UNDERSTANDING THE ROLE OF HOXA3 IN PHARYNGEAL DEVELOPMENT:

TISSUE-SPECIFIC AND TEMPORAL-GLOBAL DELETIONS

by

HEIDI ANNE TRAU

(Under the Direction of Nancy Manley)

ABSTRACT

Hoxa3 is required for the development of the third and fourth pharyngeal pouch-derived organs, formation of the cartilages of the throat and the hyoid bone, formation of the ninth and tenth cranial nerves, and in development of the soft palate. To determine when Hoxa3 is required in the formation of each of these structures, I used a globally expressed inducible Cre recombinase to temporally knock out Hoxa3 during development. To determine the role of Hoxa3 within the neural crest and endoderm, the two tissue types that give rise to all of the organs and structures affected in the Hoxa3 null mouse, as well as the parathyroid and thymus, I used tissue-specific Cre recombinases to knock out Hoxa3. Together, these experiments give us a good indication of when Hoxa3 function becomes necessary in the formation of the various pharyngeal structures, and of the role it plays in each.

INDEX WORDS: Hoxa3, Neural crest, Endoderm, Thymus, Parathyroid, Thyroid, Hyoid, Cranial nerve, Soft palate, Cre recombinase, Temporal-global, Tissue-specific

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HEIDI ANNE TRAU

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HEIDI ANNE TRAU

Major Professor: Nancy Manley

Committee: Brian Condie

James Lauderdale

Electronic Version Approved:

Maureen Grasso

Dean of the Graduate School

The University of Georgia

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CHAPTER 1

INTRODUCTION

The study of Hox genes started in the 1940's, when Edward Lewis began studying homeotic mutations in Drosophila melanogaster. These types of mutations, which transform one body segment into another and can lead to bizarre phenotypes, led to the idea that there are master regulatory genes that determine what each segment of the body will become. When it was discovered that vertebrates, including humans, also had Hox genes, and that these Hox genes were extremely well conserved between very distantly related species, it revolutionized ideas on both vertebrate development and evolution.

Since these initial discoveries, we have learned much about the structure and function of Hox genes in a diversity of species ranging from insects to fish to mammals. However, there is still much to be discovered about how these genes function. Comparatively little is known about the downstream targets of Hox proteins, what transcription factors and cofactors work in concert with Hox proteins, how these partners change both spatially and temporally, and when and where Hox genes become important in development of specific organs and structures. It was hoped that once we knew the structure of Hox genes, we could determine where and how they bound to DNA, and thus what genes were acting downstream of them. However, the similar DNA-binding mechanisms shared by all Hox proteins has thwarted these efforts, and the search for cofactors has also been slow.

With my research I attempted to better understand how and when a specific Hox gene, Hoxa3, becomes important in organogenesis in the mouse. Hoxa3 was the first Hox gene to have its null phenotype characterized in the mouse, but after 20 years of research, we still know relatively little about its roles in organogenesis. By piecing apart when Hoxa3 becomes important in the development of each structure, I can begin to understand how the requirements for this protein change over time and in different tissues as organogenesis progresses, and begin the search for possible downstream targets that may be expressed at that time and place.

Introduction to Hox Genes

Hox genes encode transcription factors that are crucial in dictating segment identity along the anteroposterior (A-P) axis of developing organisms (McGinnis, 1992). The order of Hox genes within gene clusters roughly follows their order of expression down the A-P axis, with some overlap. These clusters are very highly conserved across vastly divergent taxa. Mammals are characterized by having four Hox clusters with a total of 39 Hox genes, while fish have 7 clusters dues to an extra whole genome duplication after their split with other vertebrates (Amores, 1998, Postlethwait, 1998). It is thought that these Hox cluster duplications may have been what spurred vertebrate evolution, by allowing for the diversity of expression and the development of novel functions of Hox genes. Many of the Hox paralogs have undergone subfunctionalization, wherein they have divided up the ancestral functions among themselves (Walsh, 2003). In some cases, they may also undergo neofunctionalization, in which they start to develop new roles distinct from the ancestral functions (Walsh, 2003). None of the Hox clusters in mammals are complete, indicating that some of the paralogs were lost once duplication occurred.

Hox genes all have a common structure that is characterized by the presence of a homeodomain, and reveals their common origin. The homeodomain is a highly conserved sequence of 60 amino acids that is responsible for binding to transcriptional binding sites in DNA (Levine, 1988, McGinnis, 1992). This homeodomain contains three alpha helices that form a helix-turn-helix motif and an additional domain called the N-terminal arm that allows the protein to fit into the grooves of the DNA double helix. The helix-turn-helix fits into the major groove of the double helix, and the N-terminal arm fits into the minor groove to provide some added specificity (Qian, 1989, Piper, 1999, LaRonde-LeBlanc, 2003). The Hox3 paralogous group is unique among Hox proteins in that it is also characterized by a long C-terminal domain, which was shown by an evolutionary alleles technique to be important in their functioning (Chen, 2010).

Numerous homeodomain proteins recognize a consensus sequence called the TAAT-motif (Desplan, 1988). Since many Hox genes that recognize this sequence have vastly different effects on the cells they are active in, the discrimination of binding sites that all contain the same consensus sequence is a puzzle. It is thought that the presence of coactivator molecules may prove to be the key to this mystery. Hox genes usually don't act alone in binding to DNA sequences, but require the presence of one or more coactivators. These cofactor interactions are highly dependent on a hexapeptide sequence containing four core residues (YPWM) located just N-terminal of the homeodomain (Chang, 1995, Phelan, 1995, Shen, 1997). The heterodimer often involves a member of the PBC family of proteins, and the binding sites are often paired with Pbx/Meis binding sites (Chang, 1995, Phelan, 1995, Shen, 1997). These heterodimers with non-Hox genes yield specificity to the Hox proteins binding ability, thus allowing the Hox genes to be specifically recruited to target-specific cis-regulatory sequences.

Control of downstream targets occurs at the enhancers. The combination of Hox proteins and other transcription factors with tissue-specific proteins leads to the specific activation of downstream molecules, which are mostly transcription factors and signaling pathway components (Graba, 1997). Hox proteins are expressed in segments in developing organisms, but their targets are regulated in an organ- and tissue-specific manner within those segments (discussed in Hombria, 2003). In addition, Hox expression within a segment varies with time, creating a temporal element as well. Thus, relatively broad expression of Hox genes can lead to developmental control on a very fine scale.

Generally, Hox genes have two functions (as described in Hombria, 2003). The first is homeosis. This ensures that once the fate of a given segment has been determined by the Hox genes, it is maintained throughout development. This is achieved via a chromatin "memory mechanism", which ensures that the identity of each segment is established within almost all of the cells in the segment throughout development (Simon, 2002). The second function is the control of organogenesis and morphogenesis in the developing embryo. This function does not require continuous Hox gene expression, but instead is regulated temporally, and it is organ specific. These two functions are not mutually exclusive: there are cases of intermediate functioning where localized activation of targets occurs concurrently with the regulation of other Hox genes in neighboring segments.

Hoxa3 Expression and Function

Hoxa3 was the first Hox gene to be mutated by homologous recombination, and as such its phenotype has been very well characterized (Chisaka, 1991). Hoxa3 is has been implicated in

such processes as patterning, cell migration, proliferation, apoptosis, and differentiation. Mouse Hoxa3 is expressed in neural crest derived mesenchymal cells of the pharyngeal pouches, and in the pharyngeal endoderm. Work by Chisaka (1991), Condie (1994), and Manley (1995, 1997, 1998) has demonstrated that Hoxa3 is required for the development and migration of the third and fourth pharyngeal pouch-derived organs, formation of the cartilages of the throat and the hyoid bone, formation of the ninth and tenth cranial nerves, and in development of the soft palate. All of these structures are affected in the Hoxa3 null mice.

The thyroid, thymus, parathyroid, and ultimobranchial bodies are formed from the third and fourth pharyngeal pouches. These organs interact as they are being formed and migrating, and two processes which occur concurrently starting at E10.5 and lasting until E15.5. The thymus begins to develop at E11.0 as a proliferation of cells in the third pharyngeal pouch, which then migrate ventrally and fuse at the throat. The parathyroid develops from the third pharyngeal pouch alongside the thymus, and migrates to a final position in association with the thyroid gland in the throat. The thyroid gland is formed by the fusion of the thyroid diverticulum derived from the floor of the pharynx, and the ultimobranchial body formed from the fourth pharyngeal pouch. The diverticulum gives rise to the thyroxin-producing cells of the thyroid, and the ultimobranchial body gives rise to the parafollicular cells which produce calcitonin.

This is not the only Hox gene to be expressed in these regions. Condie (1994) initially demonstrated that Hoxa3 and Hoxd3 interact synergistically. When these genes are mutated individually, none of the affected structures overlapped. However, when they were mutated together in the same embryos, some of the defects were greatly exacerbated in a dosage-sensitive manner. Further work by Manley (1997 and 1998) demonstrated that Hoxb3 also interacts synergistically with Hoxa3 and Hoxd3, and also indicated that it may be the total number of

Hox3 genes present in a given region that is important for proper development, rather than the specific identities of the genes. Support of this idea came from Greer (2000), in which Hoxa3 and Hoxd3 were swapped so that their protein coding regions are expressed from the other gene's loci. This means that the resulting mice either lacked Hoxa3 and instead expressed Hoxd3 from both loci, or vice versa. They found that in both cases the mice developed normally. This demonstrated that Hoxd3 protein is functionally equivalent to Hoxa3 when expressed from the Hoxa3 locus, and vice versa, and thus that it is cis-regulatory changes rather than changes in the proteins themselves that are responsible for the differences between these paralogous genes. This result was particularly striking given the low level of amino acid identity between these two genes outside of the homeodomain.

Hoxa3 is Expressed in Neural Crest and Endoderm

Hoxa3 is expressed in two tissue types: the neural crest mesenchyme, and the foregut endoderm. These two tissue types interact extensively during pharyngeal organ development. Neural crest cells originate at the edges of the neural tube in transient outpocketings of the hindbrain called rhombomeres. The neural crest cells in these rhombomeres then migrate ventrally towards the pharyngeal arches and pouches, with neural crest cells from specific rhombomeres populating specific arches. Once the neural crest cells reach the pharyngeal arches, they are patterned by signals from the foregut endoderm (LeLievre, 1975, Couly, 2002 and Ruhin, 2003). Hox genes have been shown to have an important role in determining what structures the neural crest cells form. Minoux (2009) demonstrated that the ground state for all

the arches is Hox negative, and that the ground pattern is modified via Hox genes to adopt a segment-specific identity.

Early work in chick-quail chimeras by Le Lievre and LeDouarin (1975) demonstrated that neural crest cells give rise to the entire visceral skeleton, the mesenchymal components of the glandular pharyngeal derivatives, as well as much of the muscle and connective tissue in the embryo. Of the structures affected in the Hoxa3 null embryos, the hyoid bone, cranial nerves, and soft palate have neural crest cell contributions to their formation (Chisaka, 1991, and Manley, 1995). Neural crest cells also contribute extensively to the environment through which the third and fourth pouch endoderm-derived organs must migrate. This therefore indicates that Hoxa3 may play an important role in cephalic neural crest cell function (Chisaka, 1991). Only one phenotype in the Hoxa3 null mutant does not have any input from neural crest cells: the tracheal epithelium, which is endoderm-derived (Manley, 1995). This phenotype revealed that Hoxa3 has specific roles in the endoderm as well as in the neural crest.

Not all of the structures affected in the Hoxa3 mutant are derived from cells that express Hoxa3. Both the hyoid lesser horn and the soft palate are derived from second arch neural crest cells, which do not express Hoxa3 at E10.5. This observation leads us to several interesting possibilities for how Hoxa3 is acting during the formation of these structures: Hoxa3 is expressed early in these cells and then shut off; or Hoxa3 is using another molecule to signal to these tissues; or cells expressing Hoxa3 later migrate into this region; or Hoxa3 turns on de novo in these structures later. In any case, it will be important to determine how and when Hoxa3 is involved in the formation of these structures.

Constructing the Downstream Pathway

Attempts to find downstream targets by locating Hoxa3 binding sites in the genome have been ineffective to date. Despite this, several genes involved in the downstream pathway have been identified by looking at the phenotypes of double mutants, and by looking at gene expression patterns via in situ hybridizations and qRT-PCR. The genes identified to date include Tbx1, Six1, Eya1, and Pax1/9. Recently, a rudimentary pathway has been confirmed using microarrays on Tbx1 mutant embryos, that places Hoxa3 upstream of Eya1 and Six1, which then regulate the expression of the transcription factors Tbx1 and Pax1, and which in turn are upstream of organ specific genes such as Gcm2 and Foxn1 (Zou, 2006, Ivins, 2005). The transcription factor Pax9 appears to be involved in a reciprocal signaling pathway with Hoxa3, and the exact nature of their interactions is not yet understood (Peters, 1998, Zou, 2006). While we have yet to determine the mechanism by which Hoxa3 controls the expression of these genes, it is encouraging to have a foundation from which to start building pathways.

CHAPTER 2

MATERIALS AND METHODS

Generation of Mutant Mice and Genotype Analysis

The Hoxa3 null allele has been previously described (Chisaka, 1991, Manley, 1995, 1997, 1998). For both our temporal-global and tissue-specific deletions, a floxed Hoxa3 allele was used (from Mario Capecchi's lab). This allele contains a LoxP site within the intron of Hoxa3, and another just downstream of the stop codon. When recombination of this allele was induced, the entire second exon of Hoxa3 was removed, containing the homeodomain and the C-terminal domain. This recombined allele should be a null allele. The Cre strains used were the tamoxifen inducible globally expressed CAGG-CreER (Hayashi, 2002), the NCC-specific Wnt1-Cre (Danielian, 1998), the endoderm-specific tamoxifen inducible FoxA2-mcm-CreER (Park, 2008), and the parathyroid-specific PTH-Cre (Libutti, 2003).

