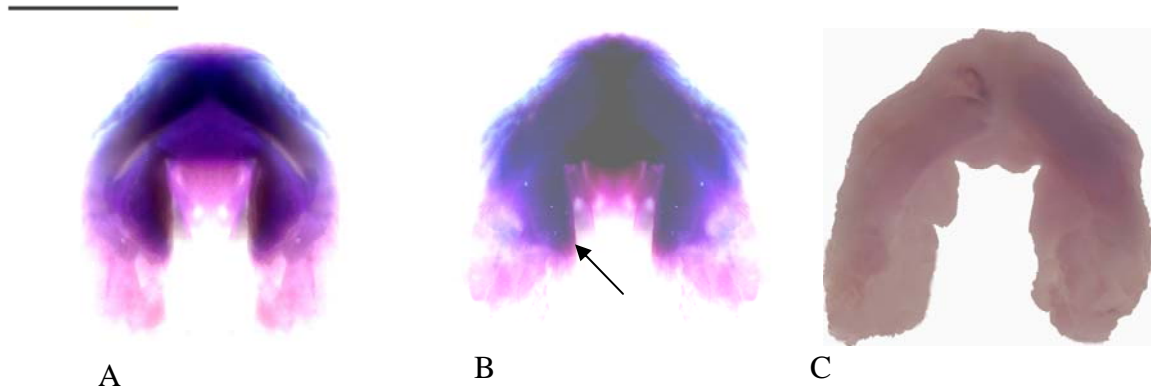


Figure 10. Exogenous *Cre* expression overlaps with endogenous *Pax2* expression in E12.5 palatal shelves. In E12.5 *Pax2Cre/Dicer^{WT}* embryos, the expression pattern of exogenous *Cre* (A) overlaps with the expression pattern of endogenous *Pax2* (B) in the palatal shelves (arrows). No probe control (C). Scale Bar = 1mm

D. Reciprocal expression patterns of *Cre* and *Dicer*

ISH expression patterns of *Cre* and *Dicer* in $Pax2Cre^+/Dicer^{LoxP}$ and WT embryos were compared using riboprobes specific for either *Cre* or *Dicer* mRNA. The riboprobe used to detect *Dicer* expression is specific for exon 2 of the *Dicer* transcript which is eliminated upon Cre-mediated recombination in the $Pax2Cre/Dicer^{LoxP}$ embryos. *Cre* expression was analyzed in both $Pax2Cre^+/Dicer^{WT}$ and $Pax2Cre^+/Dicer^{LoxP}$ embryonic brains. Results indicate that in both $Pax2Cre^+/Dicer^{WT}$ and $Pax2Cre^+/Dicer^{LoxP}$ embryos, *Cre* is expressed in the midbrain/hindbrain area (Fig. 11). Comparison of *Dicer* expression between $Pax2Cre^+/Dicer^{WT}$ and $Pax2Cre^+/Dicer^{LoxP}$ embryos shows *Dicer* mRNA is missing in embryos homozygous for floxed *Dicer* alleles, specifically in areas overlapping with *Cre* expression (Fig. 12).

Altogether, these ISH results attest to the effectiveness of *Pax2Cre* to mediate *Dicer* deletion in a spatially restricted manner. *Dicer* mRNA expression in the palatal shelves of E12.5 $Pax2Cre/Dicer^{LoxP}$ embryos was residual when compared to a WT (*Pax2Cre*-negative) littermate (Fig. 13). Consistent with our previous analysis of *Pax2* and *Pax2Cre* expression in $Pax2Cre^+/Dicer^{WT}$ mice, *Pax2Cre* was expressed in the palatal shelves.

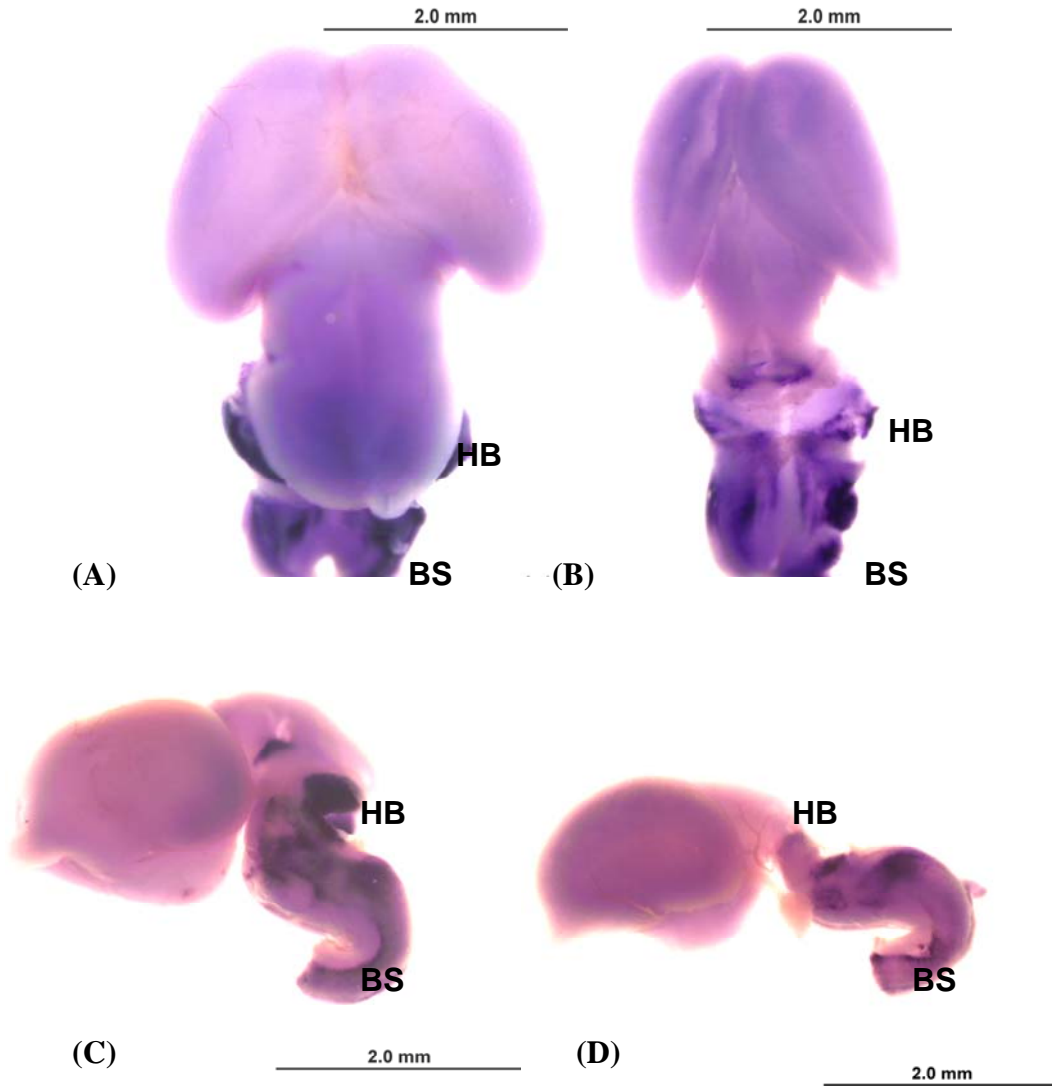


Figure 11. *Cre* expression in *Pax2Cre/Dicer*^{LoxP} and *Pax2Cre/Dicer*^{WT} E12.5 embryo brains using ISH. (A and C). Caudal and saggital views of *Cre* mRNA expression detected by ISH in *Pax2Cre/Dicer*^{WT} embryos. (B and D) Caudal and saggital views of *Cre* mRNA expression in *Pax2Cre/Dicer*^{LoxP} embryos. Abbreviations: hindbrain (HB), brainstem (BS) Scale Bar = 2mm.

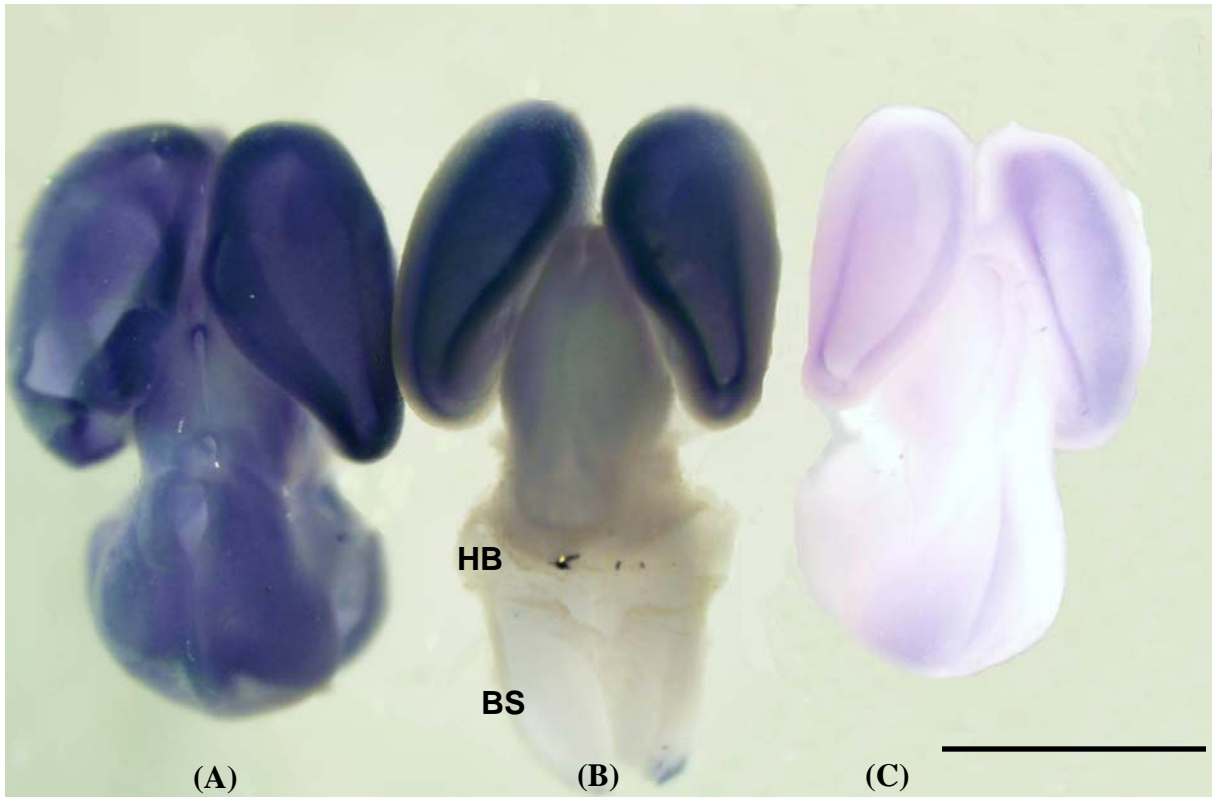


Figure 12. ISH using an antisense probe against *Dicer* mRNA in E12.5 control and *Pax2Cre/Dicer^{LoxP}* brains. WT brains show ubiquitous *Dicer* expression (A). *Pax2Cre/Dicer^{LoxP}* embryos (B) are lacking *Dicer* mRNA in tissues where *Cre* was shown to be expressed (see Fig 11), specifically, they are lacking *Dicer* mRNA detection in the hindbrain (HB) and brainstem (BS). No probe control (C). Scale Bar = 2mm

