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Development and Evolution of the Pharyngeal Apparatus

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Abstract

The oral or pharyngeal apparatus facilitates the dual functions of respiration and feeding. It develops during embryogenesis from transient structures called pharyngeal arches, which comprise a reiterated series of outgrowths on the lateral side of the head. The pharyngeal arches and their segmental arrangement are highly conserved throughout evolution from invertebrate chordates such as amphioxus, through to vertebrate agnathans including avians, squamates and mammals. The structural organization of the pharyngeal arches is also highly conserved and involves contributions from each of the three primary endoderm, mesoderm and ectoderm germ layers. The endoderm is particularly important for pharyngeal arch formation and segmentation and also plays a critical role in tissue specific differentiation. The ectoderm gives rise to neural crest cells (NCC) which provides an additional layer of complexity to pharyngeal arch development and differentiation in vertebrates compared to invertebrate chordates that do not possess NCC. Collectively the pharyngeal arches give rise to much of the neurovasculature and musculoskeletal systems in the head and neck. The complexity of development renders the pharyngeal apparatus prone to perturbation and subsequently the pathogenesis of birth defects. Hence it is important to understand the signals and mechanisms that govern the development and evolution of the pharyngeal complex.

Introduction

The oral or pharyngeal apparatus serves the dual functions of respiration and feeding in many species in the animal kingdom. In humans, the mouth, nose and associated sinus, together with the pharynx which is connected to the larynx and trachea, form the upper respiratory tract. The pharynx, also connects to the esophagus, caudal to the larynx as part of the upper digestive tract. Thus, the mouth and the pharynx serve the essential functions of both feeding and respiration, and therefore it is essential they form correctly during embryogenesis. One-third of congenital disorders affect the head and neck¹ and several syndromes such as DiGeorge syndrome² or Treacher Collins syndrome³ encompass malformations that affect the structure and function of the oral complex. Hence it is

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important to understand the molecular mechanisms and cellular processes that govern the establishment and development of the pharyngeal complex.

The pharyngeal complex forms during early embryonic development and commences as a reiterated series of outgrowths on the lateral side of the head called pharyngeal arches (PAs) (fig1 and 2a). Together with a medial outgrowth called the fronto-nasal prominence (FNP), these transient morphological structures collectively give rise to the face and the neck of an adult organism. Anatomically, the structure and organization of the PAs has been highly conserved throughout vertebrate evolution. Agnathan (jawless) fish such as lamprey possess PAs and, even invertebrate chordate species such as amphioxus exhibit pharyngeal segmentation of the head. These PAs give rise to the gills, the respiratory organs for aquatic species and are therefore referred to as branchial arches in aquatic species.

In this review we focus (i) on the importance of the PAs in formation of the face, especially the jaw and the neck; (ii) highlight emerging evidence of a role for the endoderm in their formation and differentiation; (iii) and discuss the mechanisms underpinning PA development and evolution.

1- PA Development

The human head and face are anatomically complex structures that form during embryogenesis, from the FNP and the PAs. The FNP gives rise to the forehead and the nose, while the paired PAs give rise to the lower face (the jaw), the neck and part of the upper thorax.

The number of PAs is species-dependent and may vary from 4 to 9. For example, in mammals there are 5 pairs of PAs, numbered 1, 2, 3, 4 and 6 (as the fifth PA disappears almost as soon as it forms), while in zebrafish there are 7 PAs⁴. However in each case they develop sequentially in a cranial to caudal manner and are separated by a cleft and pouch which appose each other. The 1st PA which is also called the mandibular arch, appears first followed by the 2nd PA or hyoid arch, then the others one by one. In fish, the 3rd to 7th PAs are called gill arches. For aquatic species, the number of PAs is usually higher since the gills are relatively inefficient filters. The basic structure of each PA is the same. Externally, the PAs are composed of ectoderm which forms the pharyngeal cleft or groove. Internally, the PA are lined with endoderm which forms the pharyngeal pouch (fig.1). At the junction that separates the different PAs, the endoderm contacts the ectoderm by an active movement called out-pocketing.