Mice homozygous for the floxed Hoxa3 allele (A3fx) were crossed to mice doubly heterozygous for the Hoxa3 null allele and one of the Cre recombinases. All colonies were maintained on a C57BL6 genetic background. Intraperitoneal (IP) injections of tamoxifen in corn oil were done on pregnant mothers between E6.5 and E12.5 at a concentration of 3mg tamoxifen per 40g mouse as described in Hayashi (2002). Litters were collected for analysis by cesarean section and genotyped by PCR. Embryonic age was estimated by considering noon of the day of a vaginal plug as E0.5.

mRNA Quantification

Total mRNA was collected from E11.0, E11.5, E12, and E12.5 embryos, followed by reverse transcription to form first-strand cDNA using Superscript II (Invitrogen). Quantitative PCR was performed on an ABI 7500 real-time PCR system using the default amplification program. qRT-PCR was done using the taqman gene expression assay with commercially available probes to 18S RNA and Hoxa3. The Taqman Hoxa3 primer set used was Mm01326402_m1, which amplifies a 71bp region at the end of the C-terminal of Hoxa3. Three mutant embryos from each time point were run in duplicate, along with a no RT control and an $a3^{fx/-}$;Cre^{-} control littermate.

Histology

For paraffin sectioning, embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), and embedded in Paraplast Plus. Ten micrometer sections were stained with hemotoxylin and eosin (H&E), mounted in Cytoseal 60, and photographed using an Optronics camera and a Zeiss Axioplan microscope. At each time point at least two mutant embryos were examined.

Neurofilament Staining

Neurofilament staining were performed using the 2H3 anti-155-kDa monoclonal antibody obtained from the Developmental Studies Hybridoma Bank (Dodd, 1988). Embryos were fixed

in methanol:DMSO (4:1) overnight, treated with hydrogen peroxide to inactivate endogenous peroxidases, rehydrated, and incubated overnight in the 2H3 antibody at a 1:4 dilution in PBSTMD (PBS, 0.5% Triton X-100, 2% skim milk, 1% DMSO). Embryos were washed in PBSTMD and incubated overnight with an HRP-conjugated secondary antibody at 1:100 in PBSTMD. Embryos were washed again, and color reactions were performed in diaminobenzidine and hydrogen peroxide. Embryos were cleared in 1:2 benzyl benzoate:benzyl alcohol for analysis and photographed using a Spot camera and Leica MZ125 dissecting scope. A minimum of three mutant and three control embryos were examined in each experiment.

In Situ Hybridization

Whole mount *in situ* hybridization was performed on E10.75 embryos as described (Manley, 1995, Carpenter, 1993). Embryos were fixed overnight in 4% paraformaldehyde in PBS, then dehydrated in methanol and stored at -20ºC until use. The digoxigenin-labelled RNA probes were used at 0.5µg/ml. Alkaline phosphatase-conjugated anit-digoxigenin Fab fragments were used at 1:5000. Color reactions were carried out over time periods ranging from 4 hours to overnight. Embryos were photographed without clearing, using a Spot camera and a Leica MZ125 dissecting microscope.

For sectioned *in situ* hybridization, E13.5 embryos were first embedded in paraffin and 10 µm serial sections were collected from the level of the eye through the normal level of the thymus and examined. Slides with the appropriate sections were used for *in situ* hybridization. The digoxigenin-labeled RNA probes were used at a concentration of 0.5µg/ml. Probes for Pax9 (Neubuser, 1995), PTH (Liu, 2007), Foxn1 (Gordon, 2001), Gcm2 (Gordon, 2001), and Hoxa3

(Manley, 1995) have been previously described. A minimum of two mutant and two control embryos were used for each *in situ* hybridization performed. Alkaline phosphatase-conjugated anit-digoxigenin Fab fragments were used at 1:5000. Color reactions were carried out from 1 to 3 days. The slides were then counterstained with nuclear Fast Red and mounted in Cytoseal 60 and photographed using an Optronics camera and a Zeiss Axioplan microscope.

3d Reconstruction

Sectioned in situs were photographed and the serial sections were reconstructed using Winsurf. Once reconstructed, the volume was calculated for each organ using the Volumetrics (tab delimited) function.

Skeleton Preparation

For skeleton preparations, E18.5 embryos were fixed for 5 days in 95% ethanol, followed by removal of lipids in acetone, and stained for 10 days with alcian blue 8GX and alizarin red. The skeletons were then cleared in a glycerol series and dissected to reveal the hyoid bone and throat cartilages and photographed using a Spot camera and Leica MZ 125 dissecting scope. Between one and seven mutants were examined at each time point, along with a minimum of two controls.

CHAPTER 3

TEMPORAL-GLOBAL DELETION OF HOXA3

Introduction

Hoxa3 mutant embryos exhibit a wide range of phenotypes affecting a variety of structures derived from neural crest cell (NCC) derived mesenchymal cells of the pharyngeal pouches and the pharyngeal endoderm (Chisaka, 1991, Manley, 1995, Manley, 1997, Manley, 1998). Mouse Hoxa3 is required for the development and migration of the third and fourth pharyngeal pouch-derived organs, formation of the cartilages of the throat and the hyoid bone, formation of the ninth and tenth cranial nerves, and in development of the soft palate (Chisaka, 1991, Manley, 1995, Manley, 1997, Manley, 1998). The thyroid, thymus, parathyroid, and ultimobranchial bodies are formed from the third and fourth pharyngeal pouches. These organs interact as they are being formed and migrating, two processes which occur concurrently starting at E10.5 and lasting until E15.5. The thymus begins to develop at E11.0 as a proliferation of cells in the third pharyngeal pouch, which then migrate ventrally and fuse at the throat. The parathyroid develops from the third pharyngeal pouch alongside the thymus, and migrates to a final position in association with the thyroid gland in the throat. The thyroid gland is formed by the fusion of the thyroid diverticulum derived from the floor of the pharynx, and the ultimobranchial body formed from the fourth pharyngeal pouch. The diverticulum gives rise to the thyroxin-producing cells of

the thyroid, and the ultimobranchial body gives rise to the calcitonin producing parafollicular cells.

Mouse Hoxa3 mRNA is first expressed around E7.5, with a cranial limit between rhombomeres 4 and 5 and at the third pharyngeal arch, and extending posteriorly to the tip of the tail (Gaunt, 1987). As there are no phenotypes associated with this more posterior expression of Hoxa3, it is unclear what role Hoxa3 may be playing in these tissues. In addition, once Hoxa3 expression is initiated, it continues indefinitely, and the function of the postnatal expression of Hoxa3 is also unknown.

In this study I sought to determine when Hoxa3 function becomes required for the formation of each of the organs and structures affected in the Hoxa3 null mutant embryos. To determine when Hoxa3 is required in the formation of each of these structures, I used a globally expressed inducible Cre recombinase to temporally knock out Hoxa3 during development. I found that there is about a 24 hour lag time between when Cre recombination was first induced and when the mRNA level of Hoxa3 had dropped below the critical threshold. I found that Hoxa3 is required initially for the formation of the third pouch derived organs (by analyzing embryos in which recombination was induced at E9.5), then for their separation from the pharynx (E10.5), and finally for their migration (E11). Its continued expression after the thymus and parathyroid had completed their migration was necessary for maintaining the structural integrity of these organs. The thyroid lobes and ultimobranchial body formed and migrated normally at all times points examined, but Hoxa3 expression was required for the fusion of the ultimobranchial body with the thyroid (as observed after induction at E10.5), and for proper formation of the thyroid isthmus (E9.5). Analysis of the skeleton indicated that Hoxa3 plays a very early role in the formation of the hyoid bone and thyroid cartilage, and that the skeleton is a very plastic structure

(induction at E7.5). The tracheal epithelium appeared to require Hoxa3 expression continuously in order for it to maintain its organized structure (at least until E12.5). These results led me to conclude that Hoxa3 has multiple diverse roles during embryogenesis, and that its ongoing expression functions to maintain the integrity of some of the organs in which it is expressed.

Results

Hoxa3 DNA and mRNA are Efficiently Deleted after Cre Induction

To globally delete Hoxa3 at different time points in development, I crossed homozygous floxed Hoxa3 (A3fx) mice to mice doubly heterozygous for a ubiquitously expressed tamoxifen-inducible form of Cre recombinase, CAGG-CreER and the previously characterized Hoxa3 null allele (A3bx) (Hayashi, 2002, Chisaka, 1991). Pregnant dams were given a single IP injection of tamoxifen to induce recombination. Upon Cre induction, the entire fourth exon of the floxed Hoxa3 allele, which includes the homeodomain and C-terminal domain, was removed by the Cre recombinase (Figure 3.1C-D).

Before I began analyzing the Hoxa3 phenotypes in these embryos, I wanted to determine how quickly the Hoxa3 gene was deleted after recombination was induced, and how quickly and completely the mRNA disappeared following induction. To test this, I injected pregnant mothers at E10.5 with tamoxifen to induce recombination, and then collected the embryos at 12, 24, 36, and 48 hours post-injection. PCR was then done using primers to the floxed Hoxa3 allele and the recombined allele (Figure 3.1A). I found that 12 hours after recombination was induced, the Hoxa3 gene was efficiently deleted.

The level of Hoxa3 mRNA was predicted to never reach zero, even after complete removal of the floxed allele, due to continued transcription of the null allele in these mutants. The classical Hoxa3 null allele was created by inserting a neomycin resistance cassette into the homeodomain of Hoxa3 (Chisaka, 1991). However, Hoxa3 mRNA is still produced from this mutant allele, as demonstrated by *in situ* hybridization (Manley, 1995). I performed qRT-PCR on whole embryo RNA using a C-terminal Hoxa3 probe, and found that the Hoxa3 mRNA was reduced to what I predict to be the null level by 24 to 36 hours post-injection (Figure 3.1B). Our results indicate that Hoxa3 is efficiently removed following tamoxifen injection, and is effectively gone by 24 to 36 hours post-injection.

Hoxa3 has Multiple Roles in Thymus and Parathyroid Organogenesis

The thymus and parathyroid both develop from the third pharyngeal pouch starting at about E10.5. These organs start as a thickening of the epithelium of the pouch, which then breaks free of the trachea and migrates ventrally. As they migrate, the parathyroids separate from the thymus, and become closely associated with the thyroid, while the thymus lobes continue migrating until they reside just above the heart. In the Hoxa3 null mutant, neither of these organs form, indicating that Hoxa3 plays a critical role in their organogenesis. However, it is unclear whether Hoxa3 plays any roles beyond initial organogenesis of these organs.

In order to test whether Hoxa3 plays any roles in the later development of these organs, I examined E13.5 embryos in which recombination was induced at either E9.5, E10.5, E11, or E11.5 (Figure 3.2). As I expected, embryos in which recombination was induced prior to organ formation (E9.5) were both athymic and aparathyroid and resembled the Hoxa3 null mutants

(Figure 3.2G-I, n=1). When recombination was induced at E10.5, after organogenesis has begun, the thymus was found to be ectopically attached to the trachea, unable to migrate to its normal position (Figure 3.2G-F, n=3). In order to determine whether the parathyroid remained persistently attached to the ectopic thymus, I performed *in situ* hybridization for the thymus marker Foxn1 and for parathyroid hormone (PTH) on sectioned E13.5 embryos (Figure 3.3, n=2 for each *in situ*). My results showed that the thymus was ectopically attached to the trachea (Figure 3.3D), while the parathyroid remained ectopically located near the trachea, though not necessarily attached to the thymus (Figure 3.3B). Because both of these organs were ectopic when induction occurred at E10.5, this result indicated that Hoxa3 plays a role in the separation of the third pouch from the trachea. I next sought to determine whether Hoxa3 played a role in the migration of the thymus and parathyroid. Embryos induced at E11 were found to have ectopic thymi at about the level of the ultimobranchial bodies, which suggests that Hoxa3 does play a role in the migration of these organs once they have formed and detached from the trachea (Figure 3.2C, n=4). However, it is also possible that embryos induced at E11 may have a slight delay in separation which could account for this observation, since the window for migration is quite short. When induction occurred slightly later at E11.5, the parathyroids and thymus were found at their normal positions within the embryo (Figure 3.2B, n=2).

I next sought to determine whether there were any long term effects from deletion of Hoxa3 at each of these time points. At E18.5, the anatomical positions of the thymus lobes at each time point were the same as described at E13.5, however, the thymus lobes had developed large cysts (Figure 3.4J-L). This result indicated that Hoxa3 plays a role in maintaining the structural integrity of the thymus. In contrast, the parathyroids had a much more dramatic phenotype at E18.5. While they were easily located at E13.5 after induction at E11.5 (Figure 3.2B), they were

completely gone by E18.5 (Figure 3.4B, n=1). This result was not entirely unexpected given what we know from Hoxa3; Pax1 compound mutants (Su, 2001), and from parathyroid-specific deletions of Hoxa3 (see Chapter 4, Figure 4.2). In both of these experiments the parathyroid was found to become progressively smaller as development proceeds, and in the case of the Hoxa3; Pax1 compound mutants, to eventually disappear entirely. It therefore appears that Hoxa3 is necessary for the maintenance of the parathyroid gland, and without Hoxa3 expression the parathyroid disappears over time.

Hoxa3 is Necessary for Even Spreading of the Thyroid and Fusion with Ultimobranchial Bodies

The ultimobranchial bodies form from the fourth pharyngeal pouch and fuse with the thyroid diverticulum arising from the ventral floor of the pharynx at the midline at the level of the 2nd pharyngeal pouch. The thyroid diverticulum gives rise to the thyroxin-producing cells of the thyroid, and the ultimobranchial bodies give rise to the calcitonin producing parafollicular cells. In the Hoxa3 null mutant embryos, the ultimobranchial bodies remain distinct from the thyroid, and the two cell types do not mix. The isthmus that connects the two sides of the thyroid also fails to form or is abnormal in these embryos.