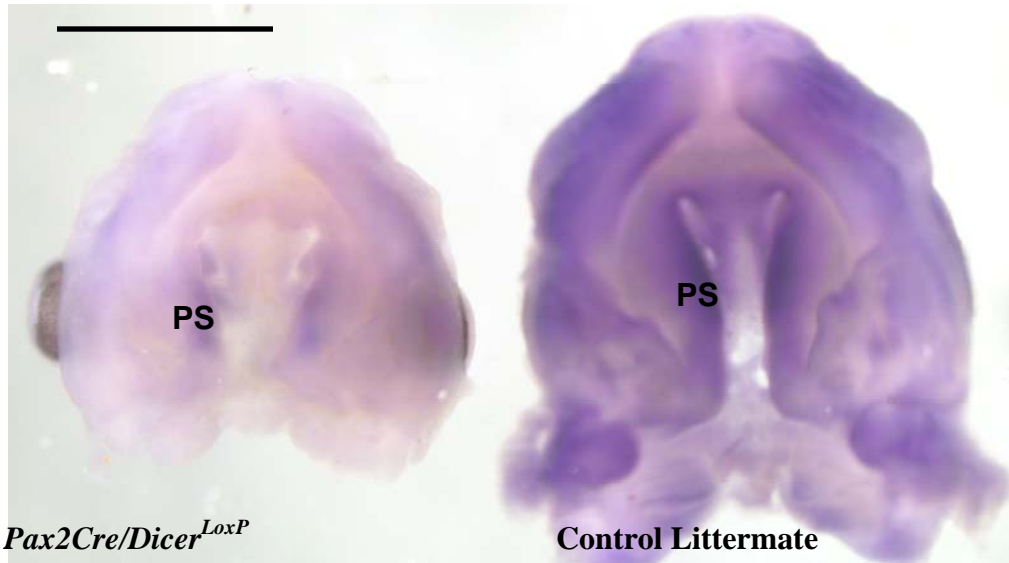


Figure 13. Decreased *Dicer* mRNA in E12.5 *Pax2Cre/Dicer^{LoxP}* palates. With the mandible removed, E12.5 *Pax2Cre/Dicer^{LoxP}* embryos have residual *Dicer* mRNA detected in the secondary palatal shelves, but there is decreased detection compared with the littermate control. Scale Bar = 2mm

E. Relative expression levels of *Bmp4*, *Fgf8* and *Fgf10* in E17.5 control and *Pax2Cre/Dicer^{LoxP}* palatal tissue

Palatogenesis is dependent on many genetic pathways and involves reciprocal regulation and signaling between the ectoderm and mesenchyme that is mediated by a variety of growth factors and signaling molecules. Prominent factors known to be involved in cleft palate belong to the BMP and FGF families. *Bmp4*, *Fgf8*, and *Fgf10* mRNA expression levels were tested between *Pax2Cre/Dicer^{LoxP}* and control littermates.

Micro-dissected palatal tissue was obtained from four E17.5 WT embryos and four *Pax2Cre/Dicer^{LoxP}* littermates. Total RNA was extracted from this tissue, and quantitative PCR (Q-PCR) performed in quadruplicate to compare the relative expression level of *Bmp4*, *Fgf8*, and *Fgf10* using *β-actin* as an internal control. No differences were found in the relative mRNA expression levels of *Bmp4*, *Fgf8*, or *Fgf10*, between control and *Pax2Cre/Dicer^{LoxP}* (Fig. 14), suggesting that the cleft of the secondary palate of the *Pax2Cre/Dicer^{LoxP}* embryos is not due to a loss of expression of these genes.

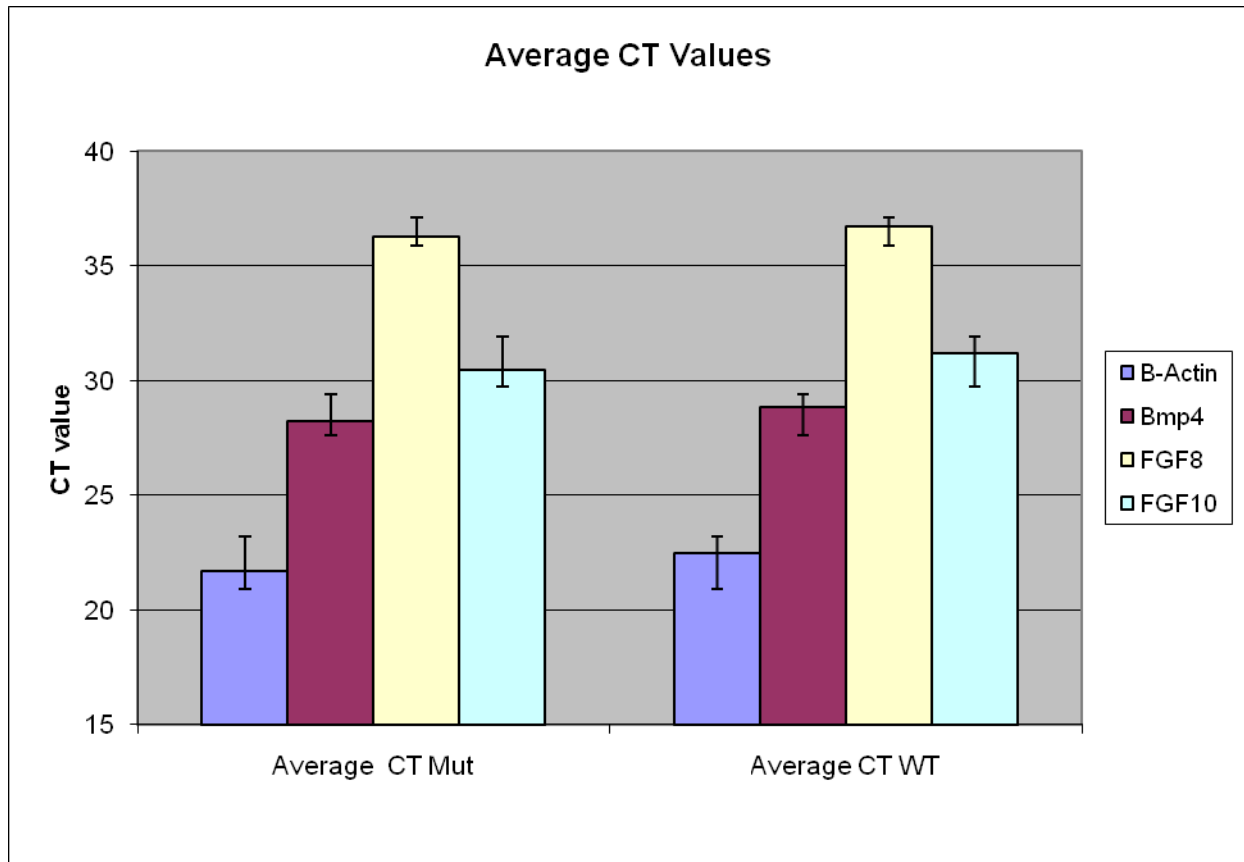


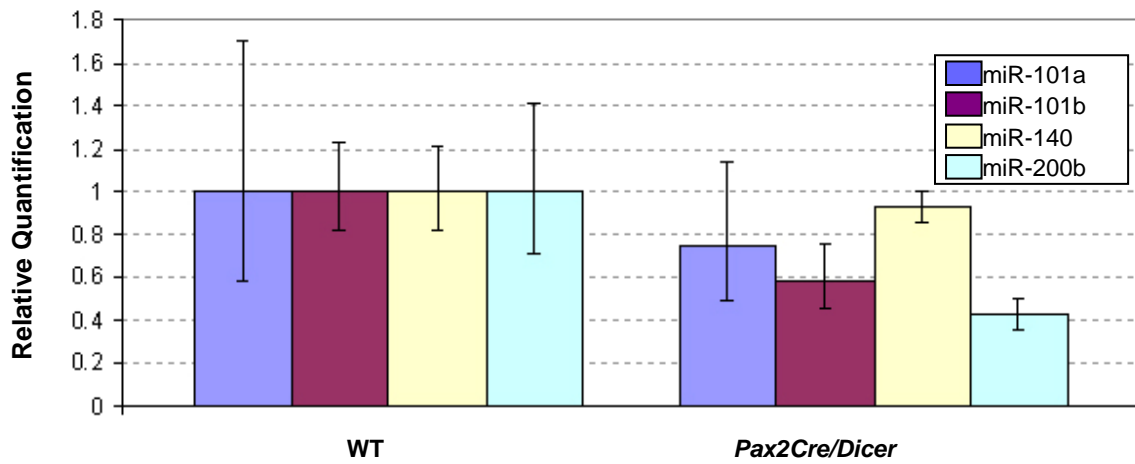
Figure 14. Q-PCR comparing relative expression of *Bmp4*, *Fgf8*, *Fgf10* between *Pax2Cre/Dicer^{LoxP}* and control embryos at E17.5. To test whether genes commonly implicated in dimorphic palate development could be positively associated with the cleft palate of *Pax2Cre/Dicer^{LoxP}* embryos, Q-PCR was used to determine the relative expression levels of *Bmp4*, *Fgf8*, and *Fgf10* in the palatal tissue (WT embryos) and palatal shelves (*Pax2Cre/Dicer^{LoxP}*) relative to β -actin. T-tests showed there was no significant difference in the mRNA expression of any of the genes examined ($P>0.05$).

F. Relative Quantification of select miRNAs

Palatal tissue RNA used for Q-PCR with *Bmp4*, *Fgf8*, and *Fgf10* was also used for Q-PCR with probes targeting miR-101a, miR-101b, miR-140, and miR-200b. These miRNA have been shown in a prior miRNA micro-array analysis to be down-regulated in E17.5 *Pax2Cre/Dicer^{LoxP}* palatal tissue, and have been suggested to have regulatory functions within gene pathways involved in craniofacial development. Q-PCR was used to confirm the microarray data, and to show that loss of *Dicer* results in loss of miRNA.

miRNA miR-101b, and miR-200b were shown to be significantly down-regulated in *Pax2Cre/Dicer^{LoxP}* palatal tissue compared with their control littermates, while miR-140 and miR-101a were down-regulated, but were not statistically significant (Fig 15).

A.



	Min RQ	Max RQ	Significance
miR-101a	0.50	1.14	No
miR-101b	0.46	0.75	Yes
miR-140	0.86	1.00	No
miR-200b	0.36	0.50	Yes

B.

