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Hox genes in the pharyngeal region: how Hoxa3 controls early embryonic development of the pharyngeal organs

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ABSTRACT The pharyngeal organs, namely the thyroid, thymus, parathyroids, and ultimobranchial bodies, derive from the pharyngeal endoderm during embryonic development. The pharyngeal region is a segmented structure comprised of a series of reiterated structures: the pharyngeal arches on the exterior surface, the pharyngeal pouches on the interior, and a mesenchymal core. It is well known that *Hox* genes control spatial identity along the anterior-posterior axis of the developing vertebrate embryo, and nowhere is this is more evident than in the pharyngeal region. Each of the distinct segmented regions has a unique pattern of *Hox* expression, which conveys crucial positional information to the cells and tissues within it. In the context of pharyngeal organ development, molecular data suggest that HOXA3 is responsible for specifying organ identity within the third pharyngeal pouch, and in its absence, thymus and parathyroid organogenesis fails to proceed normally. Recent studies comprising a series of *Hoxa3* mutations identified specific spatial and temporal roles for HOXA3 in pharyngeal organ development, including both cell-autonomous and non-autonomous functions, revealing a system that is more complex than originally thought. Here, we will review the current understanding of the role of *Hox* genes in the early embryonic development of the pharyngeal organs in the mouse, with a particular focus on the function of HOXA3 in thymus and parathyroid organogenesis.

KEY WORDS: *Hoxa3, pharyngeal development, thymus, parathyroid, mouse*

Introduction

The pharyngeal apparatus is an excellent example of an embryonic segmented structure that must be precisely patterned along the anterior-posterior (AP) axis (Frisdal and Trainor, 2014). This region is comprised of many different types of tissue, including epithelia, muscles, and nerves, and gives rise to the pharyngeal organs, namely the thymus, parathyroids, thyroid, and ultimobranchial bodies. The HOX transcription factors are classically known for their ability to control segmental patterning and specify positional identity during embryonic development (Alexander *et al.,* 2009), and their role in pharyngeal development is no different. *Hox* genes are expressed by several cell types in the pharyngeal region, including the endodermal epithelium and neural crest cells, making their role in pharyngeal organogenesis extremely complex. In this review, we will discuss the role of *Hox* genes in patterning the major structures of the pharyngeal region of the developing mouse embryo. We will introduce the pharyngeal organs and define key aspects of their embryonic development, with a specific focus on the role of *Hoxa3* in the coordinated development of the thymus and parathyroids during early mouse embryogenesis. We will also discuss the relationship between HOXA3 and other transcription factors that are present and active in the pharyngeal region during this time.

The pharyngeal region

The pharyngeal apparatus gives rise to a wide range of organs and structures of the head and neck, ranging from those required for feeding and respiration, to those that derive from the upper digestive tract (Frisdal and Trainor, 2014). During embryonic development, the pharyngeal region consists of a series of segmented structures, which are tightly organized along the embryonic anterior-posterior (AP) axis (Fig. 1).

Abbreviations used in this paper: BMP, bone morphogenetic protein; E, embryonic day; Eya, eyes absent; Fox, forkhead box; Gcm, glial cells missing; Hox, homeobox; NCC, neural crest cell; Pax, paired box; Pbx, pre-B-cell leukemia homeobox; PTH, parathyroid hormone; Six, Sine oculis homeobox; TEC, thymic epithelial cell.

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The pharyngeal arches are perhaps the most apparent of the pharyngeal structures because they are visible from the exterior of the embryo. The arches are transient structures that resemble a series of bilateral paired bulges in the face/neck region, separated by pharyngeal grooves or clefts. Mammals have five pairs of pharyngeal arches (numbered 1, 2, 3, 4, and 6; 5 regresses as soon as it forms) that form sequentially in a rostral to caudal sequence. In mice, these form between day 8 and day 11 of embryonic development (E8-E11). Arch 1 has two prominences: the upper (maxillary) prominence forms the maxilla (upper jaw), zygomatic bone and the squamous part of the temporal bone; the lower (mandibular) prominence forms the mandible (lower jaw). Arch 2 forms most of the hyoid bone, while arches 3, 4 and 6 form various neck structures, including the cartilage of the larynx. Each pharyngeal arch is composed of cells from all three germ layers: an external ectodermal layer, an internal endodermal layer, and a central mesenchymal core. In vertebrates, the core is composed of mesoderm and neural crest cells. The pharyngeal arches also have blood vessel, nerve, muscle, and cartilage components. Each arch has its own blood supply, the aortic arches; the arch arteries undergo extensive remodeling during development (Hiruma *et al.,* 2002). Each arch also has an associated nerve: the first arch has the mandibular (and maxillary) division of the trigeminal nerve (cranial nerve V); the second arch contains the facial (VII) nerve; the third arch has the glossopharyngeal (IX) nerve; and the fourth arch is innervated by the vagus (X) nerve. Precise patterning of the pharyngeal arches, and all of the cell and tissue types of which they are made up, is therefore clearly essential for their correct development.