Between the endoderm and ectoderm epithelia is a mesenchymal core. In invertebrates such as amphioxus, the mesenchymal core is composed of mesoderm. In vertebrates, the mesenchymal core is composed of both mesoderm and neural crest cells (NCC). NCC are a multipotent stem cell population that delaminate from the neural plate via an epithelial to mesenchymal transformation⁵, and migrate in discrete segregated streams, colonizing each PA (fig.1 and fig.3a). Collectively, these four tissue types: endoderm, mesoderm, ectoderm and NCC interact to give rise to the skeletal, muscular, vascular and nervous system elements of the head and the neck of vertebrates.

Briefly, the **endoderm** gives rise to viscera including the thymus, thyroid and parathyroid glands which comprise part of the endocrine system. The **mesoderm** gives rise to endothelial cells and myoblasts, the progenitors of the vasculature and musculature respectively (fig.3 b and c). The pharyngeal vascular system comprises a reiterated series of pharyngeal arch arteries (PAAs) that are present within each PA (fig.1). The **ectoderm** can be subdivided into a lateral domain of surface ectoderm and a medial domain of neural ectoderm which gives rise to the skin and nervous system respectively. Furthermore, the dorsal neural ectoderm that forms a boundary with the surface ectoderm, also generates NCC that migrate into the FNP, contributing to bones and connective tissue of the face and skull⁶. The NCC that colonize the PAs give rise to the bones of the jaw, the three small bones of the middle ear (malleus, incus and stapes) as well as the cartilages of the neck. They also contribute to the formation of the teeth, as NCC give rise to the dentin secreting odontoblasts and pulp. In contrast, the enamel is produced by oral ectoderm derived ameloblasts. Similar to the teeth, the peripheral nervous system is also of dual cellular origin. NCCs generate sensory neurons and glia that integrate with surface ectoderm derived cranial sensory placode generated neurons. NCC also form mural cells, the pericytes and smooth muscle cells that surround endothelial cells within the great blood vessels of the head, and parts of the neck and upper thorax.

2- PA Differentiation

Skeletal derivatives: the role of NCC in bone formation in the head and neck

In mammalian adults, the jaw is composed of the maxilla and dentary bones, which define the upper and lower jaw respectively. These two bones articulate to facilitate mastication, respiration and vocalization. The jaw is formed during embryogenesis primarily from the 1st PA, which is composed of two paired processes known as the maxillary and mandibular prominences. The maxillary process give rise to the upper jaw and the palate, while the mandibular process give rise to the lower jaw. Fate mapping and lineage tracing in avians, fish and mice have revealed that the bones that comprise the upper and lower jaw are derived from NCC that primarily colonize the 1st PA⁷⁻¹¹ (fig.2b, 3a). However, recent lineage tracing analyses in avian species has indicated that part of the upper jaw comes from facial prominences rostral to the the 1st PA¹².

During embryogenesis, two types of ossification, intramembranous and endochondral, underpin formation of the skull and facial skeleton. In the intramembranous ossification process, NCC differentiate directly into functional osteoblasts¹³, which induce an osteoid matrix that becomes a center of ossification. The maxilla and dentary bones undergo direct ossification of NCC and are therefore classified as membrane or dermal bones.

In contrast, during endochondral ossification, NCC initially form a cartilage template, which is subsequently replaced by osteoblasts. Two such cartilages derived from the 1st PA are Meckel's cartilage, in the mandibular prominence and palatopterygoquadrate, in the maxillary prominence. The malleus which is derived from Meckel's cartilage, together with the anterior ligament of the malleus and the sphenomandibular ligament, collectively contribute to the temporomandibular joint. The palatopterygoquadrate gives rise to the alisphenoid, a bone that is part of the orbital wall, and it also gives rise to the incus.

Reichert's cartilage of the 2nd PA forms the third middle ear bone known as the stapes (fig. 2b).

In the neck, the larynx and pharynx are composed of skeletal elements that are, in anterior to posterior order, the hyoid bone, the thyroid cartilage and the cricoid cartilage. The hyoid bone facilitates tongue movement and swallowing, and the body of the hyoid bone is derived from NCC of the 3rd PA. It is attached to the styloid process of the temporal bone by the stylohyoid ligament, and is derived from Reichert's cartilage (2nd PA). The thyroid cartilage protects the vocal cords and serves as an attachment for the laryngeal muscles. It derives from NCC of the 4th PA. Finally, the cricoid is a ring of cartilage surrounding the trachea, which supports the ligament and muscles involved in opening and closing the airway, as well as producing sound. The cricoid comes from NCC of the 6th PA. (fig.2b and table 1).