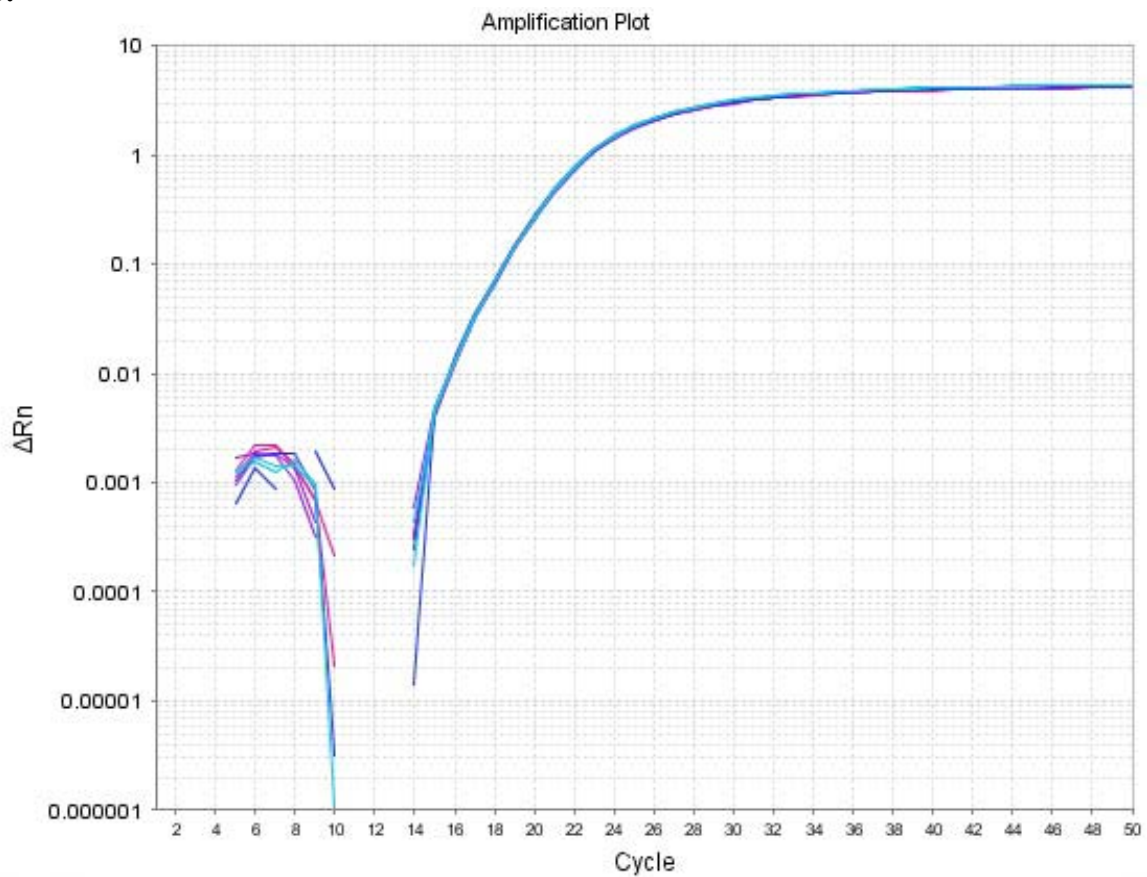


Figure 15. Relative quantification indicates a significant decrease in miRNA 101b, and 200b. A. Q-PCR indicates that in *Pax2Cre/Dicer^{LoxP/LoxP}* embryos, miRNA miR-200b and miR-101b are significantly decreased in E17.5 palatal tissue compared to their WT littermates. miRNA expression in *Pax2Cre/Dicer^{LoxP/LoxP}* was quantified relative to their littermates, Min and Max RQ values indicate a $P \leq 0.01$ confidence interval. **B.** Amplification plot of sno-202 control for both control and *Pax2Cre/Dicer^{LoxP/LoxP}* mRNA shows consistency in amplification, indicating that sno-202 is a proper control.

G. Spatiotemporal expression of *Sox9* in *Pax2Cre/Dicer^{LoxP}* and control littermates

Sox9 and *Sox10* are common molecular markers of viscerocranial contributing CNC (Mori-Akiyama et al. 2003). Contribution of CNC to the viscerocranium was examined by analyzing the expression pattern of *Sox9* in littermate control and *Pax2Cre/Dicer^{LoxP}* embryos between E9.5 and E12.5. This time period encompasses the end of CNC migration to the first pharyngeal arch, the onset of palatogenesis, and the formation of the viscerocranial skeletal precursors. At E9.5 the *Pax2Cre/Dicer^{LoxP}* embryos heads are visibly smaller, and there are morphological differences in the rhombencephalon. In both the control and *Pax2Cre/Dicer^{LoxP}* embryos *Sox9* is expressed in the first pharyngeal arch (PA1) and in the otic pit (OP). At E9.5 no difference in *Sox9* ISH staining in the first pharyngeal arch was found among control and *Pax2Cre/Dicer^{LoxP}* embryos (Fig. 16).

At E10.5, both control and *Pax2Cre/Dicer^{LoxP}* embryos express *Sox9* throughout the mandibular and maxillary processes of the first pharyngeal arch. Moreover, the *Pax2Cre/Dicer^{LoxP}* embryo shows an expanded area of *Sox9* expression in the midbrain and hindbrain region, relative to the control embryo (Fig. 17). At E11.5, *Sox9* is expressed in the mandibular and maxillary processes of first pharyngeal arch in both the control and *Pax2Cre/Dicer^{LoxP}* embryos. Similar to E10.5, *Pax2Cre/Dicer^{LoxP}* embryos at E11.5 have expanded areas of *Sox9* expression in the mid and hindbrain areas, as well as in the posterior first pharyngeal arch. In addition, other morphological differences are visible at E11.5 in the hindbrain, which is smaller in the *Pax2Cre/Dicer^{LoxP}* embryo than in its control littermate (Fig. 18).

Because body sizes are too large for whole embryo ISH, E12.5 embryos were microdissected. Whole brain, maxilla, and mandible removed to visualize the palate and the brain separately. Differences in *Sox9* expression between control and *Pax2Cre/Dicer^{LoxP}* are more conspicuous at E12.5 than at earlier ages (Fig. 19). E12.5 control embryos have *Sox9* staining detected in the hindbrain, forebrain, and brainstem, but no staining in the midbrain. E12.5 *Pax2Cre/Dicer^{LoxP}* embryos have *Sox9* staining detected in the midbrain and have no staining detected in the hindbrain. E12.5 control and *Pax2Cre/Dicer^{LoxP}* do have similar *Sox9* staining in the forebrain.

In their maxilla, both E12.5 control and *Pax2Cre/Dicer^{LoxP}* littermates have staining detected along the midline, in the tissues which will later contribute to the vomer, nasal capsule, and pre-sphenoid. Nevertheless, as seen in (Fig. 18) the overall extent of *Sox9* staining at E12.5 in the *Pax2Cre/Dicer^{LoxP}* is diminished compared to the WT.

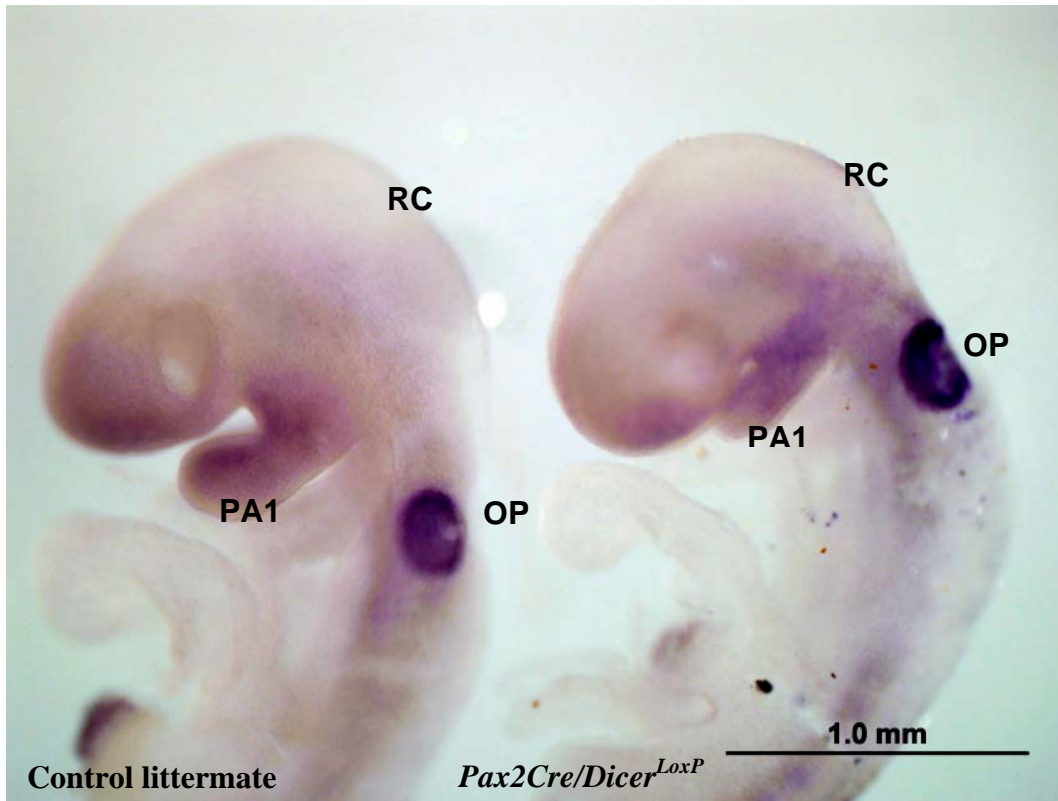


Figure 16. *Sox9* detection at E9.5 is similar in the first pharyngeal arch in control and *Pax2Cre/Dicer^{LoxP}* littermates. At E9.5 there is little difference in *Sox9* expression between the control and *Pax2Cre/Dicer^{LoxP}* embryos. *Sox9* is expressed in the first pharyngeal arch (PA1) and the otic pit (OP) in both embryos. The *Pax2Cre/Dicer^{LoxP}* rhombencephalon (RC) is smaller than the control. Scale bar = 1 mm.

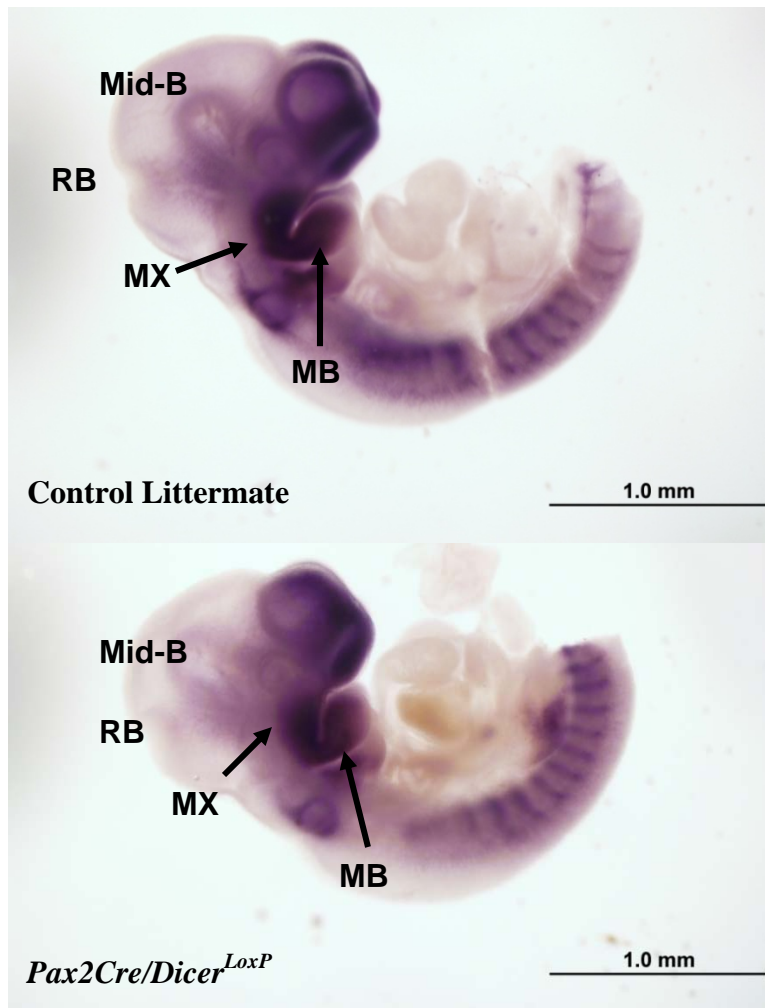


Figure 17. *Sox9* detection at E10.5 is similar between control and *Pax2Cre/Dicer^{LoxP}* littermates. At E10.5, *Pax2Cre/Dicer^{LoxP}* embryos have similar *Sox9* staining patterns in their first pharyngeal arch maxillary (MX) and mandibular (MB) processes (arrows). The *Pax2Cre/Dicer^{LoxP}* embryos have expanded areas of *Sox9* staining toward the midbrain (Mid-B) and rhombencephalon (RB) compared with their control littermate embryos. This pattern was repeated throughout our analysis. Scale bar = 1mm.