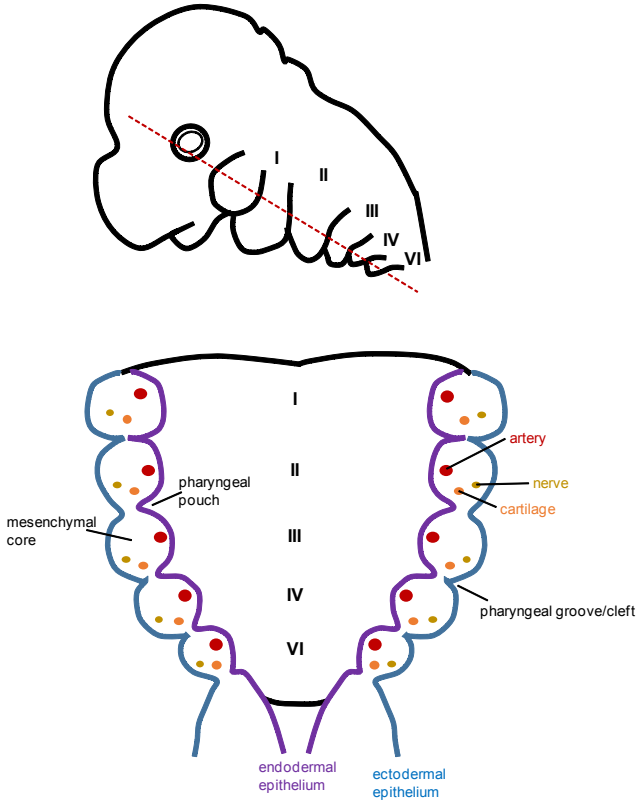

Fig. 1. Major components of the pharyngeal region. *Cross section shows that each pharyngeal arch is composed of an outer ectodermal epithelial layer (blue), an inner endodermal epithelial layer (purple) and a mesenchymal core. Each arch also has its own artery (red), nerve (tan) and cartilage component (orange).*

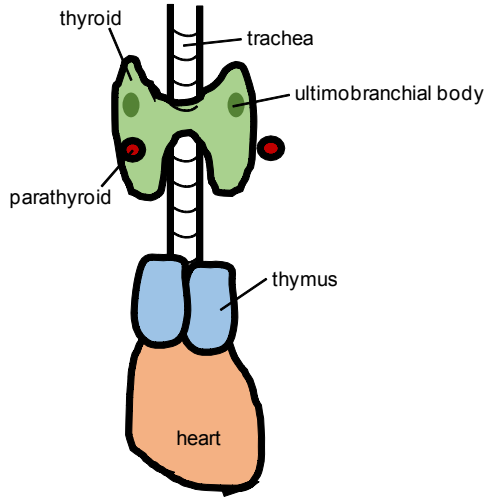

Fig. 2. Approximate locations of the pharyngeal organs in the adult. *The thymus (blue) lies on top of the heart; the ultimobranchial bodies (dark green) fuse completely with the thyroid (light green), and the parathyroids (red) are close to the lateral aspects of the thyroid lobes. Notice that sometimes the parathyroid are embedded within the thyroid, but sometimes remain as separate structures.*

On the interior of the developing embryo are another series of segmented structures called the pharyngeal pouches. These are transient paired endodermal outpocketings that form on the lateral surfaces of the pharynx and lie in between the exterior pharyngeal arches. Like the arches, they arise sequentially during development, and are numbered so that the first pouch is most anterior, closest to the head. There are four pairs of pharyngeal pouches in mice, which form between embryonic days 8 and 10 (E8 and E10). The pharyngeal endoderm will give rise to the thyroid, ultimobranchial bodies, thymus, and parathyroids, collectively known as the pharyngeal organs, and these will be the focus of this review.

Neural crest cells (NCCs) are a transient multipotent cell population that emerges from the dorsal neural tube during embryonic development (Dupin and Le Douarin, 2014). In the head, NCC migration begins before closure of the neural tube. The cells originate from hindbrain segments called rhombomeres, and move along predetermined paths to populate the pharyngeal arches (Kulesa and McLennan, 2015). NCCs give rise to a diverse range of structures and cell lineages, including melanocytes, craniofacial cartilage and bone, smooth muscle, peripheral and enteric neurons and glia (Dupin and Le Douarin, 2014).

The pharyngeal organs

The pharyngeal organs can be defined as any organ that derives from the endodermal epithelium in the anterior pharyngeal region of the developing embryo. In vertebrates, these organs include the thyroid, ultimobranchial bodies, thymus, and parathyroids (Fig. 2). The thyroid is a butterfly-shaped endocrine organ located on the ventral aspect of the pharynx, and is comprised of two lobes connected at the midline by an isthmus. The thyroid diverticulum, the precursor of the thyroid, arises from the ventral midline of the

foregut endoderm, at the level of the second pharyngeal arch, and descends to the area at the junction of the larynx and the trachea (Fagman *et al.,* 2006). The thyroid is responsible for maintaining body metabolism via the production of thyroid hormones. The ultimobranchial bodies are small organs that derive from the fourth pharyngeal pouches and give rise to the calcitonin-producing parafollicular cells of the thyroid. They fuse completely with the thyroid diverticulum-derived primordium during mid-late embryogenesis (Fagman *et al.,* 2006). The thymus is a bilobed organ located in the chest region just above the heart, and is the primary lymphoid organ responsible for T cell production. T cell precursors enter the thymus via the bloodstream and undergo a complex sequence of selection events to generate a fully functional mature T cell repertoire that is released into the periphery (James *et al.,* 2018). Thymic epithelial cells (TECs) are the major functional component of the thymus, all of which originate from the endoderm of the third pharyngeal pouch (Bennett *et al.,* 2002; Gill *et al.,* 2002; Gordon *et al.,* 2004). The thymus also has a neural crest-derived component: NCCs migrate from the hindbrain to surround and invade the developing primordium, eventually forming the thymic capsule and pericytes that surround the blood vessels (Jiang *et al.,* 2000). The parathyroid glands are a pair of small endocrine organs located in the neck region, named for their close proximity to the thyroid gland. The parathyroids regulate calcium and phosphate homeostasis via the production of parathyroid hormone (PTH). In mice, the parathyroids originate with the thymus from the third pharyngeal pouches (Manley and Capecchi, 1998). During embryogenesis, the common thymus-parathyroid primordium must undergo a complex series of morphogenetic events to detach from the pharynx and separate into distinct organ domains (Gordon and Manley, 2011). This allows the thymus to descend into the superior mediastinum towards the heart, while the parathyroids remain in the neck region, associated with the thyroid.