Vasculature: the role of mesoderm and NCC in formation of blood vessels

The vasculature comprises a network of vessels that facilitates blood circulation throughout the entire organism. Blood circulation serves several roles, it brings oxygen and nutrients to organs through the arterial system, and it removes waste products through the venal system. The vasculature regulates body temperature and also plays a role in immune defense by transporting immune cells (such as leukocytes or granulocytes). The vasculature is the first organ system to develop during embryogenesis. It is essential for normal development and undergoes considerable remodeling in association with growth and maturation of the organism.

The left and right dorsal aortae are the first major blood vessels to form in the embryo. They lie along the anterior-posterior axis of the embryo and connect to the heart and umbilical arteries. The PAAs form in concert with the PAs in a cranio-caudal gradient¹⁴ and connect the heart to the existing dorsal aorta. The PAAs comprise tubes of endothelial cells that are derived from mesoderm. They form through the process of vasculogenesis, which is defined as *de novo* formation of blood vessel from endothelial cell precursors known as angioblasts. To maintain vessel stability and quiescence, mural cells are recruited along the endothelial tube. These cells are pericytes and smooth muscle cells and are derived from NCC¹⁵. Although most of the smooth muscle cells in the heart field are derived from NCC, a small population is derived from mesoderm. Furthermore, the smooth muscle cells of the coronary arteries are thought to have a pro-epicardial origin which is derived from lateral plate mesoderm^{16, 17}.

The primitive vasculature is initially symmetrical but subsequently undergoes asymmetric remodeling to form the mature, definitive and functional vascular system seen in the adult¹⁸. The remodeling involves apoptosis mediated regression of the 1st and 2nd PAAs. The 1st PAA however, still contributes to a small portion of the **maxillary** artery that supplies the deep tissues of the face. The 2nd PAA gives rise to the **stapedial** artery, which irrigates the stapedial muscle of the inner ear. The 3rd PAA is devoted to formation of the **carotid system**. It gives rise to the left and right common carotid arteries that irrigate the head and neck. The **common carotid artery** bifurcates into the internal and external carotids. The proximal portion of the **internal carotid** is an extension of the common carotid arteries while the distal portion is derived from the dorsal aorta. The **external**

carotid arteries sprout *de novo* from the common carotid arteries by angiogenesis. The 4th PAA gives rise to the **pulmonary artery** and the definitive **aortic arch**. The 6th PAA first give rises to the pulmonary trunk, a blood vessel that emerges from the right ventricles, but which is remodeled asymmetrically. While the right 6th artery disappears, the distal portion of left 6th PA artery forms the ductus arteriosus (fig.4 and table 2). This artery is essential for fetal blood circulation as it shunts blood from the pulmonary trunk to the descending aorta. At birth, as the lungs start their function, the ductus arteriosus closes allowing circulation through to the lungs to oxygenate the blood that subsequently reaches the systemic circulation of the new born.

The peripheral nervous system (PNS) is derived from neural ectoderm, non neural ectoderm, and NCC

The PNS is an essential system that carries information between the body and the central nervous system through a complex network of nerves. For example, when pain is sensed, this information is relayed to the brain through sensory nerves and returns through the motor system to control movement of the body and protect the organism. The PNS is formed from three primary tissues: neural ectoderm, NCC and ectodermal placodes (fig.3d and e). Placodes are thickened regions of epithelial cells within the surface ectoderm of the embryo. In total there are 7 cranial placodes named adenohipophyseal, lens, olfactory, trigeminal otic, geniculate, petrosal and nodose.

The PNS comprises multiple elements including: (i) cranial nerves (neurons, axons and myelin) and ganglia (neuronal cell bodies and their nursing cells or Schwann cells), (ii) spinal nerves and ganglia, (iii) autonomic nerves and ganglia and (iv) enteric nerves and ganglia. The latter three systems constitute the trunk ganglia. NCC give rise to the all peripheral glia (Schwann cells) and neurons (autonomic neurons and sensory neurons) in the trunk.