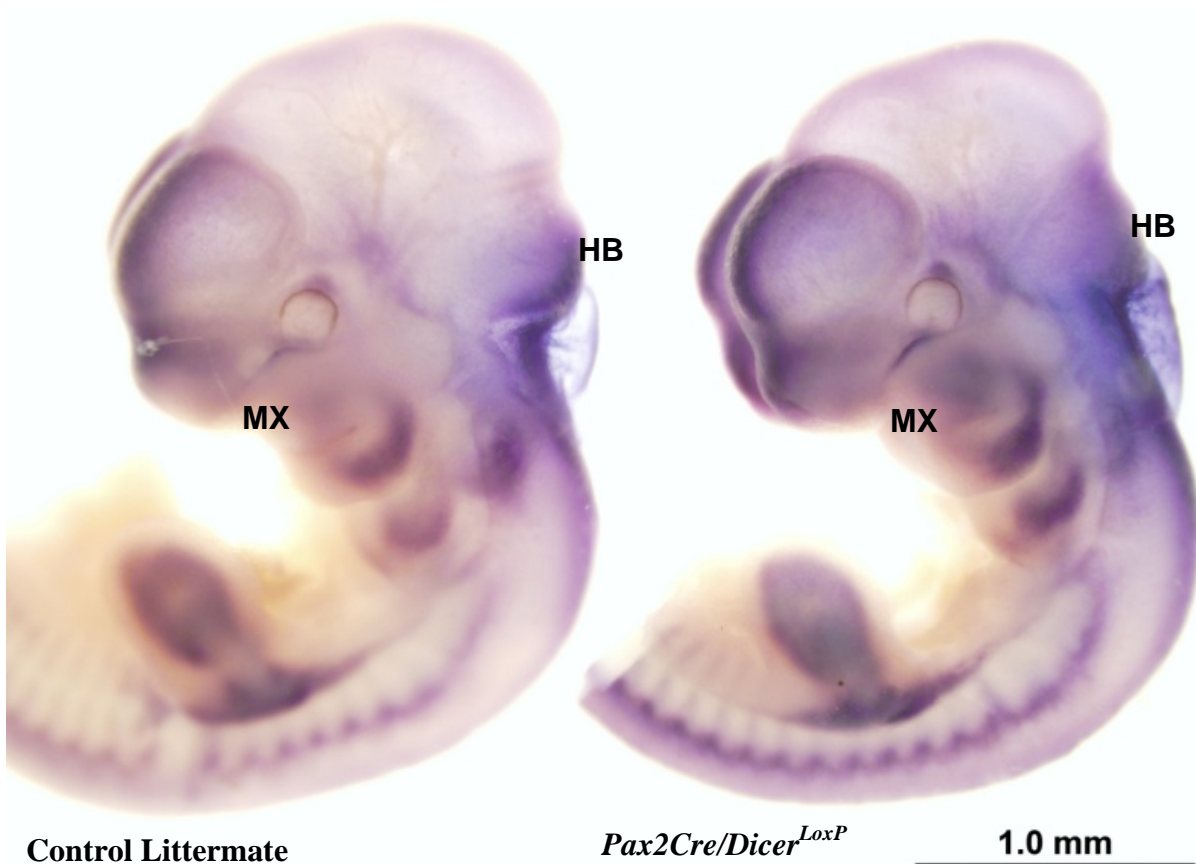


Figure 18. *Sox9* expression at E11.5. At E11.5 *Sox9* expression patterns continue to be expanded in the *Pax2Cre/Dicer^{LoxP}* embryos versus their control littermate embryos. In the *Pax2Cre/Dicer^{LoxP}* morphologic changes are visible in the rhombencephalon/mid-hind brain (HB) where expanded *Sox9* expression patterns are seen. Expanded regions of *Sox9* staining in the maxillary (MX) portion of the first pharyngeal arch are visible in *Pax2Cre/Dicer^{LoxP}*. Scale Bar = 1mm. Abbreviations: (MX) Maxillary process, (HB) Hindbrain

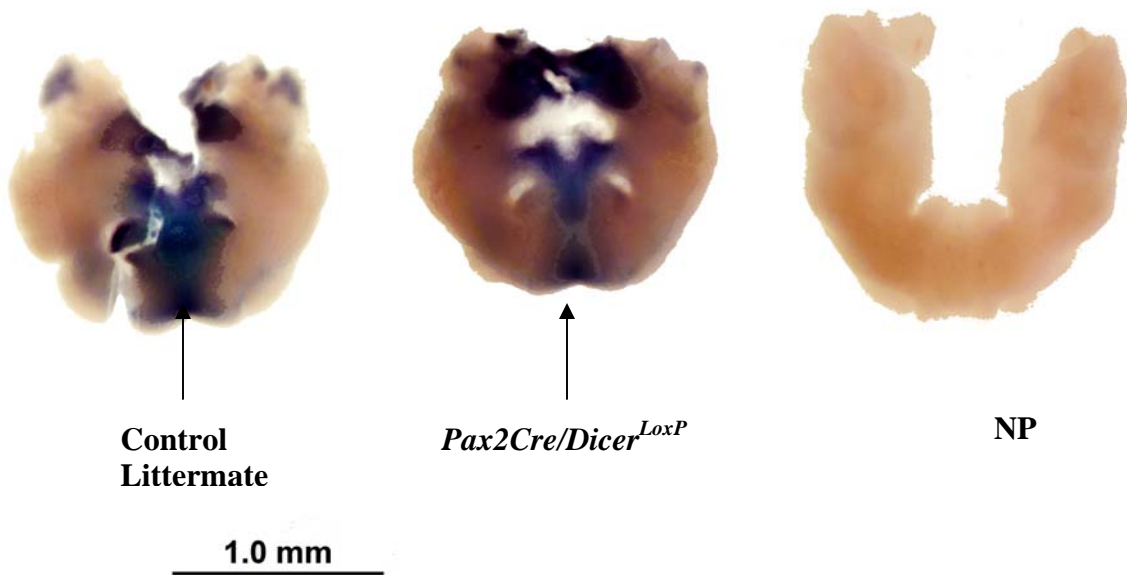
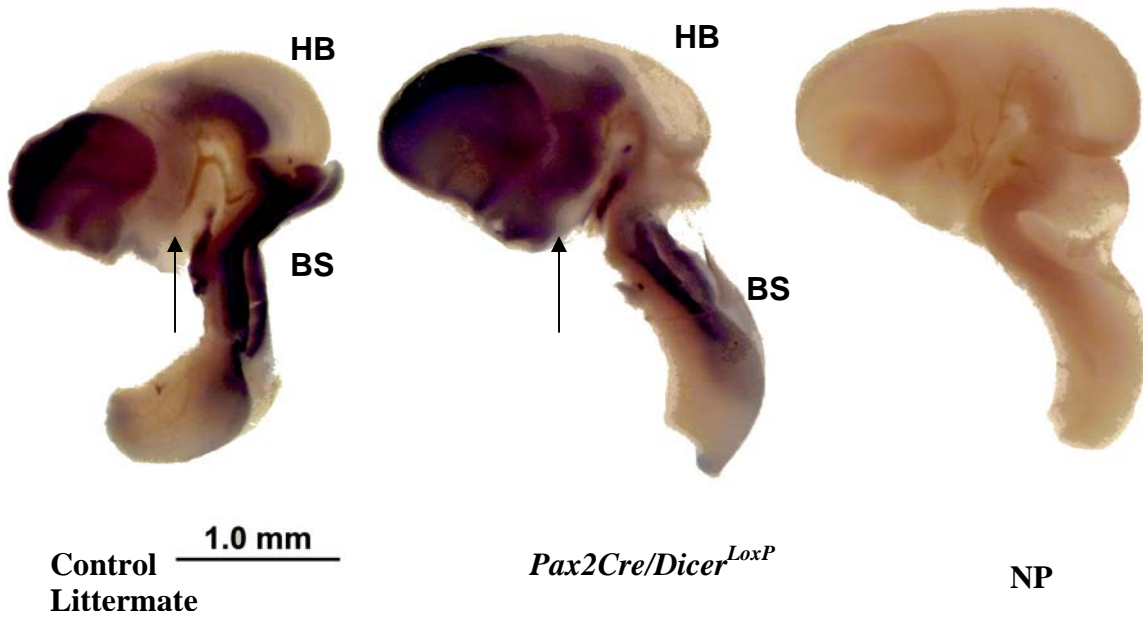


Figure 19. *Sox9* staining patterns in the brains and palates of *Pax2Cre/Dicer^{LoxP}* and control littermates. Control littermate brain shows *Sox9* expression along the caudal portion of the hindbrain (HB), and in the brain stem (BS) with no detectible expression in the midbrain (arrow). The *Pax2Cre/Dicer^{LoxP}* embryo brain shows *Sox9* detection in the midbrain (arrow) but not in the hindbrain (HB). When compared with the control, expression is similar in the brain stem (BS) of the *Pax2Cre/Dicer^{LoxP}* palate although the embryo has diminished staining in the progenitor tissue for the vomer and nasal bones (arrow). Scale bar = 1.0 mm. Abbreviations: (HB) Hindbrain, (BS) Brain Stem, (NP) No Probe Control.

III. Discussion

A. Overview

Dicer is required for proper palatal development and *Pax2Cre* is expressed in palatal progenitors. A loss of *Dicer* through *Pax2Cre* mediated recombination of floxed *Dicer* alleles results in an overall reduction in embryonic size, a cleft of the secondary palate, loss of the development of the temporal, zygomatic, and tympanic rings, hypoplastic presphenoid, basisphenoid, and nasal bones, and late embryonic lethality in *Pax2Cre+ / Dicer^{LoxP}* embryos.

B. Morphological consequence of *Dicer* deletion in developing orofacial tissue

Pax2Cre / Dicer^{LoxP} embryos are visibly smaller as early as E9.5. This pattern of overall smaller body size is consistent throughout development and may be due to an early loss of *Dicer* in progenitor cells throughout the embryo, or a loss of *Dicer* at other *Pax2* mediated growth signaling centers such as in the kidneys or the frontonasal prominence (Barembaum and Bronner-Fraser 2009; Koenig et al 2010; Chen et al 2009, Goode and Elgar 2009).

Analysis of coronal sections revealed that the first morphological differences in the developing palatal shelves among *Pax2Cre / Dicer^{LoxP}* and control littermate embryos are visible at E11.5; *Pax2Cre / Dicer^{LoxP}* embryos lack the palatal bulge expected by this stage of development. A similar feature of palatal shelf development was seen in E12.5 embryos. Control embryos have definitive palatal shelves whereas the *Pax2Cre / Dicer^{LoxP}* embryos have a flat medial maxillary process. These morphological features could be due to increased apoptosis and/or decreased proliferation in these tissues as a result of their

loss of key miRNAs, as seen in other conditional *Dicer* knockouts (Marsden and Ho 2008; Wang et al. 2008, Soukup et al. 2009). Morphologically, the palatal development of *Pax2Cre/Dicer^{LoxP}* is stopped at the equivalent of an E12.5 developmental stage.