In order to examine when Hoxa3 becomes important in these two processes, I examined E13.5 and E18.5 embryos in which recombination was induced either at E9.5, E10.5 or E11.5 (Figure 3.4 and data not shown). At E13.5, no defects were obvious in either organ after tamoxifen injection at any of these stages (Table 3.1). However, by E18.5 the null phenotypes were readily apparent (Figure 3.4A-H). The only embryos in which the isthmus was absent were those in which deletion was induced at E9.5, and one of the embryos examined also lacked a

thyroid gland on one side of the pharynx (Figure 3.4H, n=2). Hoxa3 thus appears to play a very early role in the formation of the isthmus.

At all three induction stages, the ultimobranchial bodies failed to fuse completely with the thyroid at E18.5 (Figure 3.4B-D). The most dramatic phenotype was in the embryos induced at E9.5, in which the ultimobranchial bodies were distinctly separate from the thyroid gland (Figure 3.4D, n=2). In those embryos induced at E10.5, the ultimobranchial bodies were very close to, but not fused with, the thyroid lobes (Figure 3.4C, n=1), and in those induced at E11.5 it appeared that the ultimobranchial bodies may have partially fused with the thyroid lobes (Figure 3.4D, n=1).

Hoxa3 is Required Continuously for Tracheal Epithelium Formation

The tracheal epithelium phenotype is unique among the Hoxa3 null phenotypes in being the only one that is completely NCC-independent. In normal embryos, the tissues surrounding the trachea are well organized in neat layers, with a single layer of psuedostratified columnar epithelium lining the trachea. In the Hoxa3 null embryos, the epithelium is the exact opposite, with much thicker and unorganized layers of cells, and the tracheal epithelium appears to have lost its columnar epithelial identity.

In order to determine when Hoxa3 becomes important in the formation of the tracheal epithelium, I examined E18.5 embryos in which recombination was induced at E9.5, E10.5 or E11.5 (Figure 3.5B-D, n=2 at E9.5, n=1 at E10.5 and E11.5). At none of the time points examined was the tracheal epithelium correctly organized, possibly indicating that Hoxa3 is continually required for normal epithelial development.

The Pharyngeal Skeleton has an Early and Prolonged Requirement for Hoxa3 during Development

The pharyngeal skeleton is derived from NCCs from the pharyngeal arches. The NCCs that give rise to the lesser horn of the hyoid bone originate from the second arch, while the rest of the hyoid originates from the third arch, and the throat cartilages arise from the fourth arch. It is interesting to note that Hoxa3 is only expressed in the third and fourth arches, and there are several hypotheses for how HoxA3 may affect more anterior structures. It is possible that Hoxa3 signals to the second arch NCCs to direct the formation of the lesser horn; or that Hoxa3 is transiently expressed in the second arch very early in development and subsequently turned off; or that cells expressing Hoxa3 later migrate into this region; or that Hoxa3 turns on de novo in these structures later.

I sought to determine when Hoxa3 was important in the formation of the pharyngeal skeleton by inducing recombination between E7.5 and E14.5 (Figure 3.6). Surprisingly, I did not find the clear temporal boundary between the null and wild type phenotype like I did in most of the other structures examined. Instead, I found a progressively stronger phenotype as I induced recombination earlier (Figure 3.6C-J). Embryos induced at E12.5 and later appeared phenotypically normal (Figure 3.6J, n=1 at E12.5). When induction occurred at E10.5 or E11.5, the pharyngeal skeleton appeared relatively normal, although some embryos exhibited projections from the greater horn of the hyoid bone that were never seen in the control embryos (Figure 3.6F-I, n=2 at both E10.5 and E11.5). The lesser horn was unaffected at either E10.5 or E11.5. When induction occurred at E9.5, however, the greater horn was completely fused to the

thyroid cartilage, as in the Hoxa3 null (Figure 3.6E, n=1). At this time point, the lesser horn also failed to form correctly. The underside (posterior) of the lesser horn was missing, and the resulting structure consisted of the thin projection from the body of the hyoid ending in a ball of cartilage. This phenotype was further exacerbated in the embryos induced at E8.5, which had only a thin projection of cartilage from the body of the hyoid ending in a very small ball of cartilage (Figure 3.6D, n=1). Surprisingly, in embryos induced at E7.5, this phenotype was only mildly exacerbated and did not resemble the Hoxa3 null phenotype (Figure 3.6C, n=4). Overall, these data demonstrate that Hoxa3 is required beginning very early in pharyngeal skeleton development, and that this requirement persists for several days while the skeleton is just beginning to form.

Discussion

I have used a series of temporal-global deletions of Hoxa3 to determine when Hoxa3 functioning becomes necessary for pharyngeal development. Our analysis showed that Hoxa3 has very diverse roles in pharyngeal development starting even before NCC migration, and lasting at least until birth.

Development of the pharyngeal organs hinges on the interplay of two tissue types: the NCC-derived mesenchymal cells of the pharyngeal pouches and the pharyngeal endoderm. As the pharyngeal organs develop and migrate between E10.5 and E15.5, they are surrounded by NC mesenchymal cells. These mesenchymal cells are responsible for the separation of the developing pouch derived organs from the pharynx, and possibly for their migration as well.

Hoxa3 is expressed in both of these cells types, and appears to have different roles in each (discussed further in Chapter 4).

Hoxa3 is required throughout third pouch development, being first required for initial formation and patterning of the third pharyngeal pouches, followed by their separation from the trachea and migration, and finally for maintaining their structure at least until birth. When Hoxa3 is removed while these processes are occurring, developmental defects are observed that coincide with the time of removal, thereby allowing us to demonstrate a continuous need for Hoxa3 expression while development and migration are ongoing. There is also a long-term requirement for Hoxa3 in maintaining the parathyroid glands and the thymus.

The thyroid and ultimobranchial bodies also migrate to their final positions within the embryo before fusing with each other. The thyroid diverticulum spreads to either side of the trachea, attached in the middle by a thin isthmus, followed by fusion of each thyroid lobe with an ultimobranchial body, after which the two cells types mix with each other. As in third pouch development, Hoxa3 appears to be required in both of these processes: first for proper spreading of the thyroid evenly into two lobes, and subsequently for the fusion of the ultimobranchial bodies. Unlike the thymus and parathyroid however, Hoxa3 appears dispensable for maintaining the organs once they have developed.

There is only one structure affected in the Hoxa3 null mice that is purely endodermal in origin: the tracheal epithelium. This epithelium is derived from the ventral endodermal epithelium of the embryonic pharynx, and usually forms as a single layer of columnar basal epithelial cells. In the Hoxa3 null mice, this layer of cells was poorly organized, with cells of differing shapes and sizes piling on top of each other, and creating a convoluted surface (Manley, 1995). Hoxa3 appears to be required to maintain this organization throughout development,

since removal of Hoxa3 at any time during fetal development disrupts the normal morphology of the epithelial cells that will eventually form the tracheal epithelium.

There appears to be very different requirements for Hoxa3 in the different cell types in which it is expressed. While the endoderm-derived structures appear to require Hoxa3 at different times during their organogenesis, the NCC-derived structures require Hoxa3 expression extremely early for their proper development. These results are unexpected, and hard to explain given what we know about the Hoxa3 null phenotypes. The very early requirement for Hoxa3 suggests that these phenotypes are most likely a migration defect or a patterning defect in the NCCs that give rise to the NCC-autonomous structures. While work by Manley (1995) demonstrated that NCC migration at E9.5 was grossly normal in the Hoxa3 null embryos, we cannot rule out later defects, or differences in small populations of cells. It is possible that Hoxa3 expression is required early in NCCs to make them competent to form specific structures, and this possibility will need to be further explored in the future. It is also interesting to note that even after inducing embryos extremely early in development, a Hoxa3 null pharyngeal skeleton phenotype was never observed. While this phenotype does not resemble the original published null lesser horn phenotype, they do bear a close resemblance to the lesser horns observed by Greer et. al. (1999, 2000) using a different null allele. This may be due to the way the original Hoxa3 null mice were created, by inserting a neomycin resistance cassette into the homeodomain, and it will need to be confirmed whether the skeletal phenotype observed in our temporal-global deletion mutants is not actually the null phenotype. It is also possible that there is a very low level of Hoxa3 that is not deleted after induction of recombination, and this low level of expression may account for the observed phenotype.

The pharyngeal skeletal elements appear to have a prolonged period in which Hoxa3 expression is necessary starting very early in development. In addition, there appears to be a distinct order of specification by Hoxa3, beginning with the cells that make up the upper ridge of the lesser horn of the hyoid, and gradually filling in the rest of this structure, followed by sculpting of the boundary between the greater horn of the hyoid and the thyroid cartilage. This process apparently occurs over the course of several days, and is dependent on continued expression of Hoxa3.

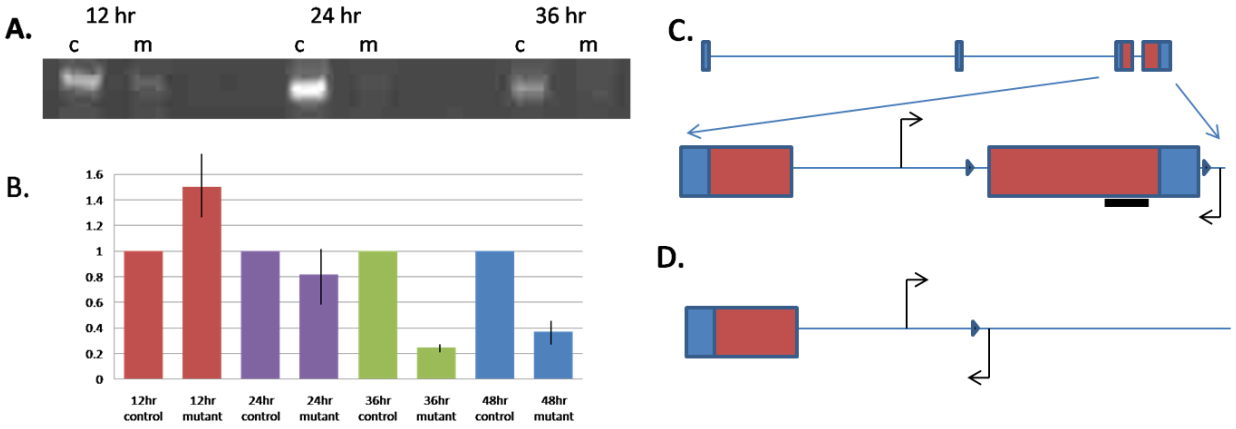

Figure 3.1: Hoxa3 DNA and mRNA are quickly lost upon activation of Cre recombinase via interperitoneal Tamoxifen injection. **A.** Genotyping gel showing the progressive disappearance of the Hoxa3 gene at 12hr, 24 hr, and 36 hr post Tamoxifen injection. c indicates control $CAGG^{+/+};a3^{fx/-}$ embryos, m indicates mutant $CAGG^{+/CreER};a3^{fx/-}$ embryo. **B.** qRT-PCR using a C-terminal Hoxa3 probe. **C.** Structure of the floxed Hoxa3 allele. Exons are denoted by the boxes, with red indicating coding and blue noncoding regions. LoxP sites are represented by the triangles. The primers used to detect the recombined allele are indicated by the bent arrows, and the probe used for qRT-PCR by the thick black band under the C-terminal domain. **D.** Structure of the floxed allele following recombination.

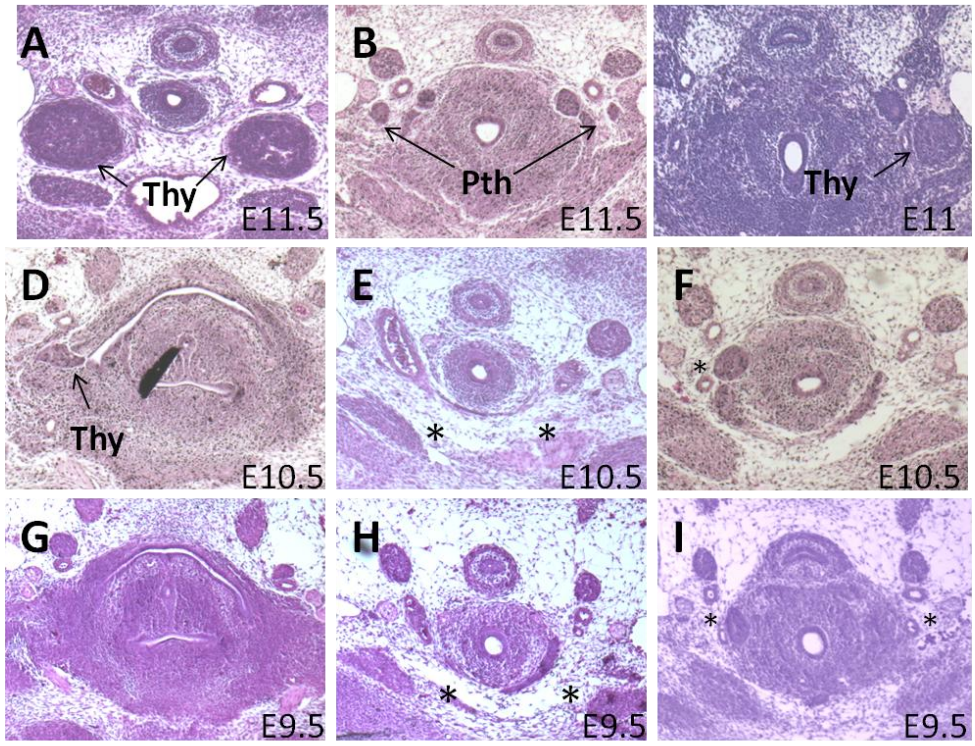

Figure 3.2: Removal of Hoxa3 early in development causes a failure of third pouch organogenesis, while later removal prohibits third pouch separation from the trachea. All embryos were collected at E13.5, and were sectioned in the transverse plane. **A, B.** Section of an embryo injected at E11.5 showing that **A** thymus and **B** parathyroid formation and migration are not affected by late deletion of Hoxa3. **C.** Section of an embryo injected at E11 showing the thymus ectopically located at the level of the ultimobranchial body. **D-F.** Section of an embryo injected at E10.5 showing that **D** the thymus is ectopically attached to the trachea and **E** not present in its normal location just anterior to the heart. **F.** Parathyroids could not be found in the normal location, and are most likely persistently attached to the ectopic thymus. **G-I.** Section of an embryo injected at E9.5 showing that **G** the thymus is neither ectopically attached to the trachea, nor **H** in its normal location. **I.** The parathyroids are also missing. Thy: thymus; Pth: parathyroid; asterisks indicate normal location of missing organ.