In humans, there are 12 pairs of cranial nerves. They are: the Olfactory n. (I), Optic n. (II), Oculomotor n. (III), Trochlear n. (IV), Trigeminal n. (V), Abducens n. (VI), Facial n. (VII), Vestibulocochlear n. (VIII), Glossopharyngeal n. (IX), Vagus n. (X), Accessory n. (XI) and Hypoglossal n. (XII). These cranial nerves are derived from both NCC and ectodermal neurogenic placodes, each of which provides neurons to the cranial nerves. The supporting cells (Schwann cells) of all cranial nerves are however all derived from NCC.

Among the 12 pairs of cranial nerves, four invade the PA to innervate muscles derived from the mesoderm core of the corresponding PA. The **trigeminal** (V), **facial** (VII), **glossopharyngeal** (IX) and **Vagus** (X) invade the 1st, 2nd, 3rd and 4th PA respectively (fig. 2c). The trigeminal comprises three branches, the ophthalmic nerve (V1), which provides sensory input to the eye muscles, and the maxillary (V2) and the mandibular nerves (V3) that innervate the maxillary and mandibular prominences respectively. The trigeminal nerve is formed from anterior hindbrain derived NCC that integrate with the trigeminal placode. The facial nerve is formed from mid hindbrain derived NCC together with the 1st epipharyngeal placode (geniculate). The glossopharyngeal nerve is derived from caudal hindbrain derived NCC and the 2nd epipharyngeal placode (petrosal). Finally, the vagus nerve is derived from trunk NCC and the 3rd epipharyngeal placode (nodose).

Muscle derivatives and their innervation

The muscles of the face control facial expression, mastication, and movement of the eyes and lips. They are derived primarily from the mesodermal cores of the corresponding PA. In adults, the muscles of the face can be categorized by their function. For example, muscles that have a role in mastication are derived from the 1st PA mesoderm, while the muscles that govern facial expression are derived from 2nd PA mesoderm. The pharynx is surrounded by 2 layers of muscle, an external layer that is derived from 4th PA mesoderm and an internal layer that is derived from 6th PA mesoderm (fig.2d).

The muscles of mastication are innervated by the maxillary branch of the trigeminal nerve (V2) and include the **Masseter** that is attached to the zygomatic process and to the zygomatic arch of the maxilla and the lateral surface of mandible. The **temporalis** is a large muscle that attaches the temporal bone and the coronoid process of mandible. Together these two muscles allow elevation of the mandible and stabilize it during mastication. The **lateral and medial pterygoid** muscles act synergistically with the masseter and facilitate depression of the mandible (or the chin). They also allow for lateral movement during chewing. Finally, the anterior belly of the **digastric muscle** acts to open the jaw (while the temporalis and masseter are relaxed) and helps during swallowing via insertion into the mandible and hyoid. The posterior belly of the digastric is derived from 2nd PA mesoderm, and inserts into the temporal bone and mastoid process. Finally, the **mylohyoid** muscle forms the floor of the oral cavity and it elevates the hyoid and tongue during swallowing. This cluster of muscles is innervated by the maxillary branch of the trigeminal nerve (V2) (table 3).

The muscles of facial expression are innervated by the facial nerve (VII). The **risorius** and **zygomaticus** (minor and major) muscles are responsible for smiling. The **orbicularis oris muscle** encircles the mouth and helps with lip movements. The **orbicularis oculi** encircles the eyes and facilitates winking. Despite its role in mastication and sucking, the **buccinator**, located in the cheek, is derived from mesoderm of the 2nd PA as well.

In the neck, the **stylopharyngeus** is a small muscle that forms the internal layer of the pharynx and helps to elevate the pharynx and the larynx during swallowing and speaking. This muscle is the only one that is derived from the 3rd PA mesoderm and it is innervated by the glossopharyngeal nerve (IX).