In addition to the developmental defects observed in palatogenesis, conditional, *Pax2Cre* mediated *Dicer* deletion results in the loss and/or malformation of several bones of the cranium, including the zygomatic, and temporal bones, and the basisphenoid, presphenoid, and palatine bones. Similar losses or developmental dimorphisms of cranioskeletal bones are seen in developmental defects collectively known as neurocristopathies, and is phenocopied in a conditional deletion of *Sox9* in CNC (Bi et al. 2001; Mori-Akiyama et al 2003; Helms et al. 2006; Passos-Bueno et al. 2009). The defects seen in the *Pax2Cre/Dicer^{LoxP}* embryos resemble those seen in *Wnt1Cre/Sox9^{LoxP}* embryos in which *Sox9* is conditionally deleted in all *Wnt1Cre* expressing cells (Crombrughe et al. 1997; Bi et al. 2001; Mori-Akiyama et al. 2003). Deletion of floxed *Sox9* alleles in *Wnt1Cre* lines of mice leads to severe cranioskeletal defects including completely absent endochondrally derived bones such as the presphenoid and basisphenoid. The absence of the presphenoid and basisphenoid in *Wnt1Cre/Sox9^{LoxP}* embryos results in a cleft of the secondary palate as a secondary effect due to a loss structures necessary to support palatal development. In humans, haploinsufficiency of *Sox9* results in a syndrome known as campomelic dysplasia, which in addition to defects in the long bones, results in craniofacial defects including a cleft of the secondary palate, due to malformation of the endochondrally derived bones of the face. Though *Dicer* is ubiquitously expressed, to date, no neurocristopathy is known to involve defects in *Dicer* or miRNA.

Pax2Cre/Dicer^{LoxP} embryos have a hypoplastic mandible; inadequate growth and development of the mandible can inhibit dropping of the tongue as the palatal shelves rotate horizontally, resulting in a secondary cleft of the secondary palate. It is possible that the *Pax2Cre/Dicer^{LoxP}* mice could experience the cleft of their secondary palate due to a physical blockage of the horizontal elevation of the palatal shelves by the tongue. In the coronal sections of E16.5 embryos, the tongue can be seen adjacent to the palatal shelves (Fig. 5), however, the tongue is not protruding into the nasal cavity which would be expected if the cleft were due to physical blockage of the palatal shelves.

Additionally, the complete absence of ossified palatine bones (Fig. 8) indicates that the cleft of the *Pax2Cre/Dicer^{LoxP}* embryos is not likely to be a secondary effect of their hypoplastic mandible, but rather a defect in CNC ability to differentiate into osteocytes.

Pax2Cre⁺/Dicer^(LoxP/WT) heterozygous knockout embryos, used as stud males for timed breeding, do not have any visible craniofacial or viscerocranial dimorphic phenotypes, suggesting the mutations seen in the homozygous null mice may be dose-dependent on the residual miRNA, Dicer enzyme, and *Dicer* mRNA. A lack of defects indicates that a single copy of the functional *Dicer* allele is sufficient for normal function. The absence of visible defects in the heterozygous knockout is consistent with observations in other conditional knockouts of *Dicer* in that the heterozygous knockout is morphologically indistinguishable from control littermates viable and can be bred in timed breeding (Marsden and Ho 2008; Wang et al. 2008, Soukup et al. 2009).

C. *Pax2Cre* expression in the cranium

In 2004, Ohyama and Groves suggested that the *Pax2Cre* line of mice could be used primarily to knockout a floxed allele in the developing inner ear (Ohyama and Groves, 2004). When they designed the bacterial artificial chromosome into which *Pax2Cre* was inserted, they included a sequence 101 kb upstream of the *Pax2* start codon, in order to try to include any promoter, enhancer, and silencing elements that were present upstream for *Pax2* transcriptional control. They also included a 20 kb segment downstream of the *Pax2* start codon. In their initial assessment of the expression pattern of *Pax2Cre* compared with native *Pax2* they found that they had replicated staining patterns in the inner ear, but they noted that a *Pax2Cre* reporter, detected by LacZ staining, was indicating *Pax2Cre* expressing tissue was present in the first pharyngeal arch. Our data, which indicate that both *Pax2* and *Cre* are expressed in the palatal shelves, and a loss of *Dicer* mRNA in the palatal shelves of *Pax2Cre/Dicer^{LoxP}* embryos, shows that *Pax2Cre* can be an effective mediator of deletion of a floxed allele in palatal precursors, and that *Pax2Cre* may be expressed in CNC.

Pax2 and *Pax2Cre* may be first expressed in the CNC that later populate the first pharyngeal arch. Evidence in the literature suggests that early in embryonic development, both *Pax2Cre* and endogenous *Pax2* are expressed in the region which later becomes the midbrain/hindbrain areas (Ohyama and Groves 2004; McMahon and Rowitch 1995). This region overlaps with the origin of CNC that populate the mesenchyme of the first pharyngeal arch, which emerge adjacent to the first two rhombomeres of the rhombencephalon (Trainor et al. 2001; Krumlauf and Trainor 2002). *Pax2* has previously been shown to be expressed in an overlapping domain with

both *Wnt-1* and *En-1* which are expressed early in presumptive CNC before they delaminate and migrate to the first pharyngeal arch (McMahon and Rowich 1995; Trainor and Krumlauf 2001; Crombrughe 2009; Zervas 2009). Ohyama and Groves (2004) also show evidence for early expression of *Pax2* and *Cre* expression in *Pax2Cre* embryos in the primitive rhombencephalon, at the 0-1 somite stage. In addition, *Wnt-1* is commonly used as a molecular marker of first arch CNC, using a *Wnt-1Cre* line to drive LacZ expression in the CNC, allowing their fate to be detected at a later date (Morki-Akiyama et al. 2003). Our data, together with the literature, indicate that *Pax2Cre* is an appropriate driver for the deletion of *Dicer* or other floxed alleles in the first pharyngeal arch palatal precursors, and that *Pax2* is expressed in the palatal shelves at E12.5.

E. Loss of miRNA mediated regulation

Several studies have shown that a loss of *Dicer* can result in a loss of miRNA mediated regulation of gene expression. Gaur et al. (2010) found that a loss of *Dicer* in adult mouse osteocytes resulted in an anabolic state in which the bones became hypertrophic. Gaur et al. (2010) correlated the anabolic state to the loss of regulatory miRNA such as miR-29b which has previously been shown to down regulate the excretion of *Colla1*, a bone matrix molecule (Gaur et al. 2010; Luna et al. 2009). In adult mice, deletion of floxed *Dicer* in osteoblasts results in an increase in the synthesis of bone matrix and thickening of cortical and trabecular bone matrix, suggesting that a loss of *Dicer* in differentiated osteoblasts results in a loss of their ability to regulate cell matrix deposition due to a loss of miRNA, such as miR-29b, to regulate these gene's expression (Gauer et al 2010).

Q-PCR and previous microarray analysis (data not shown) of miRNA expression in palatal tissues at E17.5 have shown that *Pax2Cre/Dicer^{LoxP}* embryos experience a significant loss of miRNA miR-101b, and miR-200b by E17.5 (See Fig 15). miR-200b belongs to the miR-200 family which are known to be regulators of mesenchymal-to-epithelial transition (Christoffersen, et al. 2007). miR-200b specifically has been shown by Christoffersen et al. (2007) to modulate expression of *Zfhx1b* and thereby regulate the activity of the promoter for E-cadherin. In the literature, current knowledge of miR-101b is limited to its predicted role (Bartel et al. 2007). miR-101b has been predicted to target several genes that are important to craniofacial development and palatogenesis, such as *Col10a1* which is expressed by hypertrophic chondrocytes during endochondral ossification. In humans, mutations in *COL10A1* are associated with Schmid-type metaphyseal chondroplasia, a type of familial hereditary dwarfism affecting the growth of the long bones (Lucarini et al. 2009; Megarbane et al. 2008; Wilson et al. 2002). miR-101b also has two conserved predicted target sites in the 3' UTR of *Sox9* which was used as a molecular marker of chondroprogenitor CNC in this present study (Bartel et al. 2007). The quantified decrease in miR-101b, which is a predicted down-regulatory element for *Sox9*, may be a mechanism by which *Sox9* mRNA detection was increased in the midbrain and hindbrain of *Pax2Cre/Dicer^{LoxP/LoxP}* embryos.

Analysis of relative miRNA expression levels at the onset of palatogenesis in *Pax2Cre/Dicer^{LoxP}*, and the possible roles of miR-101b in regulation of *Sox9* remain to be determined. Additionally, a quantitative analysis of protein levels is necessary to form a complete picture of the consequence of loss of miRNA biogenesis in *Pax2Cre/Dicer^{LoxP}* cranioskeletal and palatal tissues.

F. Cre – mediated *Dicer* deletion

Together, our data indicate that *Dicer* is necessary for proper palatogenesis. Comparison of *Cre* and *Dicer* expression through ISH using *Pax2Cre*^{+/+}/*Dicer*^{WT} and *Pax2Cre*/*Dicer*^{LoxP} embryos suggests that *Pax2Cre* expression within a tissue results in decreased or missing *Dicer* mRNA in *Pax2Cre*/*Dicer*^{LoxP} embryos. E12.5 ISH data, showing reduced *Dicer* mRNA detection in *Pax2Cre*/*Dicer*^{LoxP} embryos suggests that they begin palatogenesis with residual levels of *Dicer* mRNA in their palatal shelves and maxillary process compared to their control littermates.

The palates of *Pax2Cre*/*Dicer*^{LoxP} embryos do not develop past the morphological characteristics of an E12.5 control embryo, indicating an important role for *Dicer* in orofacial development. In other *Cre-LoxP* mediated transgenic lines of conditional *Dicer* deletion, decreased growth and differentiation has been associated with decreased miRNA abundance, increased apoptosis, decreased proliferation, and loss of cytoskeletal architecture (Wang et al. 2008; Mardsen and Ho 2008; Miner et al. 2008). Depletion of key miRNA, following *Dicer* deletion seems to depend on the metabolic status of the tissue in which the deletion occurs (Soukup et al. 2009; Wang et al. 2008). Deletion of *Dicer* in a quiescent cell or tissue may have a delayed effect compared with deletion of *Dicer* in a metabolically active cell which must have tight regulation on its gene expression, such as a cell that is secreting ECM, or undergoing differentiation. During palatogenesis, high rates of cell metabolism are required to allow for proliferation, apoptosis, cell phenotype transition, extracellular matrix synthesis, and cell migration (Ferguson 1988; Svoboda and Kang 2005; Chai and Maxon 2006; Clouthier et al. 2007; Gritli-Linde 2007; Clouthier et al. 2009; Passos-Buenos et al. 2009), all of which are

common processes taking place during the development of complex tissues and structures. Presumably, the changes in a cell's gene expression pattern as it undergoes differentiation requires tight control, of which miRNA may play an important part. Further studies in *Pax2Cre/Dicer^{LoxP}* embryos must be done to examine any changes in apoptosis or cell proliferation during palatogenesis in the conditional *Dicer* knockouts. Based on previous studies of *Dicer* knockout in other tissues, it would be expected that increases in apoptosis would be observed (Harfe et al. 2005; Zeheir et al. 2010). Furthermore, since the Dicer enzyme is responsible for maturation of other ds-RNA such as siRNA, investigation into the additional products of Dicer enzyme activity is necessary for complete understanding of the consequence of *Dicer* deletion (Lima et al 2009).