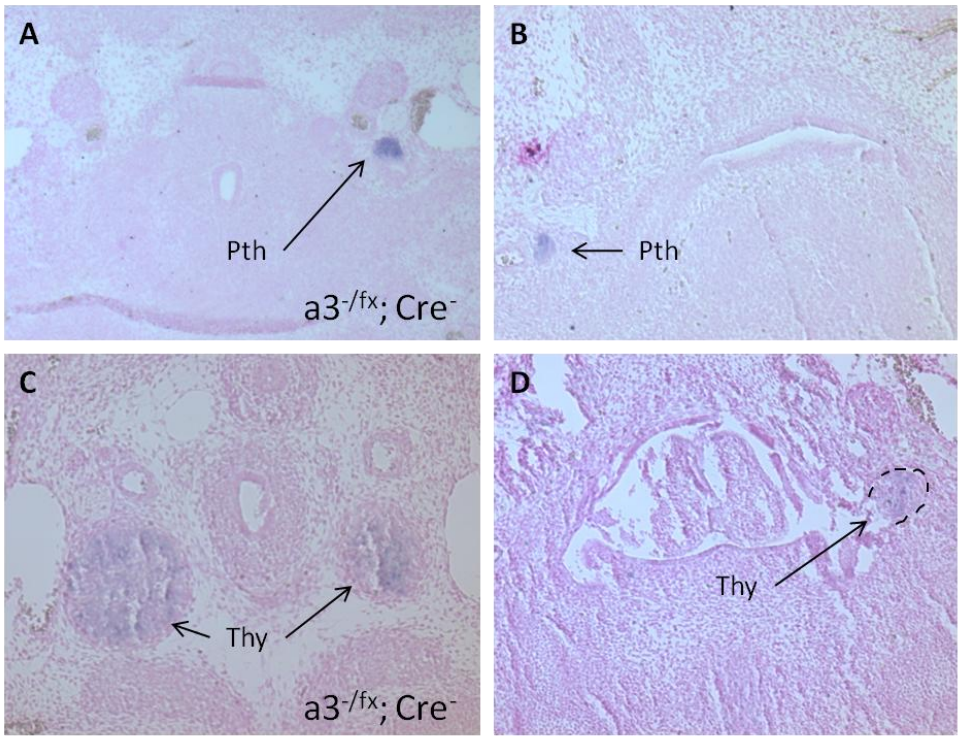

Figure 3.3: The thymus and parathyroid are ectopic after Hoxa3 induction at E10.5. **A.** Transverse PTH section in situ of a control embryo showing the normal location of the parathyroid gland at about the level of the ultimobranchial body. **B.** Mutant section in which the parathyroid is ectopically located near the trachea. **C.** Transverse Foxn1 section in situ of a control embryo showing the normal location of the thymus lobes. **D.** Mutant section in which the thymus is ectopically attached to the trachea.

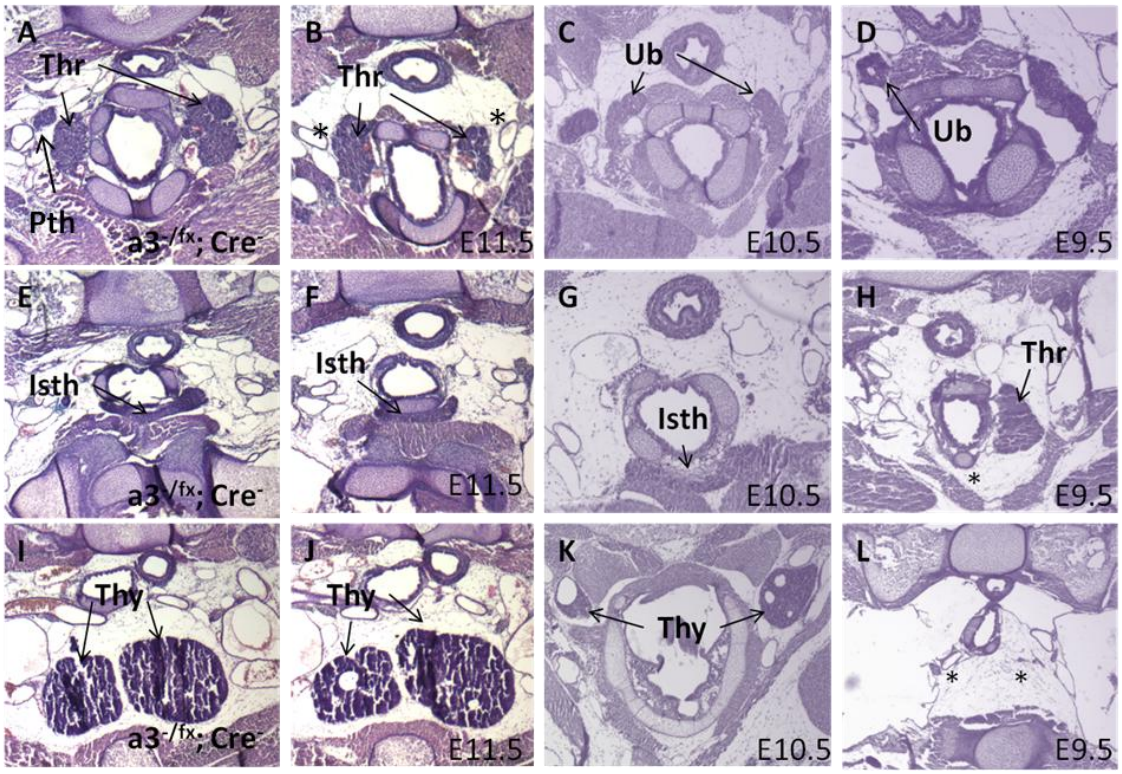

Figure 3.4: Hoxa3 is Required for Thymus Maintenance, Isthmus Formation, and Fusion of the Ultimobranchial Body. All embryos were collected at E18.5 and sectioned in the transverse plane. The time of Hoxa3 induction is indicated in the lower right. **A.** Section of a control embryos showing normal fusion of the ultimobranchial (Ub) body and thyroid (Thr). **B-D.** The Ub can still be distinguished after induction at **B.** E11.5 **C.** E10.5, and **D.** E9.5. **B.** The parathyroid is missing at E18.5 after induction at E11.5. The asterisks mark where the parathyroid glands should be. **E.** Section of a control embryo showing normal thyroid isthmus (Isth). The isthmus is present after induction at **F.** E11.5, and **G.** E10.5. **H.** It is deleted and the thyroid was present on only one side when induced at E9.5. **I.** Control section showing normal location of the thymus lobes. Thymus location was normal after induction at **J.** E11.5, ectopic at **K.** E10.5, and absent at **L.** E9.5.

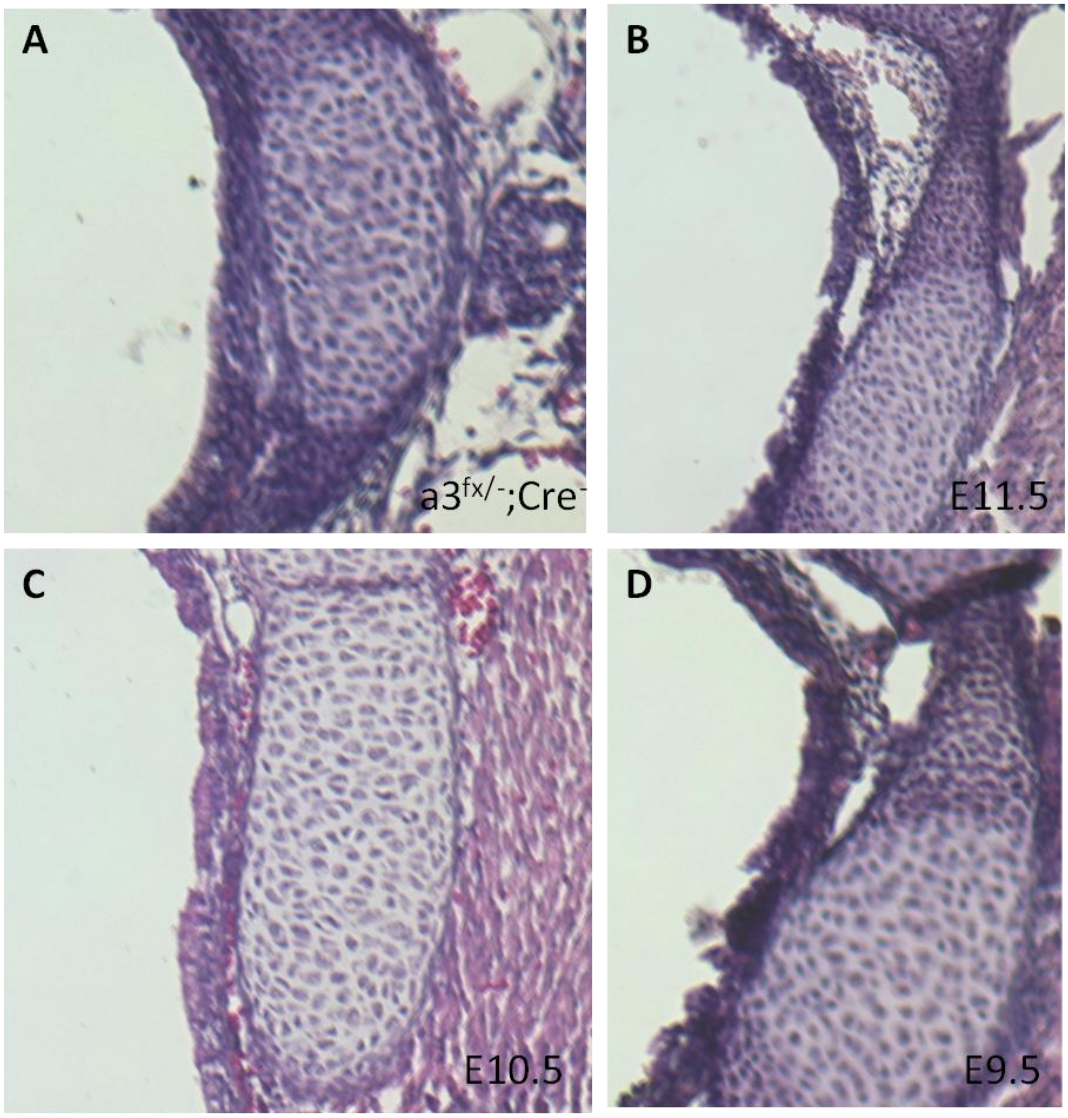

Figure 3.5: The tracheal epithelium has a continuous need for Hoxa3 expression. A. Transverse section of a control littermate showing normal tracheal epithelium structure. **B-D.** Sections from mutant embryos with Hoxs3 null phenotype in which the tracheal epithelium is disorganized. The time of Cre induction is indicated in the lower right.

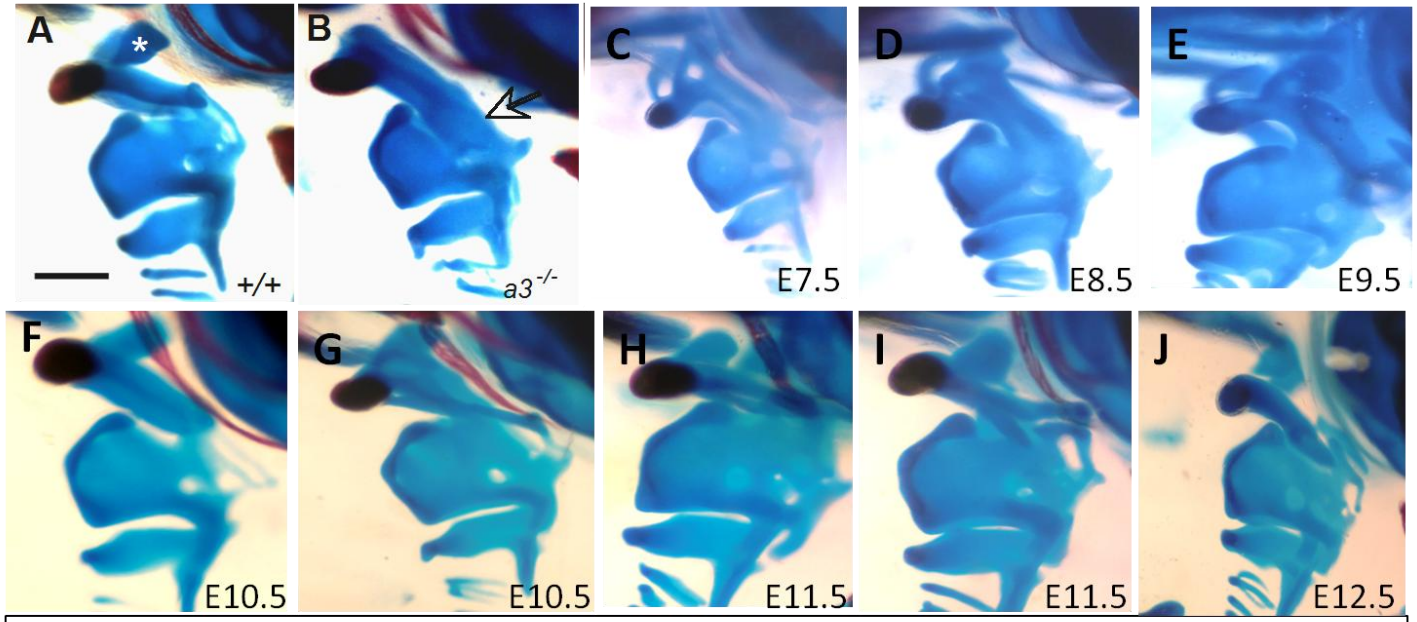

Figure 3.6: The pharyngeal skeleton has an early requirement for Hoxa3, and displays novel phenotypes after induction. E18.5 embryos were stained using alcian blue and alizarin red and cleared through a glycerol series. **A.** Wild type skeleton. Asterisk denotes the lesser horn of the hyoid bone. **B.** Hoxa3 null mutant skeleton showing deletion of the lesser horn and fusion of the greater horn to the thyroid cartilage (as indicated by the arrow). **C.** Skeleton of an embryo induced at E7.5 showing the small arch of the lesser horn and fusions with the greater horn. **D.** Skeleton of an embryo induced at E8.5 showing small arch that remains of the lesser horn, and fusion of the greater horn and thyroid cartilage. **E.** Skeleton of an E9.5 induced embryo with a thin projection of the lesser horn ending in a ball like structure, and fusion of the greater horn and thyroid cartilage. **F-I.** Skeletons of embryos in which recombination was induced at **F-G.** E10.5 and **H-I.** E11.5 have projections off the greater horn of the hyoid, but otherwise appear relatively normal. **J.** Skeleton of an embryo induced at E12.5 with normal pharyngeal skeleton formation.