The **superior, middle and inferior constrictors** form the external muscular layer of the pharynx and are derived from the 4th PA, as well as the **cricoid** and the **levator veli palatine**. They act to constrict the walls of the pharynx during swallowing. Derived from the 6th PA mesoderm, the **intrinsic muscles of the larynx** help with respiration and phonation (the movement of vocal cords to produce sound). They are each innervated by the vagus nerve (X) (table 3).

Sensory organs and their innervation

The tongue is a particular muscle that manipulates food during mastication, perceives taste, and facilitates phonetic articulation. Its musculature derives from the mesoderm of 3

different PAs: 1st PA mesoderm forms the body of the tongue, 2nd PA mesoderm is responsible for the formation of the midtongue and 3rd PA mesoderm forms the root. The neuronal component of the tongue derives from NCC. Innervation of the anterior 2/3 of the tongue comes from the lingual (mandibular division of the trigeminal nerve V3) and chorda tympani (branch of the facial VII) nerves. The posterior 1/3 of the tongue is innervated by the glossopharyngeal nerve (IX).

The ear is important for balance and hearing and it is derived in part from the otic vesicle which forms dorsal to the 2nd and 3rd PAs. The pharyngeal pouch and cleft that separates the 2nd and 3rd PAs are crucial for formation of the external and middle ear. The pouch (endoderm) gives rise to the tubotympanic recess, the epithelium of the tympanic cavity, and the Eustachian tube that links the nasopharynx to the middle ear. In addition, the first pharyngeal pouch also gives rise to the tympanus, which defines the boundary of the middle ear. In contrast, the pharyngeal cleft (ectoderm) gives rise to the external auditory canal (table 4). The vestibulocochlear nerve (VIII) is derived from the otic placode and innervates the developing inner ear.

3- Formation and Evolution of the Pharyngeal Apparatus

The origin of the pharyngeal apparatus can be traced during evolution as far back as the cephalochordates (fig.5). For example, in amphioxus, an invertebrate aquatic species (and thus not possessing NCC), segmentation can be observed in the embryonic head, which is analogous to the PAs of vertebrates¹⁹. The amphioxus respiratory system consists of perforations at the contact points between the endoderm and ectoderm that facilitate filtration of water for feeding and respiration. These contact points are formed by out-pocketing of the endoderm that evaginates to reach the ectoderm. This active movement is responsible for delimiting each segment²⁰. Thus out-pocketing of the endoderm is an important conserved event that initiates the segmental patterning of the PAs in invertebrates and in vertebrates.

Evolutionarily, NCC are thought to be a cell type characteristic of vertebrates. Within this group, some species such as lamprey are characterized by the absence of a jaw which makes them an interesting model with which to study the pharyngeal region. Lampreys possess NCC and PAs but are agnathans (jawless vertebrate) that belong to the cyclostomes, which are characterized by a round mouth. Lampreys have a toothed and tunnel-like mouth that facilitates grabbing prey and sucking their blood which is filtered through the gills. In lamprey, the 1st PAs gives rise to a rod of cartilage that fuses to form an unjointed branchial basket²¹. In gnathostomes, (jawed vertebrates), 1st PA differentiation has undergone considerable modification to form a jaw, with dorsoventral regionalization and articulation by a joint.

A “hinge and cap” model has been proposed to mechanistically describe development of the variety of jaws observed in animal kingdom. According to this model, the upper and lower jaws can be viewed as two polarized units that articulate²² and furthermore they can be classified in two distinct groups: (i) the non-mammalian jaw, where articular and quadrate elements (homologs of the incus and malleus) form the articulation; and (ii) the mammalian

jaw, where the incus and malleus make an articulation but the force bearing articulation is conveyed by the squamosal and dentary elements. The increased musculoskeletal complexity of the mammalian jaw reflects the transition from an aquatic filtering lifestyle to a more land dwelling predatory lifestyle. Furthermore, this lifestyle transition was also associated with the transformation of gills to lungs and was accompanied by a reduction in the number of caudal pharyngeal or branchial segments in avians, reptiles and mammals, compared to fish. In addition, this transition was also accompanied by creation of an air chamber in the middle ear to conduct sound. The three ossicles within the middle ear have undergone considerable transformation. In fish, Meckel's cartilage forms part of the lower jaw, while in mammals it forms the malleus. The hyomandibular bone present in fish has been transformed from a suspensory element, into a supporting element of the skull, the stapes²³. However, studies in cartilaginous fish and bony fish have illustrated the homology of the dorsal and ventral skeletal elements of the mandibular, hyoid and gill arches to the skeletal elements of the mammalian upper and lower jaw. This homology has been confirmed through conserved expression of *Dlx* genes, which play a predominant role in establishing the polarity and identity of the PAs²⁴.