Given the high level of metabolic activity during palatogenesis, the expression pattern of *Pax2Cre* in the upper rhombencephalon, and the expression pattern of endogenous *Pax2* in the neural plate prior to CNC migration, the orofacial dimorphisms in *Pax2Cre/Dicer^{LoxP}* mice may be caused by the depletion of *Dicer* mRNA in CNC at the time of their delamination and migration (Serbedzija et al. 1992; McMahon and Rowich 1995; Chai et al. 2000; Trainor et al. 2003; Ohyama and Groves 2004). Developmentally, this would mean that the CNC would have had their floxed *Dicer* alleles recombined by embryonic day E8.0, nearly four days prior to the onset of palatogenesis. Subsequent cell divisions, morphological changes, and synthesis of ECM, could dilute any residual miRNA, remaining *Dicer* mRNA, and Dicer enzyme remaining in the palatogenic tissues, and further disrupt palatogenesis.

Since miRNA are relatively stable in their miRNA-AGO2 complex, many effects of the loss of the floxed *Dicer* alleles would depend on the half life of each specific

miRNA. Recently, Kia and Pasquinelli (2010) suggested that miRNA homeostasis can be actively modulated by cis-acting modifications and trans-acting proteins, and that miRNAs can be actively degraded by endonucleases. Since miRNAs are stable and protected when bound with the argonaute protein, functional deficiencies after loss of *Dicer* may depend on the rate of loss of miRNA due to either active degradation, passive degradation, or dilution following cell division (Grosshans and Chatterjee 2009; Soukup et al. 2009). Variability in the rate of degradation of residual miRNA, *Dicer* mRNA, and *Dicer* protein has been seen in other transgenic models using the *Cre/LoxP* system for *Dicer* deletion (Wang et al. 2008). This indicates that a functional assessment of the role of specific miRNA may not be possible without tracking the rate of their loss at several time points.

G. Role of *Bmp4*, *Fgf8*, and *Fgf10* in *Pax2Cre/Dicer^{LoxP}* palatogenesis

Palatogenesis is dependent on many genetic pathways and involves reciprocal regulation and signaling between the ectoderm and mesenchyme which is mediated by a variety of growth factors, prominent factors known to be involved in cleft palate include the BMP and FGF families (Neubusster and Bachler 2001; Wilke and Morris-Kay 2001; Cobourne 2004; Nie et al. 2006).

Bmp4 has been shown to have specific expression domains that are segregated between the anterior and posterior portions of the palatal shelves; and these domains change during palatogenesis (Lee et al. 2006). By E16.5, *Bmp4* is upregulated in both the posterior and anterior palate, and is expressed in both the palatine bone and the mesenchyme (Nie X.G. 2005).

Fgf8 is known to be involved in fusion of the midfacial prominences between E9.5 and E10.5 (Gong et al. 2005), and in the palate, *Fgf8* is known to be involved with the induction of transcriptional mediators of cell fate, such as *Pax9* which may be involved with embryonic skeletal development (Lefebvre and Smits 2005; Hilliard et al. 2005). Impairment of *Fgf8* and *Fgf10* signaling has been shown to contribute to cleft lip and cleft palate, as both factors are involved in fusion of mid facial prominences (Riley et al. 2007). Data from Riley et al suggest that members of the FGF signaling pathway may be involved in 3-5% of non-syndromic cleft lip and/or palate (Riley et al. 2007).

To test whether miRNA depletion following *Dicer* deletion may have disrupted the expression of *Bmp4*, *Fgf8*, or *Fgf10* Q-PCR was performed using palatal tissue from E17.5 *Pax2Cre/Dicer^{LoxP}* and control littermate embryos. Results indicated that the expression of the mRNA of *Bmp4* and *Fgf8* and *Fgf10* family of growth factors was not involved in the cleft palate of the *Pax2Cre/Dicer^{LoxP}* embryos, as there was no significant difference in the quantitative detection of any of these mRNAs in the embryos palates. Moreover, since miRNA principally repress the translation of mRNA, proteomics or other quantification studies of relative protein levels must be done to completely test the involvement of these growth factors.

H. The role of CNC in *Pax2Cre/Dicer^{LoxP}* palatogenesis

Nearly all the disrupted cranial skeletal features of *Pax2Cre/Dicer^{LoxP}* embryos are derived from CNC, indicating that the *Pax2Cre/Dicer^{LoxP}* mice may suffer from some kind of a facial neurocristopathy which may result from failure of CNC to migrate, differentiate, or develop into facial structures. Disruption of the development of non-

CNC derived structures could be the result of loss of *Dicer* in signaling and organizing centers of the face such as the forebrain or optic stalk; both of which are known to be areas of *Pax2Cre* expression (Goode et al. 2009; Fotaki et al 2008).

Recently, Eberhart et al. (2008) showed that miRNA miR-140 was responsible for mediating part of the migration pathway for palatogenic CNC in zebrafish. In mice, CNC migrate from the rhombencephalon, past the optic stalk, and into the first pharyngeal arch, between E8.0 and E9.5. Their migration is dependent on a variety of signaling centers which release chemotactic cytokines to guide the CNC migration pathways (Krumlauf et al. 2002; Clouthier and Schilling 2004; Clouthier et al. 2007; Fotaki et al. 2008; Olesnick et al. 2009; Clouthier and Reust 2009; Killian et al. 2009). One such structure which helps to create a chemotactic gradient for CNC is the optic stalk, which is also known to express *Pax2Cre* and could be disrupted by loss of *Dicer* there (Fotaki et al. 2008; Hitchcock et al. 1998). As they migrate and respond to directional cues, CNC must express receptors for specific chemotactic molecules, and this expression has been shown to be regulated by miRNA miR-140 in zebrafish (Eberhart et al. 2008). Lack of *Dicer* and consequently, miRNAs such as miRNA miR-140 in *Pax2Cre/Dicer^{LoxP}* CNC cells, may have impaired the CNC and contributed to the cleft palate and hypoplastic bone development in *Pax2Cre/Dicer^{LoxP}* embryos.

Considering the *Pax2Cre* expression data shown by Ohyama and Groves, showing *Pax2Cre* expression in the future midbrain/hindbrain at the 0-1 somite stage, *Pax2Cre/Dicer^{LoxP}* CNC differentiation may become deregulated as soon as two days after *Dicer* deletion. *Pax2Cre/Dicer^{LoxP}* embryos begin showing misexpression of *Sox9* in the midbrain and hindbrain beginning at E10.5. *Sox9* is a molecular marker for

chondroprogenitor CNC and loss of *Sox9* in CNC has been shown to result in a loss of endochondrally derived cranioskeletal elements such as the basisphenoid and presphenoid; as well as resulting in a cleft palate as a secondary effect due to a loss of structural support upon which the palatal shelves develop (Bi et al. 2001; Mori-Akiyama et al. 2003; Gordon et al. 2009).

Dicer has recently been shown to be required for the survival of differentiating neural crest cells; but not for their migration (Zehir et al. 2010). CNC specific deletion of *Dicer* using *Wnt1Cre* transgenic mice shows that CNC lacking *Dicer* can migrate to the pharyngeal arches in the correct pattern, but once they begin their differentiation program CNC undergo increased apoptosis and a loss of CNC derived structures such as the bones and cartilage of the cranioskeleton (Zehir et al. 2010).

In *Pax2Cre/Dicer^{LoxP}* embryos, *Sox9* expression patterns become dysregulated beginning at embryonic day E10.5, when *Sox9* staining patterns are expanded in the midbrain and hindbrain. At E11.5, *Sox9* expression continues to be expanded in the hind brain, and is increased in the maxillary process. By E12.5, *Sox9* staining pattern is ectopic in the midbrain, and there is a loss of *Sox9* in the precursor to the vomer and nasal bones in the maxilla. Changes in *Sox9* expression in the future cranioskeleton may be due to a loss of miRNA mediated regulation of their differentiation pathways.

Q-PCR showed that miR-101b was downregulated in palatal tissue following *Dicer* deletion. miR-101b has a target site in the 3' UTR of *Sox9* (Bartel, et al. 2007); miss-expression of *Sox9* in the hindbrain, and later in the first pharyngeal arch may be due to a loss of miRNA mediated regulation of its expression pattern, or a loss of miRNA

mediated regulation of upstream factors such as *Shh* or *Bmp2* regulating *Sox9* expression (Park et al. 2010, Gordon et al. 2009; Nakanishi et al 2009).

Decreased *Sox9* expression in the mesenchymal condensations destined to become the vomer, nasal, and maxillary bones, in the medial portion of the maxillary process indicate that the downstream differentiation pathways of the CNC may be affected by their loss of residual miRNA such as miR-101b. Further experiments need to be done to confirm this assumption by examining, at the protein level, expression of definitive markers such as Col2a1 in chondroprogenitor CNC (Mori-Akiyama et al. 2009). Loss of *Sox9* mediated endochondral ossification may contribute to the dysmorphic features found later in the basisphenoid and presphenoid of the *Pax2Cre/Dicer^{LoxP}* embryos. Dysmorphic features of the basisphenoid and presphenoid may result in a cleft palate as a secondary effect due to a loss of support structures upon which the palate must form.

All together, these results show that residual miRNA may still provide sufficient regulation of gene expression in the CNC of *Pax2Cre/Dicer^{LoxP}* for up to three days after *Dicer* is eliminated, but that mature miRNA do become depleted at later time points shown in our miRNA Q-PCR data, earlier loss of miRNA may cause *Pax2Cre/Dicer^{LoxP}* palatogenesis to stop at E12.5 due to a loss of miRNA mediated regulation. Studies will be necessary to determine whether rates of cell proliferation and apoptosis are alter in the early development of the palatal shelves.

Further studies will investigate the involvement of CNC in the *Pax2Cre/Dicer^{LoxP}* phenotype. Moreover, ISH and Q-PCR using probes specific for miRNAs will be

performed to allow for the development of possible molecular mechanisms underlying the orofacial dimorphism in *Pax2Cre/Dicer^{LoxP}* mouse model.

IV. Conclusion

This study has been novel in two ways. First, it has shown that the *Pax2Cre* positive tissues contributing to the viscerocranium and palate development require the miRNA processing enzyme Dicer for proper formation. Second, it has definitively shown that palate and cranioskeletal precursors express *Pax2Cre* and endogenous *Pax2* which has never been directly tested. Moreover, the palatal defects observed in *Pax2Cre/Dicer^{LoxP}* embryos are not a result of miss-expression of common growth factors implicated in cleft palate, *Fgf8*, *Fgf10*, or *Bmp4* at the mRNA level. Results also point to involvement of CNC in the *Pax2Cre/Dicer^{LoxP}* cleft palate phenotype, which is observed through misexpression of *Sox9* in the conditional *Dicer* knockout mice. *Sox9* is a molecular marker of chondrogenic CNC cells, which contribute to the endochondrally derived skeletal elements of the cranial skeleton upon which palatal development is dependent for support. Embryos lacking *Sox9* in CNC have been shown to have severe craniofacial phenotypes including a cleft palate, and in humans, haploinsufficiency in *Sox9* results in a craniofacial disorder known as campomelic-dysplasia (Crombrughe et al. 1997; Bi et al. 2001; Mori-Akiyama et al. 2003; Nie 2006; Yokohama-Tamaki and Shibata 2008; Wegner et al. 2008; Gordon et al. 2009)

The present thesis is groundbreaking work for the development of further studies analyzing the role of specific miRNA involved in orofacial development. This work shows that miRNA are necessary for orofacial development, however, many questions remain to be answered, such as which specific processes, cells, or tissue interactions taking place during orofacial development require the input and fine tuning of miRNA. These and other questions will serve as the foundation upon which new studies will be

developed towards deciphering the required combination of specific miRNA necessary for viscerocranial and palatal development.