Table 3.1: Summary of Phenotypes at each Stage of Cre Induction.

Phenotype	Tissue Origin	Hoxa3 Null	E7.5	E8.5	E9.5	E10.5	E11.5
Viability		Neonatal Lethal	NA	NA	NA	NA	NA
Soft Palate	2nd Arch NCC	Truncated	NA	NA	NA	NA	Normal
Hyoid Lesser Horn	2nd Arch NCC	Absent	Reduced	Reduced	Reduced	Normal	Normal
Thyroid Isthmus	Ventral Endoderm (2nd Arch)	Absent	Absent	Absent	Absent	Normal	Normal
Tracheal Epithelium	Ventral Endoderm (3rd Arch)	Disorganized	Disorganized	Disorganized	Disorganized	Disorganized	Disorganized
Parathyroids	3rd Pouch Endoderm	Absent	Absent	Absent	Absent	Embedded in Thymus	Normal/ Degenerate
Thymus	3rd Pouch Endoderm	Absent	Absent	Absent	Absent	Ectopic/ Cystic	Normal/ Cystic
cgIX	3rd Arch NCC	Disconnected/ Fused with cgX	NA	NA	Normal	Normal	Normal
Hyoid/ Throat Cartilage	3rd and 4th Arch NCC	Reduced/Fusions	Fused	Fused	Fused	Normal/ Projections	Normal/ Projections
Ultimobranchial Body	4th Pouch Endoderm	Persistent	NA	NA	Persistent	Persistent	Normal

CHAPTER 4

TISSUE-SPECIFIC DELETION OF HOXA3

Introduction

Hoxa3 mutant embryos exhibit a wide range of phenotypes affecting a variety of structures derived from neural crest cell (NCC) derived mesenchymal cells of the pharyngeal pouches and the pharyngeal endoderm (Chisaka, 1991, Manley, 1995, Manley, 1997, Manley, 1998). The pharyngeal arches and pouches are transient structures that give rise to many of the organs and tissues of the neck region of vertebrates. NCCs migrating from transient outpocketings of the hindbrain called rhombomeres, provide mesenchymal cells to the developing arches and pouches, as well as directly contributing to the formation of some structures such as cartilage and connective tissue (LeLievre and LeDouarin, 1975). Some organs affected in the Hoxa3 mutant embryos, such as the thymus and parathyroids, require epithelial-mesenchyme interactions for their development and migration (Auerbach, 1960, Revest, 2001, Itoi, 2007). Other phenotypes appear to be due to NCC or endodermal cell autonomous roles for Hoxa3.

Recent work by the Manley lab characterized the role of Hoxa3 in the NCCs (Masuda, unpublished). In mice in which Hoxa3 is knocked out in the NCC using Wnt1-Cre, the posterior throat cartilages, cranial nerves, and secondary palate phenotypes resemble the Hoxa3 null phenotype. These structures are NCC derived, indicating that these phenotypes result from a cell-autonomous NCC defect. However, the lesser horn of the hyoid is not deleted as in the

Hoxa3 null, but is reduced, indicating that its formation may also depend on another cell type, such as the endoderm. The endoderm-derived thymus and parathyroid organs are also affected in the NCC-specific Hoxa3 deleted embryo, indicating that the NCCs have a specific role in their formation. The thymus was ectopic, and remained persistently attached to the pharynx, while the parathyroids showed delayed separation from the thymus.

It is hard to know what role Hoxa3 may play in thymus and parathyroid organogenesis beyond the initial formation of these organs due to their absence in the Hoxa3 null mutant. The role of Hoxa3 in the thymus was recently deduced by deleting Hoxa3 specifically in the thymus using Foxn1-Cre. These embryos showed mild hypoplasia of the thymus, but no other detectable defects (Masuda, unpublished). Indirect evidence for a role for Hoxa3 in maintaining the parathyroid comes from studies on compound Pax1;Hoxa3 mutant mice (Su, 2001). In these mice, the Pax1 null parathyroid phenotype is exacerbated by the removal of a single copy of Hoxa3, resulting in almost complete loss of parathyroids by E18.5. However, a direct test of Hoxa3 function in the parathyroid is still lacking.

In order to further understand the function of Hoxa3 in each of the tissues in which it is expressed, I wanted to continue characterizing tissue-specific deletions of Hoxa3 by using a floxed Hoxa3 allele and tissue-specific Cre recombinases. I first wanted to determine whether Hoxa3 played different roles in two of the tissues in which it is expressed, the endoderm and neural crest, by characterizing the phenotypes of endoderm-specific deletion mutants and comparing them to the NCC-specific deletion mutants already characterized. These two tissues, the NCCs and endoderm, are of the most interest to us because they give rise to all of the organs and structures affected in the Hoxa3 null embryos. I was also interested in what possible roles Hoxa3 may play, if any, in the parathyroid glands after they have formed, and thus tested

whether Hoxa3 is required in the parathyroid after initial organogenesis. Finally, I wanted to explore a possible mechanism underlying the lesser horn phenotype seen in the NCC-specific deletion mutants. Together, these experiments give us a good indication of the functions of Hoxa3 in the two tissues and some of the organs in which it is expressed, and help to characterize the various roles Hoxa3 plays throughout development.

Results

Hoxa3 in the Endoderm Induces Pax9 Expression

Most of the NCC derived structures affected in the Hoxa3 null had the same phenotype in the NCC-specific deletion of Hoxa3 embryos. However, there was one phenotype that didn't follow this pattern: the lesser horn of the hyoid was not deleted as in the Hoxa3 null, but was reduced, indicating that its formation may depend on another cell type, such as the endoderm. It is possible that Hoxa3 in the endoderm signals to the neural crest cells that form the lesser horn of the hyoid bone via a pathway involving an unknown signaling molecule(s). A good potential candidate component of this signaling pathway is Pax9. Pax9 is expressed in the endoderm of the pharyngeal pouches during pharyngeal development, and is known to interact with Hoxa3 (Manley, unpublished). Pax9 mutant mice have numerous defects in their craniofacial skeleton including malformed greater and lesser horns of the hyoid and the thyroid and cricoids cartilages, similar to those seen in Hoxa3 mutant mice, indicating that Pax9 expression in the endoderm is required for proper patterning of the neural crest cells that give rise to the skeleton (Peters,

1998). In addition, the endoderm has previously been shown to be crucial for directing proper craniofacial skeletal development (Couly, 2002, Ruhin, 2003).

In order to determine whether Pax9 plays a role in the malformation of the lesser horn of the hyoid in the Hoxa3 null and NCC-specific knockout mice, I did whole mount *in situ* hybridizations on E10.75 $a3^{-/-}$ and $a3^{fx/-}$;Wnt1-Cre^{+} embryos using a Pax9 probe (Figure 4.1, n=3 for each genotype). In the Hoxa3 null embryos, Pax9 expression is reduced specifically in the third pharyngeal pouch, but appears unchanged in the other pouches, somites, and anterior forelimb (Figure 4.1C). This result is consistent with a role for Pax9 in signaling from the third pouch endoderm to the neural crest cells that give rise to the lesser horn of the hyoid bone. In contrast, the $a3^{fx/-}$;Wnt1-Cre^{+} embryos had no reduction in Pax9 staining in the third pouch (Figure 4.1B). These results are consistent with a model in which Hoxa3 in the pharyngeal endoderm regulates Pax9, which in turn signals to the NCCs that become the lesser horn of the hyoid bone.

Hoxa3 Expression is Important for Parathyroid Maintenance

In order to detect any possible roles for Hoxa3 after initial organogenesis in the parathyroid, I used tissue-specific deletions of Hoxa3 within this organ using PTH-Cre. PTH-Cre is first expressed around E11.5, after formation of the third pharyngeal pouch derived organs has begun and just before the organs start to migrate (Libutti, 2003). Initially, there was no noticeable change in the parathyroids of wild type and $a3^{fx/-}$;PTH-Cre^{+} mutant embryos at E13.5 (Masuda, unpublished, data not shown). However, by E15.5, the mutant embryos had parathyroid glands that were significantly reduced (41.3% smaller) in overall size compared to control littermates as

determined by measuring the volume of reconstructed parathyroids (Figure 4.2, n=2). It therefore appears that the parathyroids either begin to shrink or else fail to grow after removal of Hoxa3. In the first case I would expect the parathyroid to eventually disappear completely, whereas in the second I would expect to find small parathyroids at each developmental stage examined. In embryos in which Hoxa3 was globally deleted at E11.5, the parathyroid appeared to form and migrate normally by E13.5, but had completely disappeared by E18.5 (Chapter 3, Figure 3.2 and 3.4). Similarly, in Hoxa3;Pax1 compound mutants, the parathyroid disappeared by E18.5 (Su, 2001). These data are all consistent with the conclusion that Hoxa3 is required after initial organ formation to maintain the parathyroid.

Deletion of Hoxa3 in the Endoderm has Minimal Effects on Development

The organs affected in the Hoxa3 null mouse, namely the thymus, thyroid isthmus, ultimobranchial bodies, and parathyroids, as well as the tracheal epithelium, are all derived from the endoderm. While the NCCs that surround these tissues appear to have a role in the separation of the third pouch derived organs from the pharynx, and possibly their migration, it is likely that their initial formation depends primarily on the endoderm. To test the function of Hoxa3 specifically in endoderm, I used an endoderm-specific, tamoxifen-inducible Cre recombinase, FoxA2-mcm-CreER, to delete Hoxa3 specifically in endoderm (Park, 2008).

I first looked at whether initial third pouch formation and patterning occurred normally in these mutants. I did whole mount *in situ* hybridization on E10.5 embryos for Gcm2, which is an early parathyroid marker, and found that it was expressed in the third pouch in the mutants, though possibly at a lower level than in the controls (Figure 4.3A-B, n=2). It therefore appears

that initial patterning of the pouch may occur normally. I next looked at the structure of the pouch at E11.5, and found that this was also normal (Figure 4.3C-D, n=2). Together these results indicated that patterning and initial organ development of the third pouch are normal in these embryos, contrary to our expectations.

My original hypothesis was that deletion of Hoxa3 in the endoderm would result in failure of formation of endoderm-derived organs, such as the thymus and parathyroid, if their formation was endodermal cell autonomous. When I looked at these organs at E13.5 however, I found the thymus and parathyroids were missing from their normal locations (Figure 4.4C-D, n=2) and that there was what appeared to be an ectopic thymus located at about the level of the ultimobranchial bodies (Figure 4.4E). When I looked several days later in development at E18.5, the thymus was still ectopically located in the throat, and the parathyroid was missing (Figure 4.5C-D, n=1). These results indicate that Hoxa3 in the endoderm is required for organ migration. At this later stage I was also able to examine the thyroid and ultimobranchial body defects. The ultimobranchial bodies fuse with the thyroid lobes normally, (Figure 4.5E) and the thyroid isthmus was unaffected by the deletion of Hoxa3 (Figure 4.5F).

The only structure in the Hoxa3 null embryos that does not have any input from NCCs is the tracheal epithelium. In the Hoxa3 null mice, this layer of cells is poorly organized, with cells of differing shapes and sizes piling on top of each other, and creating a convoluted surface (Manley, 1995). When I examined the tracheal epithelium at E18.5 following deletion of Hoxa3 in the endoderm, I found that the epithelium was disorganized as seen in the null embryos (Figure 4.6B, n=1). This result indicates that Hoxa3 plays an endodermal cell autonomous role in the formation of this epithelium.

The NCC autonomous phenotypes characterized in the NCC-specific null mice, such as the pharyngeal skeleton phenotypes and cranial nerves, were found to be normal in the endoderm-specific Hoxa3 null mice (Figure 4.7, n=8 for skeletons, n=3 for neuro). These results confirm that the endoderm is not required for the formation of these neural crest cell derived structures.