Tightly regulated positional information is essential to convey specific pouch identity onto the pharyngeal endoderm. This in turn will transfer organ-specific identity to epithelial cells that make up the pouch. Furthermore, in another level of complexity, the third pouch must form both a thymus lobe and a parathyroid gland. How this spatial patterning is achieved within an initially uniform epithelial structure has been the focus of much research in the field of developmental biology.

Coordinated thymus and parathyroid organogenesis

The development of the thymus and parathyroids is particularly intriguing because despite the fact that the organs have very different adult structures, locations, and functions, they in fact share a common embryonic origin: the third pharyngeal pouches (Manley and Capecchi, 1998). Each pouch will form a single endodermal epithelial primordium that is surrounded by neural crest-derived mesenchymal cells, and will eventually give rise to one thymus lobe and one parathyroid gland. The primordia must therefore be precisely patterned such that the epithelial cells are directed towards either a thymus or parathyroid fate. Interestingly, while the neural crest-derived mesenchyme may be required for fine-tuning of this patterning (Griffith *et al.,* 2009), the positional information is all contained within the pharyngeal endoderm from the earliest stages of pouch formation (Gordon *et al.,* 2004). In short, the process of thymus-parathyroid organogenesis is a highly dynamic process that can be thought of in terms of a series of tightly coordinated events: pouch formation, pouch outgrowth, pouch patterning into specific organ domains, detachment from the pharynx, organ separation, and, finally, organ migration (Fig. 3 and 4), all of which occur over a relatively short period of developmental time.

The third pharyngeal pouches arise on day 9 of mouse embryonic development (E9.0). At this time, they comprise a single epithelial cell layer surrounding a central lumen that is continuous with that of the pharynx. By E10.5 the pouches have begun to increase in size but are still uniform in structure and remain attached to the lateral surfaces of the pharynx. More significant and dramatic changes occur over the next 24 hours, on both a cellular and a molecular level. Differential proliferation within the primordium results in a larger prospective thymus (ventral) domain relative to the parathyroid (dorsal) domain at E11.5. As a result of the increased proliferation, the epithelial cells within the primordium begin to lose the organized single cell layer morphology and assume a more three-dimensional network-like architecture. This growth increase continues over the next few days, such that the central lumen closes

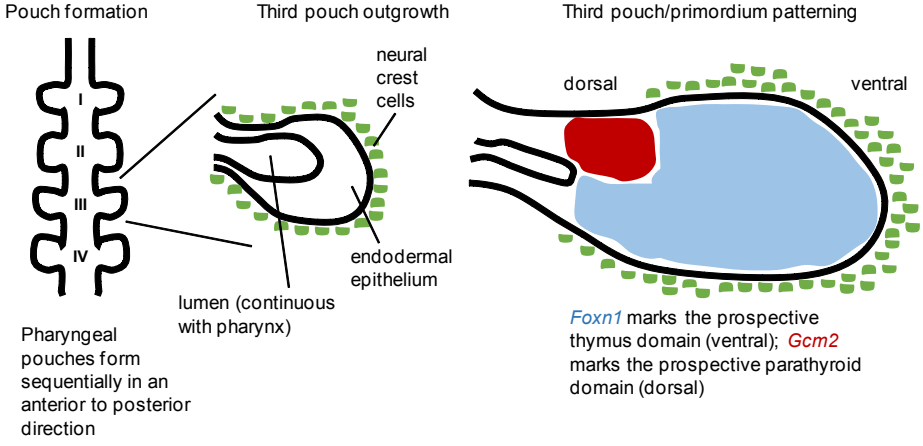

Fig. 3. Formation, outgrowth and patterning of the third pharyngeal pouch. *The pharyngeal pouches form as paired outpocketings on the lateral surfaces of the pharynx. As the pouches grow, they become surrounded by neural crest cells (green) that migrate into the pharyngeal arches from the hindbrain. The third pharyngeal pouch will form a common primordium that gives rise to the thymus and parathyroids. The primordium is patterned into the specific organ domains by the expression of* Foxn1 *(thymus; blue) and* Gcm2 *(parathyroid; red).*

and the organs assume a more rounded appearance. During this time, cell fate is established within the primordium, as is made evident by the expression of organ-specific molecular markers. The parathyroid domain is marked by the expression of *Gcm2*, the mammalian homolog of the *Drosophila Glial cells missing* gene, beginning at E10.5 (Gordon *et al.,* 2001). Continued *Gcm2* expression is required for parathyroid maintenance and survival (Gunther *et al.,* 2000; Liu *et al.,* 2007). However, the first known molecular marker for the thymus domain is not seen until E11.25 (Gordon *et al.,* 2001). Foxn1 is a member of the forkhead transcription factor family and is required for TEC differentiation and proliferation (Nehls *et al.,* 1994). Thus, at E11.5, organ domains within the common primordium can be identified by the expression of the transcription factors *Foxn1* (ventral thymus domain) and *Gcm2* (dorsal parathyroid domain) (Gordon *et al.,* 2001), indicating that the cells within the primordium have assumed either a thymus or parathyroid fate (Fig. 3).

Epithelial-mesenchymal interactions between the endoderm and surrounding neural crest are a key aspect of early pharyngeal organ development. Ablation of the neural crest prior to migration leads to defects in thymus and parathyroid organogenesis (Bockman *et al.,* 1990). In the *Pax3* null (Splotch) mutant, a deficiency in neural crest cells was shown to cause a shift in the boundary between the organ domains such that the prospective thymus domain is increased at the expense of the parathyroid domain (Griffith *et al.,* 2009). This demonstrates a role for the neural crest-derived mesenchyme in correct patterning of the third pouch into the thymus and parathyroid domains. More recent work using tissue-specific Bmp4 mouse mutants suggest that the interactions between the endodermal epithelium and neural crest-derived mesenchyme may be mediated at least in part by Bmp signaling (Gordon *et al.,* 2010). Later in thymus organogenesis, neural crest cells are thought to provide essential proliferative signals to the TECs (Revest *et al.,* 2001; Teshima *et al.,* 2016).