VI. Citations

- Ambros, V., Lee, R.C., Feinbaum, R.L. (1993) The *C. elegans* Heterochronic Gene *lin-4* Encodes Small RNAs with Antisense Complementarity to *lin-14*. *Cell* (75) 843-854
- Barenbaum, M., Bronner-Fraser, M. (2009) Pax2 and Pea3 synergize to activate a novel regulatory enhancer for *spalt4* in the developing ear. *Developmental Biology*
- Bartel, D.P., Burge, C.B., Lewis, B.P., Shih, I., Jones-Rhoades, M.W. (2003) Prediction of mammalian MicroRNA targets. *Cell* (115) 787 – 798
- Bartel, D.P., Lim, L.P., Grimson, A., Farh, K.K.H, Johston, W.K., Garrett-Engele, P. (2007) MicroRNA targeting specificity in mammals: Determinants beyond seed pairing. *Molecular Cell* (27) 91 - 105
- Basch, M.L., Bronner-Fraser, M., Garcia-Castro, M.I. (2006) Specification of the neural crest occurs during gastrulation and requires Pax7. *Nature* (441) 218-222
- Bi, W., Huang, W., Whitworth, D.J., Dent, J.M., Zhang, Z., Behringer, R.R. de Crombrughe, B. (2001) Haploinsufficiency of *sox9* results in defective cartilage primordial and premature skeletal mineralization. *PNAS* (98) 12: 6698 - 6703

- Bian, Z., Meng, L., Torensma, R., Von den Hoff, J.W., (2009) Biological Mechanisms in Palatogenesis and Cleft Palate. *Journal of Dental Research* 88(1) 22-33
- Brugmann, S.A., Tapadia, M.D., Helms, J.A. (2006) The molecular origins of species-specific facial pattern. *Current Topics in Developmental Biology* (73) 1-42
- Briscoe, J., Cheung, M. (2003) Neural crest development is regulated by the transcription factor Sox9. *Development* (130) 5681-5693
- Busslinger, M., Bouchard, M., Pfeffer, P. (2000) Functional equivalence of the transcription factors Pax2 and Pax5 in mouse development. *Development* (127) 3703 – 3713
- Chai, Y., Jiang, X., Ito, Y., Bringas, P., Han, J., Rowitch, D.H., Soriano, P., McMahon, A.P., Sucov, H.M. (2000) Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* (127) 1671-1679
- Chai, Y., Ito, Y., Y, J.Y., Chytil, A., Han, J., Bringas, P., Nakajima, A., Shuler, C.F., Moses, H.L. (2003) Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects. *Development and Disease* (130) 5269 - 5280

- Chai, Y., Maxon, R.E. (2006) Recent Advances in Craniofacial Morphogenesis. *Developmental Dynamics* (235):2353-2375
- Chen Q., Degraff, D.J., Sikes R.A. (2009) The developmental expression profile of PAX2 in the murine prostate. *Prostate* (DOI 10.1002/pros.21099)
- Chen, Y., Zhang, Z. Song, Y., Zhao, X., Zhang, X., Fermin, C. (2002) Rescue of cleft palate in *Msx1*-deficient mice by transgenic *Bmp4* reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development* (129):4135-4146
- Christoffersen, NR., Silaharoglu, A., Orom, UA., Kauppinen, S., Lund, AH. (2007) miR-200b mediates post-transcriptional repression of ZFHX1B. *RNA* (8) 1172-1178
- Clouthier, D.E., Reust, L.B., (2009) Elucidating the timing and function of endothelin-A receptor signaling during craniofacial development using neural crest specific gene deletion and receptor antagonism. *Developmental Biology* (328) 94-108
- Clouthier, D.E. (2008) MicroRNAs in facial development. *Nature Genetics* (40) 3: 268 – 269

- Clouthier, D.E., Abe, M., Ruest, L-B. (2007) Fate of cranial neural crest cells during craniofacial development in endothelin-A receptor deficient mice. *International Journal of Developmental Biology* (51) 97 - 105
- Cobourne, Martyn. (2004) The complex genetics of cleft lip and palate. *European Journal of Orthodontics* (26) 7-16
- Couly, G., Creuzet, S., Bennaceur, S., Vincent, C., Le Dourain, N.M. (2002) Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head. *Development* (129) 1061-1073
- Creuzet, S., Couly, G., Le. Douarin, N.M (2005) Patterning the neural crest derivatives during development of the vertebrate head: insights from avian studies. *Journal of Anatomy* (207) 447-459
- Davidson, B.L., Borchert, G.M., Lanier, W. (2006) RNA polymerase III transcribes human microRNAs. *Nature Structural and Molecular Biology* 13(12):1097-1101
- DeMeyer, W., Zeman, W., Palmer, (1964) The face predicts the brain: diagnostic significance of facial anomalies for holoprosencephaly. *Pediatrics* (34) 256-263

Dijke, P.T., Heldin, C.H., Miyazono, K. (1997) Tgf-B signaling from cell membrane to nucleus through SMAD proteins. *Nature* (340) 465-471

Eberhart, J.K., He, X., Swartz, M.E., Yan, Y.L., Song, H., Boling, T.C., Kunerth, A.K., Walker, M.B., Kimmel, C.B., Postlethwait, J.H. (2008) MicroRNA Mirn140 modulated Pdgf signaling during palatogenesis. *Nature Genetics* (40) 3: 290 – 298

Ferguson, M.W.J. (1988) Palate Development. *Development* (103) 41-60

Ferguson, M.W.J., Taya, Y., O’Kane, S. (1999) Pathogenesis of cleft palate in TGF-B3 knockout mice *Development* (126) 3869 – 3879

Fotaki, V., Price, D.J., Mason, J.O. (2008) Newly identified patterns of Pax2 expression in the developing mouse forebrain. *Developmental Biology* 8(79) doi: 10.1186/1471-213X-8-79

Gaur, T., Hussain, S., Mudhasani, R., Parulkar, I., Colby, J.L., Frederick, D., Kream, B.E., van Wijnen, A.J., Stein, J.L., Stein G.S., Jones, S.N., Lian, B.J. (2010) Dicer inactivation in osteoprogenitor cells compromises fetal survival and bone formation, while excision in differentiated osteoblasts increases bone mass in the adult mouse. *Developmental Biology*. doi: 10.1016/j.ydbio.2010.01.008

Gong, S.G., Gong, T.W., Shum, L. (2005) Identification of markers of the midface.

Journal of Dental Research. (84) 69-72

Goode D.K., Elgar G. (2009) The PAX258 gene subfamily: a comparative perspective.

Developmental Dynamics (12) 2951-2974

Gordon, C.T., Tan, T.Y., Benko, S., FitzPatrick, D., Lyonnet, S., Farlie P.G. (2009)

Long-range regulation at the SOX9 locus in development and disease. *Journal of Medical Genetics* 46(10) 649-655

Grosshans, H., Chatterjee, S. (2009) Active turnover modulates mature microRNA

activity in *Caenorhabditis elegans*. *Nature* (461) 546-549

Gritli-Linde, A. (2007) Molecular control of secondary palate development.

Developmental Biology (301) 309 - 326

Harfe, B.C., McManus, M.T., Mansfield, J.H., Hornstein, E., Tabin, C.J. (2005) The

RNaseIII enzyme *Dicer* is required for the morphogenesis but not patterning of the vertebrate limb. *PNAS* (102) 31: 10898 – 10903

- Harfe, B.D., O'Rourke, J.R., Swanson, M.S. (2006) MicroRNAs in mammalian development and tumorigenesis. *Birth Defects Res C. Embryo Today* 78 (2) 172-179
- Hilliard, S.A., Yu, L., Gu, S., Zhang, Z., Chen, Y.P. (2005) Regional regulation of palatal growth and patterning along the anterior-posterior axis in mice. *Journal of Anatomy* 207 (5): 655-667
- Hitchcock, P.F., Otteson, D.C., Shelden, E., Jones, J.M., Kameoka, J. (1998) Pax2 Expression and Retinal Morphogenesis in the Normal and Krd Mouse. *Developmental Biology* (193) 209-224
- Hoess, R.H., Abremski, K., Wierzbicki, A., Frommer, B. (1986) Bacteriophage P1 Cre-loxP Site-specific Recombination. *The Journal of Biological Chemistry*. (261)1:391-396
- Honein, M.A., Rasmussen, S.A., Reefhuis, J., Romitti, P.A., Lanmer E.J., Sun. L., *et al.* (2007) Maternal smoking and environmental tobacco smoke exposure and risk of orofacial clefts. *Epidemiology* (18) 226 – 233
- Hunt, P., Wilkinson, D., Krumlauf, R. (1991) Patterning the vertebrate head: murine hox2 genes mark distinct subpopulations of pre-migratory and migrating neural crest. *Development* (112) 43-50

- Iseki, S., Yoshida, T., Vivatbutsiri, P., Morriss-Kay, G., Saga, Y. (2008) Cell lineage in mammalian craniofacial mesenchyme. *Mechanisms of Development* (125) 797 - 808
- Izaurrealde, E., Eulalio, A., Huntzinger, E. (2008) Getting to the root of miRNA – mediated gene silencing. *Cell* (132) 9-14
- Izaurrealde, E., Behm-Ansmant, I., Rehwinkel, J. (2006) MicroRNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay. *Cold Spring Harbor Symposium of Quantitative Biology*. (71) 523-530
- Jeong, J., Li, X., McEvelly, R.J., Rosenfeld, M.G., Lufkin, T., Rubenstein, J.L.R. (2008) Dlx genes pattern mammalian jaw primordium by regulating both lower jaw-specific and upper jaw-specific genetic programs. *Development* (135) 2905-2916
- Jiang, R., Lan, Y. (2009) Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development* (136) 1387-1396
- Joshua-Tor, L., Song, J.J., Smith, S.K., Hannon, G.J. (2004) Crystal Structure of Argonaute and Its Implications for RISC Slicer Activity. *Science* (305) 1434 - 1437

- Kia, Z.S., Pasquinelli, A.E. (2010) MicroRNA assassins: factors that regulate the disappearance of miRNAs. *Nature Structural and Molecular Biology* (17) 5-10
- Kim, V.N. (2005) MicroRNA biogenesis: coordinated cropping and dicing. *Nature Reviews. Molecular Cellular Biology* (6) 376 – 385
- Kim, V.N., Han, J., Lee, Y., Yeom, K-H., Nam, J.W., Heo, i., Rhee, J-K., Sohn, S.Y., Cho, Y., Zhang, B-T. (2006) Molecular Basis for the Recognition of Primary microRNAs by the Drosha-DGCR8 complex. *Cell* (125) 887-901
- Koenig S.F., Brentle S., Hamdi K., Fichtner D., Wedlich D., Gradl, D. (2010) En2, Pax2/5 and Tcf-4 transcription factors cooperate in patterning the Xenopus brain. *Developmental Biology* (DOI 10.1016/j.ydbio2010.01.011)
- Krumlauf, R., Trainor, P.A., Sobieszczuk, D., Wilkinson, D. (2002) Signalling between the hindbrain and paraxial tissue dictates neural crest migration pathways. *Development* (129) 433-442
- Krumlauf, R., Trainor, P.A. (2002) Riding the Crest of the Wnt Wave. *Science* (297) 781-783