Deletion of Hoxa3 in both Neural Crest and Endoderm Partially Recreates the Null Phenotype

While many of the null phenotypes can be recreated by deletion of Hoxa3 in either NC or endoderm, there are several phenotypes that cannot. In order to determine whether Hoxa3 expression in these two tissue types has a redundant role in the formation of these organs, I examined embryos that expressed both the endoderm-specific FoxA2-mcm-CreER and the NCC-specific Wnt1-Cre. I found that at E18.5, the $a3^{bx/fx}$;FoxA2-Cre^{+};Wnt1-Cre^{+} embryos were both athymic and aparathyroid (Figure 4.8E-F, n=2), as seen in the Hoxa3 null. This result indicates that Hoxa3 expression in either the NC or endoderm is sufficient to direct initial organogenesis, and that expression in at least one of these tissues is required for organogenesis. The ultimobranchial bodies appeared to be associated with, but not completely fused with the thyroid lobes, and this may indicate that mixing of the two cell types is incomplete as seen in the Hoxa3 null (Figure 4.8C). Interestingly, the thyroid isthmus was unaffected in these mutants, possibly indicating that another tissue type, such as the mesoderm, plays a role in directing the even spreading of the thyroid gland.

I next sought to determine whether deletion of Hoxa3 in both the endoderm and NCC could recreate the hyoid lesser horn phenotype seen in the null embryos. Endoderm-specific deletion had no effect on formation of the lesser horn, whereas NCC-specific deletion resulted in a

reduced lesser horn, though not in its complete deletion as seen in the null embryos. In the $a3^{bx/fx}$;FoxA2-Cre^{+};Wnt1-Cre^{+} embryos, the lesser horn was still not deleted as seen in the Hoxa3 null embryos, however, it was further reduced than in the NCC-specific deletion mutants (Figure 4.9B, n=3).

Discussion

I have continued work determining the role of Hoxa3 in two of the tissue types in which it is expressed using endoderm and NCC-specific deletions either singly or in combination with each other. I have also continued work characterizing the roles Hoxa3 plays in the thymus and parathyroid beyond initial organogenesis. Our analysis demonstrated that some phenotypes are NCC or endodermal cell autonomous, while others are dependent on both cell types, and one may be dependent on Hoxa3 expression in another cell type, such as the mesoderm. I have also demonstrated that continued Hoxa3 expression is critically important for the perseverance of the parathyroid gland.

The organs affected in the Hoxa3 null mouse, namely the thymus, thyroid isthmus, ultimobranchial bodies, and parathyroids, as well as the tracheal epithelium, are all derived from the endoderm. Normally during development, the thyroid diverticulum spreads to either side of the trachea, attached in the middle by a thin isthmus, followed by fusion of each thyroid lobe with an ultimobranchial body, after which the two cells types mix with each other. Both the thyroid and ultimobranchial bodies were unaffected by deletion of Hoxa3 in either the NC or endoderm. However, deletion in both of these tissues simultaneously resulted in ultimobranchial bodies that failed to fuse completely with the thyroid lobes, as observed in the null. Surprisingly,

the thyroid isthmus was still present in these mutants, indicating that Hoxa3 from another source, such as the mesoderm, plays a role in its formation. Further work will need to be done to test this hypothesis.

My initial hypothesis was that while the neural crest cells that surround the third pouch derived organs are required for their separation from the pharynx and each other and for organ migration, their initial formation depends primarily on the endoderm. In sharp contrast to this prediction, the thymus and parathyroid were not absent after deletion of Hoxa3 in the endoderm. Instead, they were found ectopically located near the ultimobranchial bodies. This phenotype is remarkably similar to that seen after removal of Hoxa3 from the NCCs. In the NCC-specific deletion mutants, the third pouch derived organs remained persistently attached to the trachea, even up until birth (Masuda, unpublished).

Instead, my data indicated that Hoxa3 expression in the NCCs and endoderm plays redundant roles in the formation of the third pouch derived organs. Embryos in which Hoxa3 was deleted in both the endoderm and NC were found to be both athymic and aparathyroid. This result demonstrates that Hoxa3 in either the endoderm or NC is sufficient to direct initial organogenesis, and that Hoxa3 expression in at least one of these two tissues is required for initial organogenesis. Furthermore, the separation of the third pouch from the trachea requires Hoxa3 expression in the NCCs, whereas migration following this separation requires Hoxa3 expression in the endoderm.

It is likely that Hoxa3 in both the NC and endoderm controls the expression of signaling molecules, and that disruption in these signaling pathways due to the deletion of Hoxa3 could account for both the separation and migration defects observed. Recent work by Kameda et. al. (2009) found that deletion of the FGF receptor, FRS2α, resulted in ectopic third pouches that

remained persistently attached to the pharynx indefinitely. The parathyroid was also persistently attached to the thymus, indicating that their separation was inhibited as well. These embryos had highly elongated third pouches that appear as though they tried to migrate, but couldn't due to the persistent attachment at their anterior end. This result indicates that FGF signaling may play a role in the separation of the thymus and parathyroid from the pharynx.

Once the third pouch has separated from the pharynx, the endoderm appears to be important for directing the migration of these organs to their normal anatomical locations within the embryo, as demonstrated by my endoderm-specific deletion of Hoxa3 results. The thymus and parathyroid are composed of epithelial cells, which are non-migratory, and therefore are most likely not moving on their own. Instead, these epithelial cells probably signal to the surrounding NCC-derived capsule, since NCCs are migratory by nature, and in this way direct the migration of these NCCs.

Two signaling pathways appear to play a role in the migration of the third pouch derived organs once they have separated from the pharynx: ephrins and BMPs. These two signals appear to play slightly different roles within the embryo with regards to organ migration. Deletion of BMP4 with Foxg1-Cre, which is active in the third pouch and in some of the surrounding mesenchymal cells, resulted in ectopic third pouch-derived organs which detached from the pharynx normally, but subsequently showed delayed separation from each other and failed to migrate (Gordon, 2010). These mutants also have a disrupted thymic capsule, and the authors concluded that BMP4-mediated signaling from the epithelium to the surrounding mesenchymal NCCs was required for normal capsule formation. It is unclear whether the disrupted capsule is the primary cause of the migration defects, or whether other defects within the NCCs are responsible. A very similar phenotype was observed in mutants in which ephrin-B2 was deleted

on NCCs (Foster, 2010). These embryos also have an ectopic thymus that detaches from the trachea normally and delayed separation from the parathyroids. However, in this case the lack of signaling was found to cause drastic changes in cell motility leading to a significant defect in cell motility. It therefore appears that the epithelium signals to the NCCs to direct capsule formation and to make them capable of migrating.

The tracheal epithelium is derived from the ventral endodermal epithelium of the embryonic pharynx, and usually forms as a single layer of psuedostratified columnar basal epithelial cells. In the Hoxa3 null, this cell layer is disorganized, forming pockets in places and building up and sloughing off in other places. In addition, the cells appear to have lost their columnar epithelial identity, and have irregular shapes. Not surprisingly, I found that deletion of Hoxa3 in the endoderm yielded an identical phenotype to that seen in the null embryos. This result indicates that Hoxa3 in the endoderm is required for proper formation of the tracheal epithelium, and that this is an endodermal cell autonomous role for Hoxa3.

While the endoderm-derived organs exhibited some surprising phenotypes, the NCC-derived structures proved much more straightforward. Masuda (unpublished) identified several structures whose development demonstrated a NCC-autonomous role for Hoxa3. For two of these, the ninth cranial nerve and greater horn of the hyoid bone, I confirmed that the endoderm does not play a role in their organogenesis via the endoderm-specific deletion mutants.

Following NCC-specific deletion of Hoxa3, the lesser horn of the hyoid is not deleted as in the Hoxa3 null, but is reduced, indicating that its formation may depend on another cell type, such as the endoderm (Masuda, unpublished). The lesser horn was also unaffected by endoderm-specific deletion of Hoxa3. However, when Hoxa3 was deleted in both tissues simultaneously, a somewhat more reduced lesser horn was seen that most closely resembles those seen after

temporal-global deletion of Hoxa3 at E7.5 (see Chapter 3 and Figure 3.6). While this phenotype still does not resemble the original published null lesser horn phenotype, they do bear a close resemblance to the lesser horns observed by Greer et. al. (1999, 2000) using a different null allele. A likely reason for this is the way the classical Hoxa3 null mouse was created: by insertion of a neomycin resistance cassette into the homeodomain. The presence of this cassette may be causing or exacerbating some of the phenotypes observed in the null embryos, and it will be important to create a null mouse line that lacks this cassette in order to determine what phenotypes are actually due to deletion of Hoxa3 and which are not.

I further demonstrated that Pax9 expression is not affected by deletion of Hoxa3 in the NC, consistent with a model in which Hoxa3 in the pharyngeal endoderm regulates Pax9, which in turn signals to the neural crest cells that become the hyoid bone. However, it is also possible that Hoxa3 expressed in either the endoderm or NC can induce Pax9 expression in the endoderm, which would account for the more severe phenotype observed in the $a3^{bx/fx}$;FoxA2-Cre^{+};Wnt1-Cre^{+} mutants. Further work is needed to distinguish between these two options.

Previous work by Masuda (unpublished) demonstrated that when Hoxa3 is removed in the thymus after organogenesis, the resulting thymi have only mild hypoplasia and no other detectable phenotypes. In striking opposition, removal of Hoxa3 from the parathyroid after organogenesis resulted in rapid shrinking of the parathyroid glands. It is unclear why these two organs, which share a common primordium and develop concurrently, should have such different responses to the removal of Hoxa3. It is possible that another Hox3 paralog is functionally substituting for Hoxa3 after its removal in the thymus, which would account for the mild phenotype. This possibility was also explored by Masuda (unpublished), who removed one copy of Hoxd3 from embryos in which Hoxa3 was deleted from the thymus. These doubly mutant

embryos still showed no phenotype beyond the initial hypoplasia, indicating that Hoxd3 is not functionally substituting for Hoxa3 in the thymus. Together these results indicate that while Hoxa3 is required for maintenance of the thymus, it is not required in the thymic epithelial cells, but in some other tissue such as the NC.

One striking difference between the thymus-specific and endoderm-specific deletion mutants is the presence of an ectopic thymus only in the endoderm-specific deletion embryos. This is surprising given that Hoxa3 is being deleted from the same set of thymic epithelial cells in each case. The key difference between these two Cre recombinases is the time they are initially expressed: FoxA2-mcm-CreER is induced by tamoxifen injection at E6.5, whereas Foxn1-Cre does not become expressed until around E11.25-11.5. My time course results in the previous chapter (see Chapter 3, Figure 3.1) indicate that Hoxa3 is effectively deleted between 24 and 36 hours post-injection, which in the case of FoxA2-mcm-CreER is between E7.5 and E8.0, and in the case of Foxn1-Cre is E12.25 to E13.0. In the first case, Hoxa3 is being deleted well before organogenesis has begun, while in the second, Hoxa3 is being deleted just after the third pouch separates from the pharynx and has at least begun its migration. As a result, separation and migration are not affected by deletion with Foxn1-Cre, since these events occur just before Hoxa3 is completely deleted.

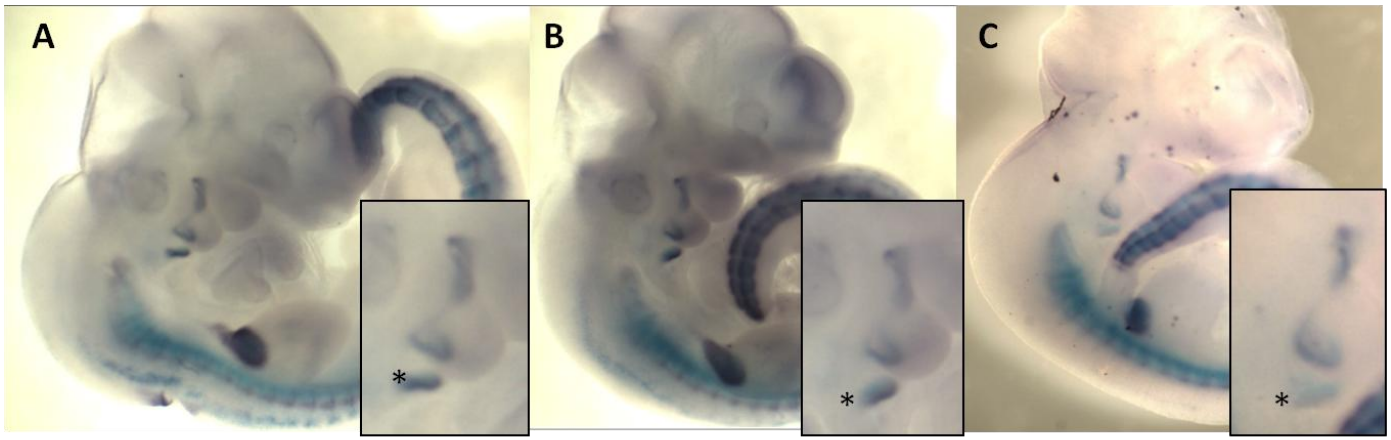

Figure 4.1: Pax9 expression in neural crest cell-specific Hoxa3 deletion embryos. A. NCC-specific mutant with identical Pax9 expression to the control littermate. Insert is a close-up view of the first three pouches, with the third pouch indicated with an asterisk. **B.** Control $a3^{fx/-}$; Cre^{-} littermate showing normal Pax9 expression. **C.** Whole mount in situ with a Pax9 probe in an $a3^{-/-}$ mutant embryo at E10.75 with reduced third pouch expression. Both A and B are at the 36 somite stage, C is at the 35 somite stage.