The endoderm as an organizer of the pharyngeal apparatus

It was once prominently thought that NCC were essential for PA formation and patterning. However, NCC ablation experiments performed in avian embryos have demonstrated that PAs form even in absence of NCC²⁵. Furthermore, mouse genetic mutants in which specific streams of NCC are absent, also exhibit normal PA formation and basic patterning²⁶. Collectively, this challenged the notion that NCC were required for the initial formation and patterning of the PAs. Consistent with this idea, amphioxus exhibit branchial segmentation indicating that pharyngeal segmentation preceded the appearance of NCC. Thus NCC are not necessary for PA formation.

Filtration in amphioxus is facilitated by perforation at contact points between the endoderm and ectoderm. Therefore it is the out-pocketing of endoderm that is critical for respiratory and feeding functions, and this has remained a conserved integral component of PA development in both invertebrates and vertebrates. In support of this idea, the zebrafish mutant *vgo*, which carries a mutation in *tbx1*, a transcription factor that is expressed in endoderm, does not form proper endodermal pouches and subsequently exhibits caudal pharyngeal arch agenesis and defects in cartilage formation²⁷. The same is also true in *Tbx1* mutant mice²⁸. This suggests the endoderm is not only required for pharyngeal arch formation but also for pharyngeal arch differentiation. Consistent with this idea, transplantation of wild-type endodermal cells into the PAs of *vgo/tbx1* mutant zebrafish can rescue cartilage formation. Similarly, endoderm ablation experiments in avian embryos also result in agenesis of cartilages of the head, including Meckel's, quadrate and articular cartilages, and can be restored through endoderm xenografts in quail/chicken chimeras²⁹. These experiments demonstrate the importance of the endoderm in PA formation, and differentiation of the NCC-derivative skeleton²⁷.

Retinoic acid (RA), the active form of vitamin A, is a diffusible morphogen that is important for patterning many tissues along the antero-posterior axis of embryos including the

pharyngeal arches. Mice mutant for *Rdh10* or *Aldh1a2*, the two key enzymes responsible for the oxidation of vitamin A (retinol) to retinal and then to RA respectively, both exhibit defects in 3rd to 6th PA formation^{30 31}. Interestingly, in avian models of vitamin A deficiency (and thus deficiency for endogenous RA), *Tbx1* expression in the ectoderm was decreased demonstrating that RA regulates the expression of *tbx1*³². Therefore, RA signaling is required for caudal endodermal pouch segmentation, and furthermore the pharyngeal pouch endoderm is very sensitive to the levels of RA³³. However, while RA is required for endodermal pouch formation and thus pharyngeal segmentation, retinoid signaling is not required for specification of the pharyngeal pouch endoderm along the anterior-posterior axis³³.

Fibroblast Growth factor (Fgf) signaling is also known to play a key role in endoderm pouch formation and differentiation. In wild-type zebrafish, clusters of endodermal cells migrate laterally to form the pharyngeal pouches. However, in zebrafish mutant for both *fgf3*^{-/-} and *fgf8*^{-/-}, endodermal cells are present but their migration is disorganized resulting in a failure of the pharyngeal pouches to form which manifests as a fusion of the PAs³⁴. These alterations in pouch development correlate with severe cartilage formation defects³⁴. Although the cartilages are derived from NCC, signaling from the endoderm is needed to induce NCC to form cartilage. This is mediated at least in part via fibroblast growth factors such as *fgf3* and *fgf8*, which are expressed in the endoderm, together with fgf receptors such as *fgfr1* which is expressed in NCC. Consistent with data in zebrafish, a hypomorphic *Fgfr1* mutant mouse also exhibits impaired PA formation and development³⁵.