To complete the developmental process, the organs must undergo a complex series of morphological changes in order to lose their connection to the pharynx, separate from each other and move to their final location in the adult animal (Gordon and Manley, 2011)(Fig. 4). The process of detachment from the pharynx is mediated by coordinated apoptosis within the endodermal epithelium (Gordon *et al.,* 2004). In contrast, not much is known about the process that separates the organs from each other, although there is evidence to suggest that it may be driven by a wedge of surrounding neural crest-derived mesenchyme that pushes between the organ domains (Gordon *et al.,* 2010). As for what controls the migration of the thymus lobes, there is evidence from the mouse to support the notion that NCCs drive this process, such that migrating NCCs 'drag' the thymus lobes through the tissue (Foster *et al.,* 2010); however, what controls the directionality of this migration is not yet known.

Thus, the coordinated development of the thymus and parathyroids is an extremely dynamic and complex process. First, positional information must be present within the pharyngeal endoderm such that third pouch identity is established. A second layer of regional specification is then required within the pouch-derived primordium that allows the endodermal epithelium to form a discrete thymus lobe and parathyroid gland.

The pharyngeal *Hox* code

Many different gene families play important roles in patterning the pharyngeal region, and perhaps the most famous of these are the *Hox* genes. *Hox* genes are classically known to be primary regulators of anterior-posterior and dorso-ventral patterning during the embryonic development of many tissues and organs, and the pharyngeal region is an excellent example of this. Here, as in other areas, the *Hox* genes display segmentally restricted domains of expression, which allows them to convey specific positional information to the region (Hunt *et al.,* 1991). In vertebrates, the *Hox1*, *Hox2* and *Hox3* paralagous groups, often referred to as the anterior *Hox* genes, are expressed in distinct expression patterns in the pharyngeal region (Fig. 5) (Parker *et al.,* 2016). *Hoxa2* is the most anteriorly expressed of the vertebrate *Hox* genes. *Hox2* group genes are expressed in the second and more posterior arches, while *Hox3* and *Hox4* group genes are expressed in the third and fourth arches, respectively. Note that the first pharyngeal arch does not express *Hox* genes (Parker *et al.,* 2016).

While it is beyond the scope of this review, it would be impossible to discuss *Hox* genes in the context of pharyngeal development without acknowledging the major role they play in directing

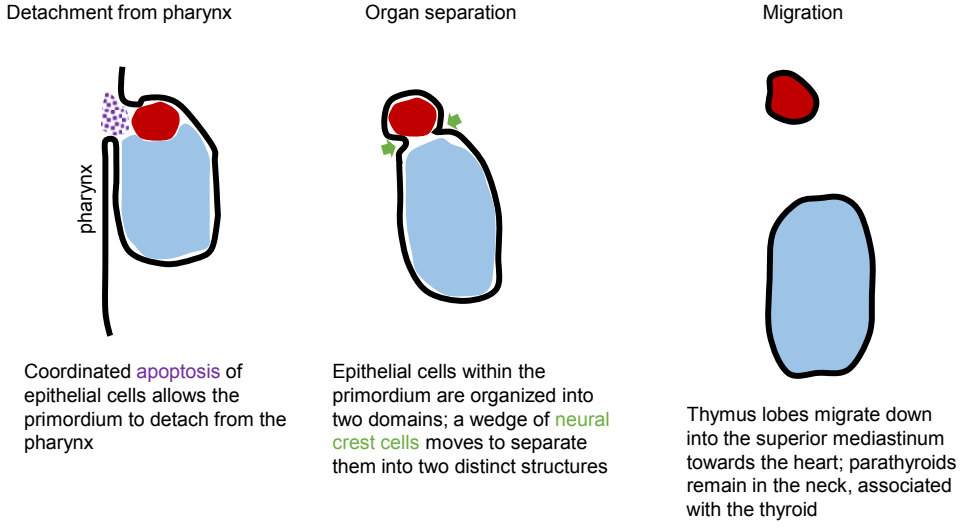

Fig. 4. Key events in thymus-parathyroid morphogenesis. *Detachment from the pharynx is mediated by coordinated apoptosis of endodermal cells (purple dots), while organ separation is thought occur via the movement of a wedge of neural crest cells (green) between the domains. After separation, the thymus (blue) moves down into the chest cavity, while the parathyroids (red) remain in the neck region, close to the thyroid lobes.*

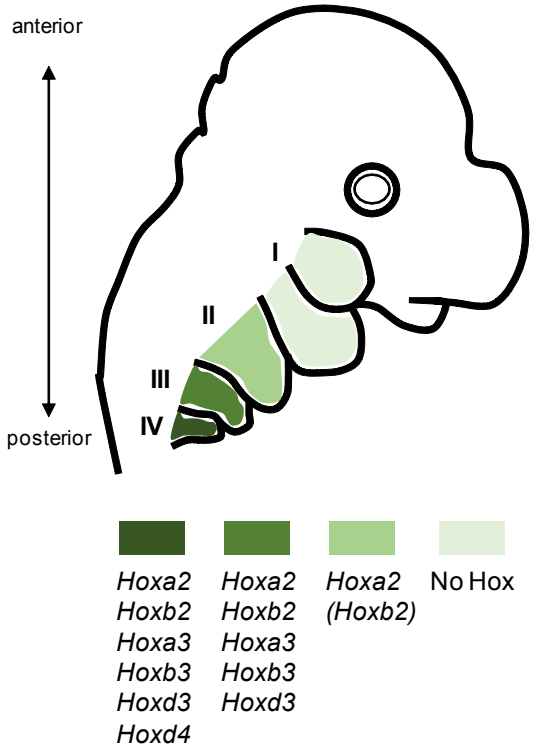

Fig. 5. The pharyngeal *Hox* code. *Nested domains of* Hox *gene expression convey positional information onto the pharyngeal region.*