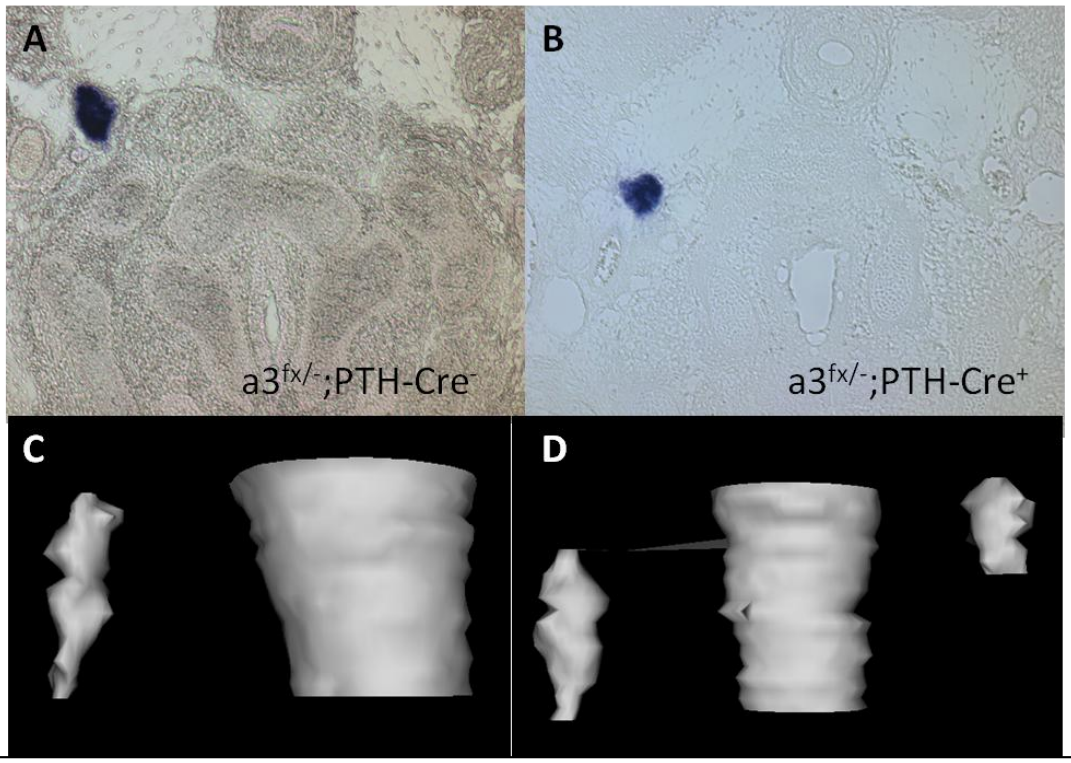

Figure 4.2: The parathyroid gland is affected by deletion of Hoxa3. Sectioned in situ using a PTH probe. **A.** Section from a $a3^{fx/-}$;Cre^{-} control littermate showing normal positioning of the parathyroid gland at E15.5. **B.** Section from a mutant embryo showing relatively normal positioning of the parathyroid gland. The parathyroid appears to be considerably smaller than the control. **C-D.** 3D reconstructions of the in situs confirmed that **D.** mutant parathyroids are on average 58.7% the size of the **C.** control parathryoids.

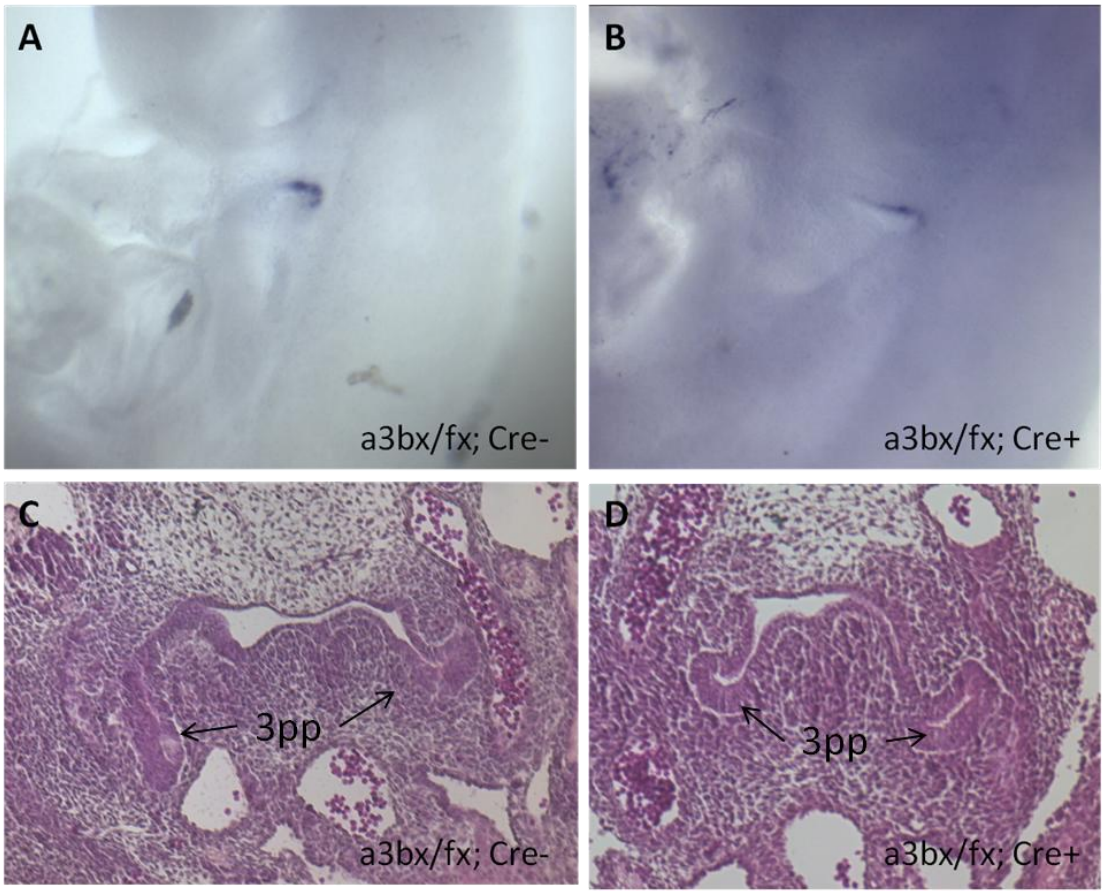

Figure 4.3: Initial patterning occurs normally following induction. A. Gcm2 whole mount in situ on E10.5 control and **B.** mutant embryos. Gcm2 is expressed in the mutants, but possibly at a lower level than in controls. **C.** Transverse sections of E11.5 control and **D.** mutant embryos showing the third pharyngeal pouch. Pouch size and shape appear similar between mutants and controls. 3pp: third pharyngeal pouch.

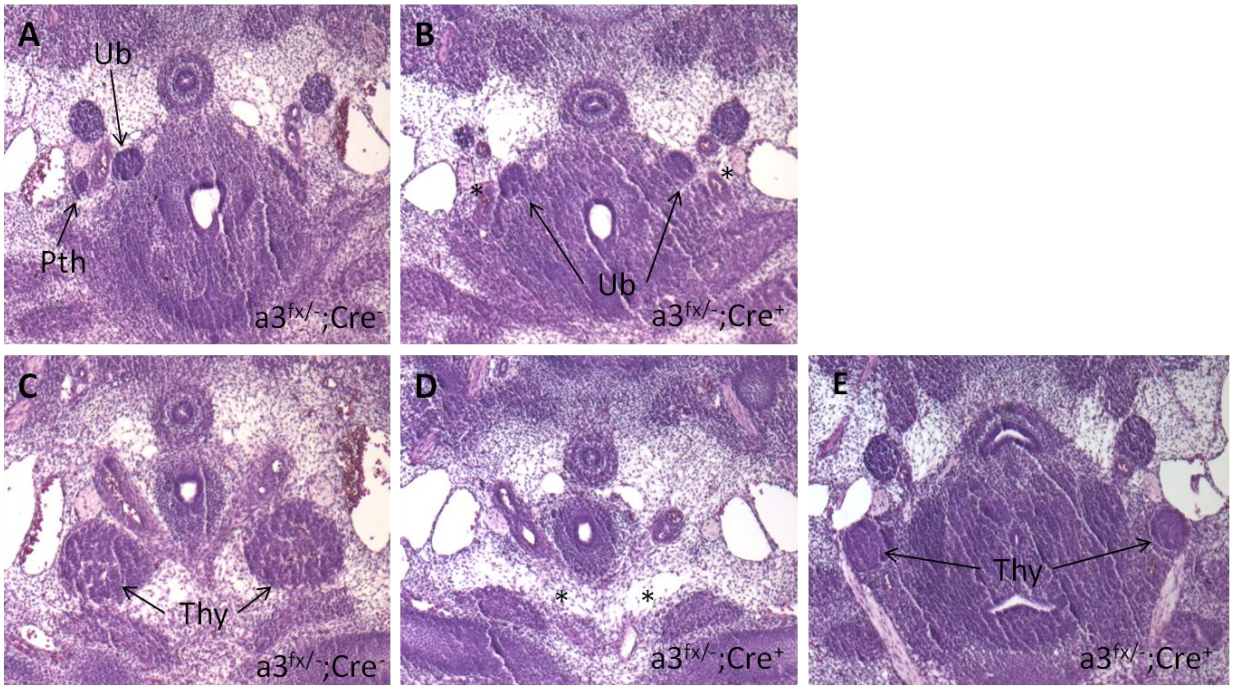

Figure 4.4: Deletion of Hoxa3 in the Endoderm Results in Ectopic Thymi. All embryos were collected at E13.5 and sectioned in the transverse plane. **A.** Control section showing normal ultimobranchial body and parathyroid gland. **B.** Section of a $a3^{fx/-};Cre^{+}$ mutant embryo with normal ultimobranchial body, but missing parathyroids. Asterisk indicates normal parathyroid position. **C.** Control section showing normal location of thymus lobes. **D.** Section from a mutant embryo with missing thymus lobes. Asterisks indicate where thymus lobes would normally be located. **E.** Possible ectopic thymi were located about the level of the ultimobranchial bodies in the mutant embryos.

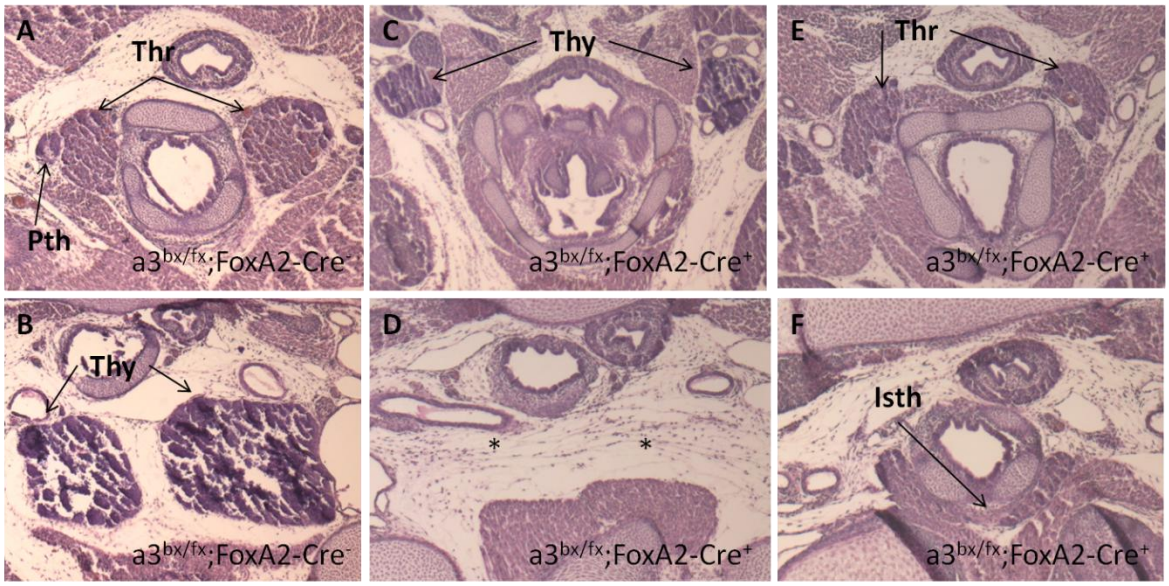

Figure 4.5: Deletion of Hoxa3 in the Endoderm does not Affect Thyroid Development. All embryos were collected at E18.5 and sectioned in the transverse plane. **A.** Control section showing normal thyroid gland, which has completely fused with the ultimobranchial bodies, and normal location of the parathyroid. **B.** Control section showing normal positioning of the thymus. **C-D.** In the mutants, the thymus is **C** ectopicly located in the throat, and **D** not found in its normal location. Asterisks mark the normal location of the thymus lobes. **E.** The ultimobranchial bodies appear to have fused with the thyroid lobes, but not as completely as in the control sections. **F.** The thyroid isthmus was also normal in mutants. Thy: thymus, Pth: parathyroid, Thr: thyroid, Isth: thyroid isthmus.

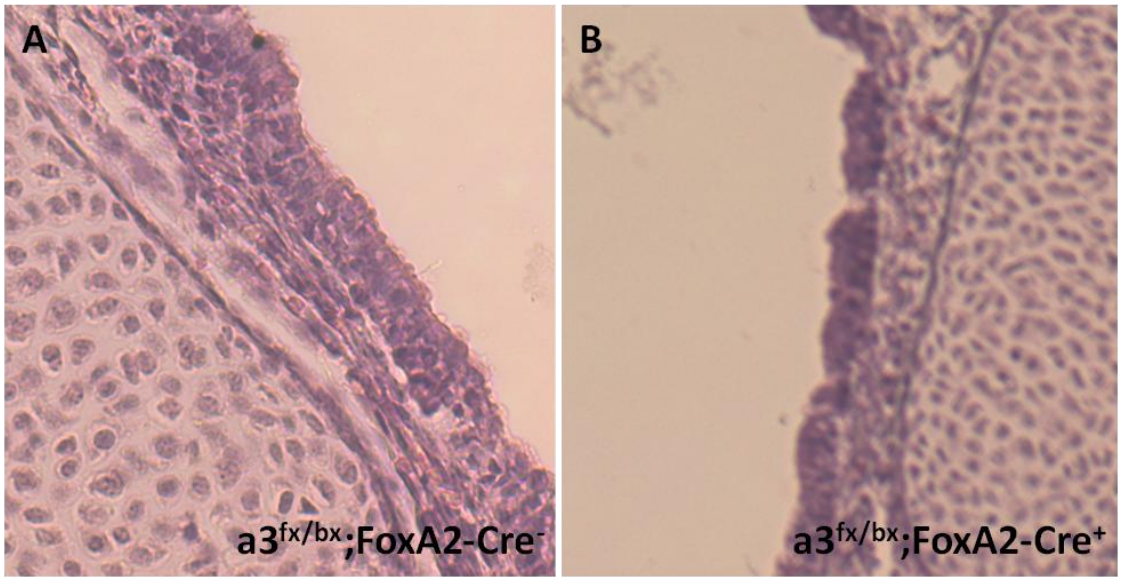

Figure 4.6: Tracheal Epithelium is Disorganized after Deletion of Hoxa3 in the Endoderm. **A.** Control section showing normal organization of the tracheal epithelium. **B.** Mutant section showing disorganization of the epithelium.