Kurihara, H., Sato, T., Kurihara, Y., Asai, R., Kawamura, Y., Tonami, K., Uchijima, Y., Heude, E., Ekker, M., Levi, G. (2008) An endothelin-1 switch specifies maxillomandibular identity. *PNAS* (105) 48: 18806 – 18811

Lee, J.M., Kim, J.Y., Cho, K.W., Lee, M.J., Cho, S.W., Zhang, Y., Byun, S.K., Yi, C.K., Jung, H.S. (2006) Modulation of cell proliferation during palatogenesis by the interplay between *Tbx3* and *Bmp4*. *Cell and Tissue Research* (327) 2: 285-292

Lefebvre, V., Smits, P. (2005) Transcriptional control of chondrocyte fate and differentiation. *Birth Defects Research Part C*. (75) 3: 200-212

Lima W.F., Murray, H., Nichols, J.G., Wu, H., Sun, H., Prakash, T.P., Berdeja, A.R., Gaus, H.J., Crooke, S.T. (2009) Human Dicer binds short single-strand and double-strand RNA with high affinity and interacts with different regions of the nucleic acids. *Journal of Biological Chemistry* 284 (4) 2535-2548

Livingston, D.M., Rajewsky, K., Kanellopoulou, C., Muljo S.A., Kung, A.L., Ganesan, S., Drapkin, R., Jenuwein, T. (2005) Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes and Development* (19) 489 -501

Lucarini, L., Evangelisti, L., Attanasio, M., Lapini, I., Chiarini, F., Porciani MC., Abbate, R., Gensini, G., Pepe, G (2009) May TGFBR1 act also as a low penetrance allele in Marfan syndrome? *International Journal of Cardiology* **131**(2): 281-284

Lufkin, T., Mark, M., Hart, C. P., LeMeur, M., Chambon, P. (1992) Homeotic transformation of the occipital bones of the skull by ectopic expression of a homeobox gene. *Nature* **359**, 835-841

Luna, C., Li, G., Qui, J., Epstein, D.L., Gonzalez, P. (2009) Role of miR-29b on the regulation of the extracellular matrix in human trabecular meshwork cells under chronic oxidative stress. *Molecular Vision* **15**, 2488-2497

Massague, J., Lagna, G., Hata, A., Hemmati-Brivanlou, A. (1996) Partnership between DPC4 and SMAD proteins in Tgf-b signaling pathways. *Nature* 832-836

Marcucio, R.S., Hu, D. (2009) A SHH-responsive signaling center in the forebrain regulates craniofacial morphogenesis via the facial ectoderm. *Development* (136) 107-116

Mardsen, P.A., Ho, J.J.D. (2008) Dicer Cuts the Kidney. *Journal of the American Society of Nephrology* (19):2043-2048

- Mayor, R., Steventon, B., Araya, C., Linker, C., Kuriyama, S. (2009) Differential requirements of BMP and Wnt signaling during gastrulation and neurulation define two steps in neural crest induction. *Development* (136) 771-779.
- McMahon, A.P., Rowitch, D.H. (1995) Pax-2 expression in the murine neural plate precedes and encompasses the expression domains of *Wnt-1* and *En-1*. *Mechanisms of Development*. (52) 3-8
- Megarbane, A., Chouery, E., Ghanem, I. (2008) A multiplex family with possible metaphyseal Spahr-type dysplasia and exclusion of RMRP and COL10A1 as candidate genes. *American Journal of Medical Genetics A* (14) 1865-1870
- Miner, J.H., Harvey, S.J., Jarad, G., Cunningham, J., Goldberg, S., Schermer, B., Harfe, B.D., McManus, M.T., Benzing, T. (2008) Podocyte-Specific Deletion of Dicer Alters Cytoskeletal Dynamics and Causes Glomerular Disease. *Journal of the American Society of Nephrology* 19(11):2150-2158
- Moreno-Pelayo, M.A., Mencia, A., Modamio-Hoybjor, S., Redshaw, N., Morin, M., Mayo-Merino, F., Olavarrieta, L., Aguirre, L.A., Castillo, I., Steel, K.P., Dalmay, T., Moreno, F. (2009) Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss. *Nature Genetics* (41) 5: 609-613

- Mori-Akiyama, Y., Akiyama, H. Rowitch, D.H, Crombrughe, B. (2003) Sox9 is required for the determination of chondrogenic cell lineage in the cranial neural crest. *PNAS* (100) 16: 9360-9365
- Mishra, P.J. (2009) MicroRNA polymorphisms: a giant leap towards personalized medicine. *Future Medicine*. 6(2):119-125
- Murray, J.C. (2002) Gene/environment causes of cleft lip and/or palate. *Clinical Genetics* (61):248-256
- Murray, J.C., Christensen, K., Juel, K., Herskind, A.M. (2004) Long term follow up study of survival associated with cleft lip and palate at birth. *British Medical Journal* 1-4
- Nakinishi S., Sakamoto, K., Yoshitake, H., Kino, K., Amagasa, T., Yamaguchi, A. (2009) Bone morphogenic proteins are involved in the pathobiology of synovial chondromatosis. *Biochemical and Biophysical Research Communications* 379 (4): 914-919
- Neubuser, A., Bachler, M. (2001) Expression of members of the Fgf family and their receptors during mid-facial development. *Mechanisms of Development* (100) 313-316.

- Nie, X.G. (2005) Differential expression of Bmp2, Bmp4, and Bmp3 in embryonic development of mouse anterior and posterior palate. *Chinese Medical Journal* (20) 1710-1716
- Nie, X., Luukko, K., Kettunen, P. (2006) BMP signaling in craniofacial development. *International Journal of Developmental Biology* (50) 511 – 521
- Olesnicky, K.E.C, Birkholz, D.A., Artinger, K.B. (2009) A role for chemokine signaling in neural crest migration and craniofacial development. *Developmental Biology* 333 (1) 161-172
- Ohyama, T., Groves, A.K. (2004) Generation of Pax2-Cre Mice by Modification of a Pax2 Bacterial Artificial Chromosome *Genesis* (38) 195 - 199
- Passos-Bueno MR., Ornelas CC, Fanganiello RD. (2009) Syndromes of the first and second pharyngeal arches: A review. *Am J Med Genet Part A* (149A) 1853-1859
- Parker, R., Liu, J., Valencia-Sanchez, M.A., Hannon, G.J. (2005) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nature Cell Biology* 7(7):719-723

- Park J., Zhang, JJ, Moro, A, Kushida, M., Wegner, M., Kim, P.C. (2010) Regulation of Sox9 by Sonic Hedgehog (Shh) is essential for patterning and formation of tracheal cartilage. *Developmental Dynamics* 239(2):514-526
- Pillai, R.S., Bhattacharyya, S.N., Filipowicz, W. (2007) Repression of protein synthesis by miRNAs: how many mechanism? *Trends in Cell Biology* 3(17):118-127
- Raible, D. W. (2006) Development of the neural crest: achieving specificity in regulatory pathways. *Current Opinion in Cell Biology* (18) 6, 698-703.
- Rijli, F.M., Ren, S.Y., Minoux, M., Santagati, F. (2005) Temporal requirement of Hoxa2 in cranial neural crest skeletal morphogenesis. *Development* (132) 4927 - 4936
- Riley, B.M., Mansilla, M.A., Ma J. Daack-Hirsch, S., Maher, B.S., Raffensperger, L.M., Russo E.T., Vieira, A.R., Dode', C., Mohammadi, M., Marazita, M.L., Murray, J.C. (2007) Impaired FGF signaling contributes to cleft lip and palate. *PNAS* 104 (11) 4512-4517
- Shomron, N., Levy, C. (2009) MicroRNA-Biogenesis and Pre-mRNA splicing Crosstalk. *Journal of Biomedicine and Biotechnology* doi: 10.1155/2009/594678

Soukup, G.A., Weston, M.D. (2009) MicroRNAs sound off. *Genome Medicine*. (1) 6: 59, 1-4

Soifer, H.S., Rossi, J.J., Sætrom, P. (2007) MicroRNAs in Disease and Potential Therapeutic Applications. *Molecular Therapeutics* (15)12:2070-2079

Serbedzija, G. N., Bronner-Fraser, M., Fraser, S. E. (1992) Vital dye analysis of cranial neural crest migration in the mouse embryo. *Development* (116) 297-307

Svoboda, K.K.H., Kang, P. (2005) Epithelial-Mesenchymal Transformation during Craniofacial Development. *J Dent Res* 84(8):678-690

Tanaka, T., Haneda, S., Imakawa K., Sakai, S., Nagaoka, K. (2009) A microRNA, miR-101a, controls mammary gland development by regulating cyglooxxygenase-2 expression. *Differentiation* 77(2): 181-187

Tannyhill, L. A. (2008) To Adhere or Not to Adhere: The role of cadherins in neural crest development. *Cell Adh. Migr.* 2(4): 223-230

Trainor, P., Krumlauf, R. (2002) Riding the crest of the Wnt signaling wave. *Science* (297) 781-783.

- Tumpel S., Wiedemann, L.M., Krumlauf R. (2009) *Hox* Genes and Segmentation of the Vertebrate Hindbrain. *Current Topics in Developmental Biology* (88) 103-137
- Vieira A.R. (2008) Unraveling Human Cleft Lip and Palate Research. *J. Dent. Res.* 87(2) 119-125.
- Von den Hoff, J.W., Meng, L., Bian, Z., Torensma, R. (2009) Biological Mechanisms in Palatogenesis and Cleft Palate. *J Dent Res* 88(1): 22-33
- Wang, D-Z., Chen J-F., Murchison, E.P., Tang, R. Callis, T.E., Tatsuguchi, M., Deng, Z., Rojas, M., Hammond, S.M., Scheider, M.D., Selzman, CH., Meissner, G., Patterson, C., Hannon, G.J. (2008) Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. *PNAS* (105)-6 2111-2116
- Wegner, M., Lommese, P., Stolt, C.C., Finzsch, M. (2008) Sox9 and Sox10 influence survival and migration of oligodendrocyte precursors in the spinal cord by regulating PDGF receptor alpha expression. *Development* (135) 637 – 646
- Weiss, S., Hennig, T., Bock, R., Steck, E., Richter, W. (2010) Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells. *Journal of Cellular Physiology* (1) 84-93

- Wilson, R. Freddi, S., Bateman, J.F. (2002) Collagen X chains harboring Schmid metaphyseal chondrodysplasia NC1 domain mutations are selectively retained and degraded in stably transfected cells. *Journal of Biological Chemistry* (15) 12516-12524
- Yokohama-Tamaki, T., Shibata, S. (2008) An *in situ* hybridization study of *Runx2*, *Osterix*, and *Sox9* in the anlagen of mouse mandibular condylar cartilage in the early stages of embryogenesis. *Journal of Anatomy* (213) 274-283
- Zamore P.D., Haley, B. (2005) Ribo-gnome: the big world of small RNAs. *Science* (309) 1519 – 1524
- Zervas, M., Ellisor, D., Koveal, D., Hagan, N., Brown, A. (2009) Comparative analysis of conditional reporter alleles in the developing embryo and embryonic nervous system. *Gene Expr Patterns* (7):475-489
- Zhang, L., Li, C., Feng, Y., Coukos, G. (2009) Therapeutic MicroRNA Strategies in Human Cancer. *Journal of the American Association of Pharmaceutical Scientists*
DOI : 10.1209/s12248-009-0145-9