the coordinated migration of NCCs through the region (Gavalas *et al.,* 2001; Minoux and Rijli, 2010; Trainor and Krumlauf, 2000). NCCs originate from rhombomeres in the hindbrain, and migrate into the pharyngeal arches along predetermined paths according to the *Hox* genes that they express (Trainor and Krumlauf, 2000). The vast amount of work that has been done in this area has demonstrated the existence of two distinct *Hox* codes: one that patterns the hindbrain segments and directs NCC migration, and one that the cells assume upon reaching their intended location in the pharyngeal region (Parker *et al.,* 2018). *Hox* gene expression is thus maintained in the NCCs that come to surround the pharyngeal pouches. In this review, we will describe the role of *Hox* genes in conveying regional specification to the pharyngeal region, with a specific focus on the developing pharyngeal organs. In particular, *Hoxa3* in the pharyngeal endoderm and surrounding neural crest-derived mesenchyme, and the associated downstream transcription factor network/cascade, has been shown to play a key role in the coordinated embryonic development of the thymus and parathyroids.

Hoxa3 is a major player in pharyngeal organ development

Hoxa3 was the first *Hox* gene to be deleted in mice by homologous recombination (Chisaka and Capecchi, 1991) and, together with *Hoxa1* (Chisaka *et al.,* 1992), provided the first evidence that suggested a role for *Hox* genes in pharyngeal region development. *Hoxa3* plays many important roles during early mouse embryonic development, including controlling processes such as cell migration, proliferation, differentiation and apoptosis. Within the developing pharyngeal region, *Hoxa3* is expressed by NCCs that originate from rhombomeres r5, r6, and r7, and populate the third and fourth pharyngeal arches (Chisaka and Kameda, 2005; Manley and Capecchi, 1995; Watari-Goshima and Chisaka, 2011). *Hoxa3* is also expressed by cells from all three germ layers that make up the arches, including endodermal, ectodermal, and mesodermal tissues (Chisaka and Capecchi, 1991; Gaunt, 1987; Gaunt, 1988; Manley and Capecchi, 1995). As will be described below, *Hoxa3* plays an important role in the differentiation and development of the organs that derive from the third and fourth pharyngeal arches and pouches.

A detailed analysis of *Hoxa3* expression in the third pharyngeal pouch and developing organ primordia was performed in an effort to better understand its role in thymus and parathyroid organogenesis (Chojnowski *et al.,* 2014). *Hoxa3* expression levels were found to be similar in the endodermal epithelium and surrounding neural crest-derived mesenchyme at E10.5. However, by E11.5, *Hoxa3* expression is reduced in the endoderm and pharyngeal pouches but is maintained at high levels in the surrounding NCCs until E12.5. *Hoxa3* expression is then undetectable in either the endoderm or surrounding NCCs at E13.5. These data imply an early role for *Hoxa3* in the endoderm, perhaps in tissue patterning, and a later role for *Hoxa3* in the neural crest-derived mesenchyme, perhaps in tissue morphogenesis. *Hoxa3* is also expressed in the developing ultimobranchial body, and is maintained even after its fusion with the thyroid (Chojnowski *et al.,* 2016). Thus, *Hoxa3* is expressed in multiple cell types during the embryonic development of the pharyngeal region, and plays distinct roles in the embryonic development of the pharyngeal organs. It is therefore perhaps no surprise that mutations in *Hoxa3* lead to a range of abnormalities.

The *Hoxa3* null phenotype has been well characterized (Chisaka and Capecchi, 1991; Chojnowski *et al.,* 2014; Chojnowski *et al.,* 2016; Manley and Capecchi, 1995; Manley and Capecchi, 1998; Su *et al.,* 2001). *Hoxa3* homozygous null mutant mice die at or shortly after birth, and exhibit a wide range of abnormalities in neck and craniofacial structures, including the throat cartilage and cranial nerves (Chisaka and Capecchi, 1991; Kameda *et al.,* 2002; Manley and Capecchi, 1995). Within the pharyngeal region, deletion of *Hoxa3* leads to athymia, aparathyroidism, thyroid hypoplasia, and persistence of the ultimobranchial bodies (Kameda *et al.,* 2004; Manley and Capecchi, 1995). More in-depth analysis of the pharyngeal organs in *Hoxa3* null mutants revealed that pouch formation and organ development are initiated normally, but subsequent organogenesis is severely affected, such that the thymus and parathyroids are absent at later stages (Chojnowski *et al.,* 2014).

Consistent with the absence of the thymus at later stages in *Hoxa3* null mutants is the fact that FOXN1 is not expressed in the primordium at E11.5 (Chojnowski *et al.,* 2014). What is surprising, however, is the discovery that FOXN1 expression could be detected, albeit at low levels, at E12. Furthermore, FOXN1 expression reaches normal levels in the developing thymus by E12.5, suggesting that the loss of *Hoxa3* leads to a delay in the initiation of *Foxn1* expression, rather than a complete inhibition. However, despite this initiation in development, the thymus is completely absent at E13.5, suggesting that HOXA3 may play an additional role in organ survival. Indeed, apoptosis is increased in the *Hoxa3* mutant primordium between E11.5 and E13.5 (Chojnowski *et al.,* 2014), and includes both the thymus and parathyroid domains, which explains the absence of both organs at later stages. In contrast to FOXN1, however, the

parathyroid marker GCM2 is properly expressed in the third pouch at E10.5, but then its expression becomes markedly reduced so that by E11.5 no GCM2 expression can be detected in the third pharyngeal pouch (Chojnowski *et al.,* 2014). This suggests that, unlike the thymus, parathyroid formation is initiated at the correct time, but then further differentiation and expansion are halted in the absence of *Hoxa3*. Thus, in the context of thymus-parathyroid organogenesis, *Hoxa3* appears to function in the specification of organ fate within the third pharyngeal pouch, which is consistent with the role of *Hox* genes in specifying positional identity during embryonic development.