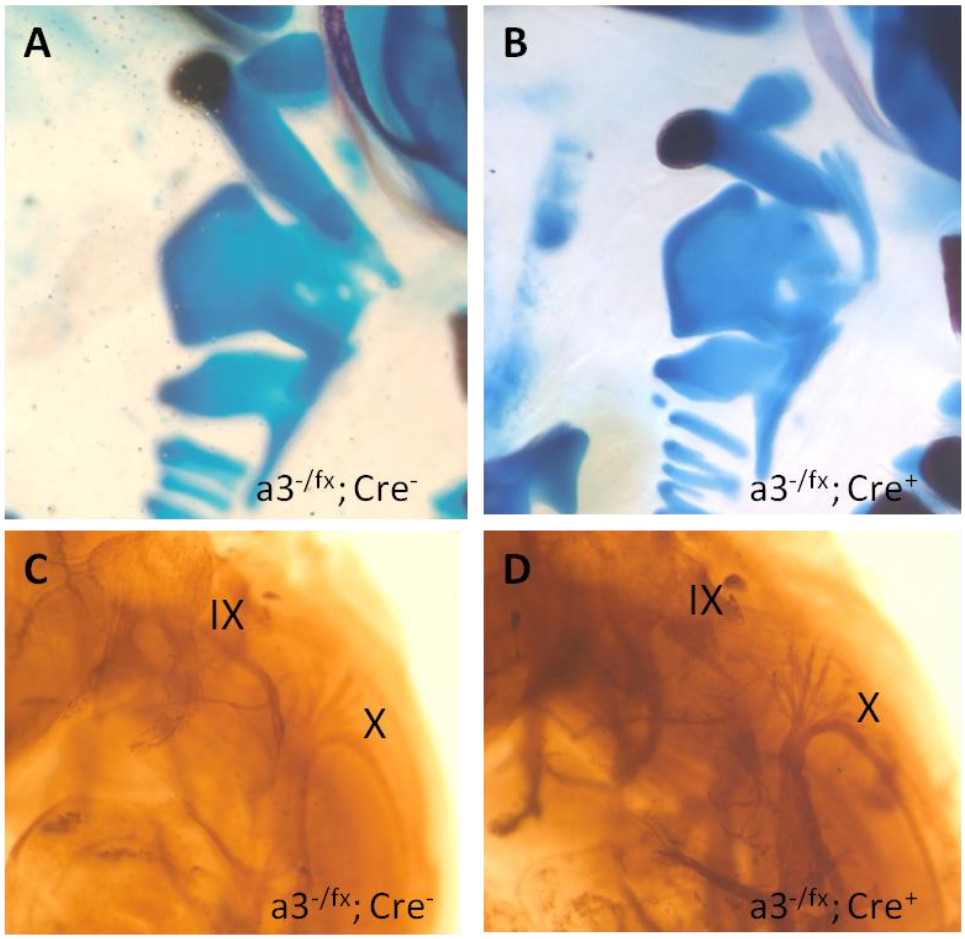

Figure 4.7: NCC-derived structures are unaffected by removal of Hoxa3 in the endoderm. For skeleton preps, E18.5 embryos were stained using alcian blue and alizarin red and cleared through a glycerol series. For neurofilament staingings, E10.5 embryos stained in whole mount with α-neurofilament antibody. **A.** Wild type skeleton showing the hyoid bone and thyroid cartilages. **B.** The mutant skeleton resembles wild type. **C.** Neurofilament staining in a control embryo showing the IX and X cranial nerves. **D.** These nerves are unaffected in the mutant.

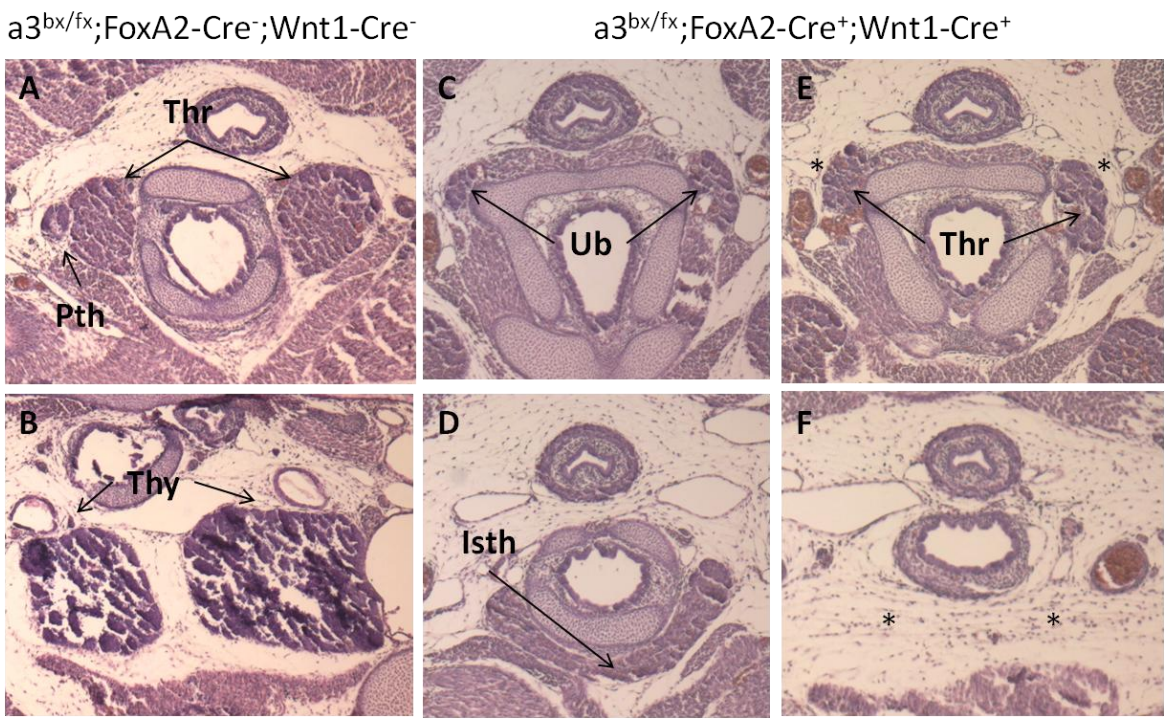

Figure 4.8: Deletion of Hoxa3 in both NCC and Endoderm Recreates some of the Null Phenotypes. All embryos were collected at E18.5 and sectioned in the transverse plane. **A.** Control section showing the normal location of the thyroid and parathyroid and **B.** the thymus. **C.** The ultimobranchial bodies are not fused with the thyroid lobes. **D.** The isthmus is still present, and may depend on another tissue type for its development. **E.** Parathyroid and **F.** thymus are both absent from their normal positions, and are not ectopic. Asterisks mark their normal locations.

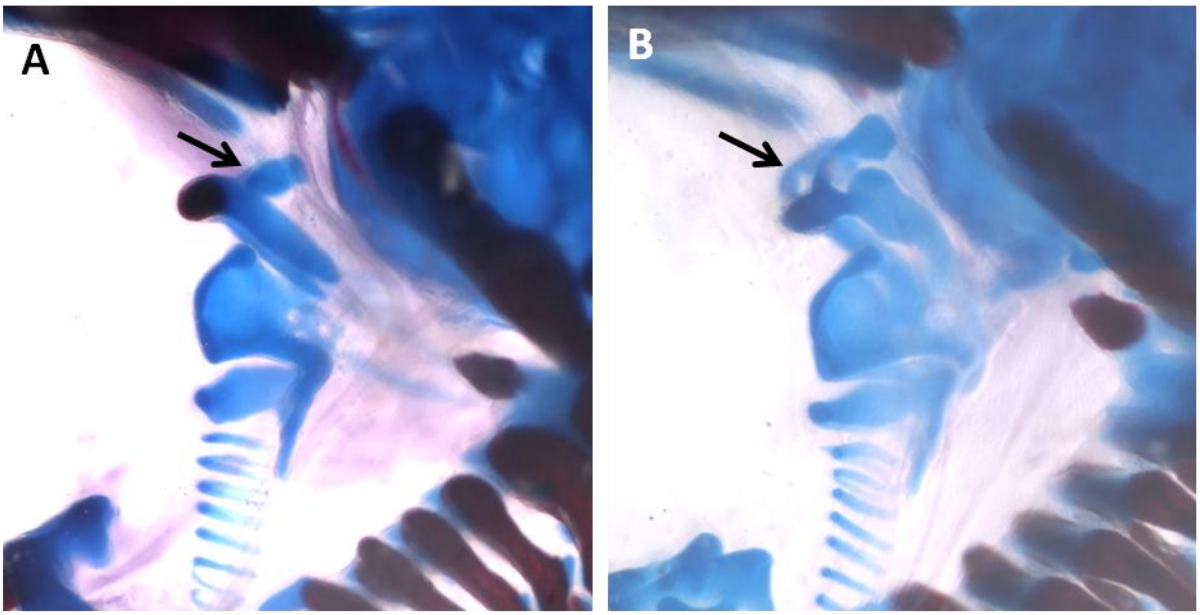

Figure 4.9: Deletion of Hoxa3 Simultaneously in both Endoderm and NCC Results in Malformation of the Hyoid Lesser Horn. E18.5 embryos were stained using alcian blue and alizarin red and cleared through a glycerol series. **A.** Control embryo and **B.** mutant embryo with the hyoid lesser horn indicated by an arrow.

Table 4.1: Summary of phenotypes in NCC-specific and endoderm-specific deletion mutants.

Null defect	Tissue origin	a3bx/fx;Wnt1-Cre	a3bx/fx;FoxA2-Cre	a3bx/fx;Wnt1-Cre; FoxA2-Cre
Truncated palate	2nd arch NCC	Truncated	NA	Truncated
Hyoid lesser horn absent	2nd arch NCC	Reduced	Normal	Further reduced
Thyroid isthmus absent	2nd arch ventral endoderm	Normal	Normal	Normal
Tracheal epithelium disorganized	3rd arch ventral endoderm	Normal	Disorganized	Disorganized
Aparathyroid	3rd pouch endoderm	Delayed separation from thymus	Ectopic	Aparathyroid
Athymic	3rd pouch endoderm	Persistent attachment to trachea	Ectopic	Athymic
cgIX fused to cgX/disconnected	3rd & 4th arch NCC	Same as null	Normal	Same as null
Hyoid/Throat cartilage reduced, fusions	3rd & 4th arch NCC	Similar to null	Normal	Same as null
Ultimobranchial body persistent	4th pouch endoderm	Normal	Normal	Persistent

CHAPTER 5

FUTURE DIRECTIONS

While I have managed to create a fairly complete profile of when Hoxa3 expression becomes important in each tissue type and organ in which it is expressed, there are still gaps in our story that will need to be filled. First, I would like to finish characterizing the phenotypes of the temporal-global Hoxa3 deletion mutants. The only phenotypes not examined were the formation of the ninth and tenth cranial nerves and the soft palate. I would also like to examine whether the follicular and parafollicular cells of the thyroid and parathyroid become mixed in mutant embryos by performing calcitonin and thyroglobulin immunohistochemistry. I would also like to further explore the mechanism underlying the pharyngeal skeleton phenotype, and hopefully distinguish whether it is due to a NCC migration defect, or a patterning defect either within the NCCs or arch endoderm. Finally, I am in the process of repeating the time course knockdown of Hoxa3 using additional time points, and including wild type embryos and Hoxa3 null embryos in addition to the mutant and control embryos used in the initial experiment. This refined experiment should help us determine what the background level of Hoxa3 null mRNA is, and should allow us to more accurately determine how the levels of Hoxa3 expression are changing after induction.

I would also like to finish my work on the endoderm-specific and dual endoderm/NCC-specific deletion mutants. While I finished most of the gross phenotypic examination on these embryos, there are a few gaps left to be filled. I would like to do *in situ* hybridizations for PTH

in endoderm-specific deletion mutants to determine whether the parathyroid is ectopically embedded in the thymus. I would also like to perform antibody stains for calcitonin and thyroglobulin on both endoderm-specific and endoderm/NCC-specific embryos to determine whether the ultimobranchial bodies and thyroid lobes completely fuse in these mutants. I would also like to start testing the role of signaling molecules in the separation and migration of the thymus lobes by doing some *in situ* hybridizations for candidate signaling molecules such as FGFs, Ephrin-B2, and BMP4.

Once I have a complete understanding of when and where Hoxa3 expression is needed during development, I can use this information to try to find downstream targets of Hoxa3 that play roles in the formation of each of these structures. There are multiple approaches that can be taken to address this question. The easiest is a literature search for candidate genes that are expressed in the tissue of interest at time points coinciding to when Hoxa3 expression is known to be critical, as well as for mutant phenotypes that resemble the Hoxa3 null phenotype in the tissue of interest. The candidates identified by this approach will have to be further verified and characterized. One powerful mechanism that may eventually be useful for us is ChIP-seq, which could help us determine what enhancers Hoxa3 binds in each of the tissues in which it is expressed. However, we are limited by the amount of tissue available in early stage embryos, and our ability to dissect out each individual tissue at these early stages. Another exciting prospect for unraveling the downstream pathways include ChIP on chip, which could help us identify where in the genome Hoxa3 is binding.

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