The ultimobranchial bodies derive from the fourth pharyngeal pouches in mouse embryonic development. They begin to detach from the pharynx at E11.5, then migrate towards the thyroid diverticulum at E13.5, finally fusing with the lateral lobes of the thyroid at E13.5-E14.5 (Fagman and Nilsson, 2011). In *Hoxa3* null mutants, the ultimobranchial bodies migrate normally but then fail to fuse with the thyroid lobes, often becoming cystic (Manley and Capecchi, 1995; Manley and Capecchi, 1998). In addition, despite that fact that *Hoxa3* is not expressed in the developing thyroid (Chojnowski *et al.,* 2016), *Hoxa3* null mutants do exhibit some defects in thyroid development, including organ migration and isthmus integrity. This suggests that HOXA3 may also play some cell non-autonomous roles during pharyngeal organ development.

Hoxa3 is expressed in the pharyngeal endoderm, including the pharyngeal pouches, and in the surrounding neural crest-derived mesenchyme, and the null mutant displays deficiencies in both of these cell types. This dynamic spatial and temporal expression of *Hoxa3* is perhaps an indication that its role in pharyngeal organ development may be more complex than originally thought. To address this, Chojnowski and colleagues performed a series of mouse genetic studies using endoderm- and neural crest-specific deletions of *Hoxa3* (achieved using *Foxa2CreERT2* and *Wnt1Cre* alleles, respectively) (Chojnowski *et al.,* 2014). Their findings are summarized in Table 1. Perhaps the most striking phenotype in both the endoderm- and NCC-specific *Hoxa3* mutant embryos is the presence of small, ectopic thymi and parathyroids at E17.5 (Chojnowski *et al.,* 2014). This is in contrast to the complete absence of these organs in the *Hoxa3* null mutant, when *Hoxa3* is deleted from both cell types, and indicates that HOXA3 in either cell type is sufficient for thymus and parathyroid organogenesis. Endoderm-specific deletion of *Hoxa3* also causes a delay in the initiation of FOXN1 expression, but no subsequent apoptosis (Chojnowski *et al.,* 2014). The fact that size of the third pouch is normal at E10.5 suggests that the thymic hypoplasia seen in these mutants is a result of a delay in the initiation of TEC proliferation, due to the late onset of FOXN1 expression. GCM2 expression is also delayed in the endoderm-specific *Hoxa3* mutant embryos, consistent with the reduced parathyroid size (Chojnowski *et al.,* 2014). In contrast, GCM2 expression is not affected in the NCC-specific *Hoxa3* mutants, and initial thymus organogenesis is also normal, indicating that *Hoxa3* in NCCs is not required for the initiation or specification of thymus or parathyroid organogenesis. However, deletion of *Hoxa3* from the neural crest-derived mesenchyme does cause later defects in organ separation and detachment of the primordium from the pharynx, which has knock-on effects on thymus migration. While in the endoderm-specific *Hoxa3* mutants organ separation is normal, detachment from the pharynx is delayed (Chojnowski *et al.,* 2014). These data suggest that HOXA3 is required in both the endoderm and neural crest-derived mesenchyme for thymus-parathyroid morphogenesis, but for slightly different processes. Furthermore, deletion of *Hoxa3* from both the endoderm and neural crest recapitulated the null mutant phenotype (Chojnowski *et al.,* 2014), confirming that these are the only two cell types in which HOXA3 is acting during pharyngeal organogenesis.

HOXA3 is not required later in embryonic development for

TABLE 1

EFFECTS OF HOXA3 MUTATIONS ON PHARYNGEAL ORGANS

Mutation	Organ	Phenotype	Reference
Null	Thymus	Athymia	(Chisaka and Capecchi, 1991; Manley and Capecchi, 1995; Manley and Capecchi, 1998)
Null	Thymus	Delay in FOXN1 initiation, then loss	(Chojnowski *et al.,* 2014)
Null	Parathyroid	Aparathyroidism	(Kameda *et al.,* 2004)
Null	Parathyroid	Normal GCM2 initiation, then loss	(Chojnowski *et al.,* 2014)
Null	Thyroid	Hypoplasia; Reduced follicular and parafollicular cells; Large, disorganized follicles; Displaced or absent isthmus	(Manley and Capecchi, 1995)
Null	Ultimobranchial body	Persistence; Failure to fuse with thyroid	(Manley and Capecchi, 1995)
Endoderm-specific	Thymus	Small, ectopic; Delayed detachment from pharynx; Impaired migration	(Chojnowski *et al.,* 2014)
Endoderm-specific	Thymus	Delayed FOXN1 initiation	(Chojnowski *et al.,* 2014)
Endoderm-specific	Parathyroid	Small, ectopic	(Chojnowski *et al.,* 2014)
Endoderm-specific	Parathyroid	Delayed GCM2 initiation	(Chojnowski *et al.,* 2014)
Neural crest-specific	Thymus	Small, ectopic; Failed organ separation; Failure to detach from pharynx	(Chojnowski *et al.,* 2014)
Neural crest-specific	Parathyroid	Small, ectopic; Failed organ separation	(Chojnowski *et al.,* 2014)

maintenance of the thymus and parathyroids. This was shown by deleting *Hoxa3* using either *Foxn1Cre* (thymus) or *PthCre* (parathyroid) strains, both of which are expressed after E11 (Gordon *et al.,* 2007; Libutti *et al.,* 2003). Thus, the role of HOXA3 in thymus-parathyroid organogenesis is restricted to the early patterning, differentiation, and morphogenesis stages, again consistent with the classical role of *Hox* genes in development.

In a follow-up study, Chojnowski and colleagues (Chojnowski *et al.,* 2016) sought to further define the temporal and cell type-specific roles of HOXA3 in pharyngeal organ development using a series of temporal deletions of *Hoxa3* from either the endoderm or surrounding neural crest. This work identified a discrete temporal window up to E11 when HOXA3 is required for thymus and parathyroid survival. A second temporal window during which time HOXA3 is required for morphogenetic events such as organ separation and detachment from the pharynx was identified between E12 and E12.5. This work also revealed that proper incorporation of the ultimobranchial bodies into the thyroid lobes requires the presence of HOXA3 in NCCs prior to E12, which is before the fusion of the two organs happens at E13.5 (Chojnowski *et al.,* 2016).

Interestingly, and perhaps surprisingly, HOXA3 also appears to function in a cell non-autonomous manner during pharyngeal organ development (Chojnowski *et al.,* 2016). Despite the fact that the anterior limit of *Hoxa3* expression is pharyngeal arch 3, many of the structures affected in *Hoxa3* mutants derive from the second pharyngeal arch, including the hyoid bone, soft palate, and thyroid. Defects in thyroid development have been observed in *Hoxa3* mutant embryos, despite the fact that detailed gene expression and lineage analyses clearly demonstrate the absence of *Hoxa3* expression in all thyroid epithelial cells (Chojnowski *et al.,* 2016). *Hoxa3* is, however, expressed in NCCs associated with the developing thyroid, suggesting an indirect role in its development. It is worth noting here that HOXA5 is known to be important for thyroid organogenesis; a mutation in *Hoxa5* results in transient structural disorganization of the developing thyroid during late gestation (Meunier *et al.,* 2003). It could therefore be tempting to speculate that HOXA3 and HOXA5 are working together to control thyroid development. However, while none of the reports of *Hoxa3* mutants assessed changes in *Hoxa5* expression, *Hoxa3* expression was unaffected by the loss of *Hoxa5* (Meunier *et al.,* 2003), which would argue against a functional relationship between the two genes.

In summary, HOXA3 is involved in the development of the pharyngeal organs to a degree that is much more complicated than originally appreciated. This is in part consistent with its dynamic expression pattern in this region, as well as the fact that it appears to play both cell-autonomous and non-autonomous roles in pharyngeal organogenesis. In addition to the more classical role for *Hox* genes in cell-type specification, HOXA3 has now been shown to function in cell survival and tissue morphogenesis during embryonic development of the pharyngeal organs, most particularly in thymus and parathyroid organogenesis.

Interactions between *Hoxa3* and other homeobox genes function to control pharyngeal organ development

All of the current evidence suggests that, in terms of *Hox* genes, *Hoxa3* is the major player in pharyngeal organogenesis. Indeed, *Hoxa3* is the only one of the mammalian *Hox* group 3 paralogs that is expressed in the pharyngeal endoderm; *Hoxb3* and *Hoxd3* expression is restricted to the neural crest-derived mesenchyme. Furthermore, single mutations in either *Hoxb3,* and *Hoxd3* do not affect pharyngeal organ development but a genetic analysis of the three *Hox* group 3 genes revealed that all are in fact important for morphogenesis (Manley and Capecchi, 1998). The *Hoxa3* null ultimobranchial body phenotype is exacerbated in both *Hoxa3-/-;Hoxb3-/-* and *Hoxa3-/-;Hoxd3-/-* double mutants. Furthermore, while the *Hoxb3-/-;Hoxd3-/-* double mutants do not exhibit defects in thymus-parathyroid development, the removal of only one copy of *Hoxa3* (to generate *Hoxa3+/-; Hoxb3-/-; Hoxd3-/-* mutants) results in a failure of the organs to migrate. Thus, the *Hox* group 3 paralogs have highly overlapping functions in mediating the migration of pharyngeal organ primordia during embryonic development (Manley and Capecchi, 1998).

There is additional evidence to suggest that the *Hox* genes may be working at least in part with *Pbx1* to coordinate the development of the pharyngeal organs. PBX1 is a TALE-class homeodomain protein that functions in part as a cofactor for HOX proteins. *Pbx1* is expressed in the developing pharyngeal region, and *Pbx1* null mutants exhibit defects in the pharyngeal pouches and associated organs (Schnabel *et al.,* 2001; Selleri *et al.,* 2001). The thymus, parathyroids, and ultimobranchial bodies are hypoplastic in *Pbx1* null mutants and organ-specific differentiation is delayed (Manley *et al.,* 2004). This is strongly reminiscent of the phenotypes seen in *Hox* group 3 mutant embryos, supporting the existence of an interaction between *Pbx1* and the *Hox* genes, and also providing evidence that *Hox* genes are not functioning alone during pharyngeal organ development.

A *Hoxa3–Pax1/9–Eya1–Six1/4* regulatory pathway controls pharyngeal organ development

It is no surprise that *Hox* genes are not the only transcription factors that play a role in pharyngeal region patterning and development. Evidence from mouse genetic analyses revealed that HOXA3 is part of a regulatory pathway that specifies regional identity and differentiation in the third pharyngeal pouch.

The *paired box* genes are a family of transcription factors that are known to be involved in segmentation during embryonic development (Dahl *et al.,* 1997). *Pax1* and *Pax9* are expressed in the early pharyngeal region, including the third pouch (Peters *et al.,* 1998; Wallin *et al.,* 1996). A mutation in *Pax1* does not affect initial pouch patterning and organogenesis, but does result in mild thymus hypoplasia (Wallin *et al.,* 1996). *Pax9* null mutants display more severe thymus hypoplasia, as well as a failure to separate from the pharynx (Hetzer-Egger *et al.,* 2002; Peters *et al.,* 1998). There is likely to be some redundancy between *Pax1* and *Pax9*, however the pharyngeal phenotype in *Pax1;Pax9* double mutants has not been reported. *Pax1* expression is reduced in *Hoxa3* null mutants (Manley and Capecchi, 1995), and an analysis of *Hoxa3+/-;Pax1-/-* compound mutants confirmed that these genes function in the same genetic pathway (Su *et al.,* 2001; Su and Manley, 2000). *Hoxa3+/-;Pax1-/-* compound mutants display thymus hypoplasia and a failure to separate from the pharynx. Furthermore, *Hoxa3* expression is normal in *Pax1;Pax9* double mutant embryos (Zou *et al.,* 2006), further suggesting that Hoxa3 is independent of *Pax1* and *Pax9*.

The *eyes absent* gene, *Eya1,* is a transcriptional coactivator that is expressed in the third pharyngeal pouch endoderm and

surrounding mesenchyme at E10.5 (Xu *et al.,* 2002). The *Eya1* null mutant phenotype is strikingly similar to that of *Hoxa3*, where thymus and parathyroid organogenesis fails to initiate and no *Foxn1* or *Gcm2* expression is present in mutant embryos (Xu *et al.,* 2002). The pharyngeal arches are also hypoplastic in these mutants, which could indirectly result in smaller than normal pharyngeal pouches. *Eya1* is also expressed in the fourth pharyngeal pouches and developing ultimobranchial bodies (Xu *et al.,* 2002). In *Eya1* null mutants the ultimobranchial bodies fail to fuse with the thyroid lobes, and both organs are hypoplastic, again reminiscent of the *Hoxa3* null mutant phenotype (Manley and Capecchi, 1995; Manley and Capecchi, 1998). Interestingly, *Hoxa3* and *Pax1* expression in the pharyngeal endoderm is normal in *Eya1* null mutants (Xu *et al.,* 2002), suggesting that EYA1 is downstream of both HOXA3 and PAX11 in a genetic cascade that controls pharyngeal organ differentiation.

Six1 and *Six4* are members of the homeobox *Six* gene family homologous to the *Drosophila sine oculis (so)* gene (Kawakami *et al.,* 2000). Like *Eya1*, *Six1* is expressed in the third pharyngeal pouch and surrounding mesenchyme, while *Six4* is present in the endoderm only (Zou *et al.,* 2006). In both *Six1* single mutants and *Six1;Six4* double mutants, thymus and parathyroid organogenesis is initiated, but then the primordium undergoes apoptosis, resulting in a lack of both thymus and parathyroids (Zou *et al.,* 2006). *Six1* has been shown to interact with *Eya1*, however *Eya1* expression is normal in *Six1* single and *Six1;Six4* double mutants, indicating that *Eya1* acts upstream of *Six* genes during early thymus-parathyroid development (Zou *et al.,* 2006). *Hoxa3* expression is also normal in the pharyngeal endoderm and mesenchyme in *Six1* single and *Six1;Six4* double mutants, again providing further evidence that HOXA3 is upstream of SIX1 and SIX4 in third pharyngeal pouch development (Zou *et al.,* 2006). Finally, despite the fact that *Six1* is not expressed in the developing thyroid, the thyroid and ultimobranchial bodies are small in *Six1* mutant embryos (Zou *et al.,* 2006). *Six1* is, however, expressed in surrounding neural crest-derived mesenchymal cells, suggesting that it may be mediating proliferative signals to the endodermal epithelium during normal development.

Taken together, all of this genetic evidence points to the existence of a regulatory cascade involving *Hoxa3*, *Pax1*, *Pax9*, *Eya1*, *Six1* and *Six4*, that controls pharyngeal pouch development and thymus-parathyroid organogenesis. *Hoxa3* is unaffected by mutations in *Eya1*, *Pax1*, *Pax9*, *Six1* or *Six4*, or combinations thereof, placing it at the top of this pathway. These data further suggest a role for HOXA3 in fate specification of the pharyngeal organs, particularly those derived from the third and fourth pouches, rather than in pouch formation itself.

Summary and perspectives

The role of *Hox* genes in pharyngeal organ development is a classic example of transcription factor regulation of patterning during embryogenesis. *Hoxa3* is the most well-characterized *Hox* mutant with defects in pharyngeal organogenesis, most notably in thymus and parathyroid development. The presence of *Hoxa3* early in development is required to specify the organ domains within the primordium, as well as for morphogenetic events and for organ survival. Many abnormalities are associated with defects in pharyngeal-derived structures, therefore understanding the molecular mechanisms that control the development of the pharyngeal complex is extremely important.

In summary, it is clear that the *Hox* transcription factors play a central role in the development of the pharyngeal organs, but the details of their relationship with other transcription factors and signaling molecules that are acting at the same time remain a mystery. For example, can the *Hox–Pax–Eya–Six* gene pathway be integrated into a larger regulatory network that is important for thymus-parathyroid organogenesis? What is regulating the Hox genes themselves? Are the cells and tissues of the pharyngeal region sensitive to specific levels of Hox gene expression? With the development of more and more sophisticated molecular techniques, perhaps we are finally getting closer to fully understanding how the pharyngeal region is patterned, and specifically the role that Hox genes play in this complex process.

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