A recent study in lamprey revealed evolutionary conserved roles for Fgf signaling in pharyngeal segmentation. In lamprey, *fgfs* are expressed in the endoderm while the *fgfr* is expressed in NCC. Incubation of lamprey embryos in an inhibitor of *fgfr*, resulted in loss of pharyngeal pouch formation, and agenesis of pharyngeal derived cartilages. Therefore, the results obtained in lamprey are consistent with results obtained in others species, indicating that the role of Fgf signaling in endodermal pouch formation and skeletogenesis is highly conserved throughout vertebrate evolution³⁶.

Thus the endoderm is an important organizer of the pharyngeal region. It initiates segmentation in the head region by undergoing evagination to reach the ectoderm in a process termed outpocketing. This outpocketing of endoderm is central to PA formation and is regulated in part through *Tbx1* and RA signaling. Furthermore, pharyngeal endoderm is a source of Fgf signaling and interactions between endoderm and NCC facilitate formation and development of NCC-derived cartilages.

Wnt signaling may also be a critical driver of endodermal pouch formation and pharyngeal segmentation. Recently, *wnt11r* and *wnt4a*, which are respectively expressed in the head mesoderm and ectoderm of zebrafish embryos, were shown to play distinct roles in the reiterated formation of pharyngeal pouches³⁷. Time lapse imaging in combination with Wnt signaling mutants suggest that Wnt signaling governs two separate events. Initially, *wnt11r* and *rac1* destabilize the endodermal epithelium to promote the lateral movement of pouch-forming cells. Subsequently, *wnt4a* and *cdc42* signaling induces the rearrangement of pouch cells into bilayers through restabilizing adherens junctions³⁷. How this newly identified,

Wnt mediated, dynamic control of epithelial morphology intersects with Fgf or RA signaling remains to be examined. However, in the future it will be important to explore these interactions and also to determine whether Wnt mediated regulation of the pharyngeal endoderm represents an evolutionary conserved mechanism underpinning pharyngeal formation and segmentation.

4- Regulation of PA Identity and Development

Many different gene families play important roles in PA patterning. Here we focus on the *Dlx* and *Hox* genes, which are the primary regulators of anterior-posterior and dorso-ventral patterning of the PAs.

A *Dlx* code for PA Patterning

Dlx genes are homeodomain transcription factors that establish pattern and polarity of the 1st PA, and they are also important for regulating the identity of NCC-derived bones of the face. In mammals, there are 6 *Dlx* genes, and they exhibit nested domains of expression in the PAs which is critical for specifying the identity of the upper and lower jaw. The six *Dlx* genes are conserved and arose as a result of cis duplication (first degree paralogues *Dlx1* and *Dlx2*) and trans duplication (second degree paralogues *Dlx1*, *Dlx6* and *Dlx4* (and *Dlx 2*, 5, 3)) during evolution³⁸. In mouse the six *Dlx* genes are expressed in the ectomesenchyme of the pharyngeal arches. Cartilaginous fish like skate and shark, and bony fish like paddlefish also possess 6 *dlx* genes similar to mammals, however, only one *dlx* gene has been found in amphioxus²⁴.

Targeted single and compound deletions of *Dlx1* and *Dlx2* revealed that these two genes are specifically required for proximal-distal identity of the 1st PA. Mouse *Dlx1* and *Dlx2* are broadly expressed in the mandible and maxillary prominences of the 1st PA, as well as in the 2nd PA (fig.6). *Dlx1*^{-/-} mutant mice exhibit a mild phenotype with a small stapes and alisphenoid bones. In contrast, *Dlx2*^{-/-} mutants display more extensive defects in proximal skeletal elements derived from the 1st and 2nd PAs such as the alisphenoid, incus and stapes. More distal elements such as the malleus, mandible and the Meckels' cartilage were apparently unaffected. *Dlx1/2*^{-/-} double mutant mice were phenotypically similar to the *Dlx2*^{-/-} mutant^{39 40} (table 5). Taken together these results demonstrate that *Dlx2* primarily, but also *Dlx1*, are required for proper differentiation of the proximal structures that are derived from the 1st PA.

Dlx5 and *Dlx6* are expressed in nested domains within the *Dlx1* and *Dlx2* expression territories (fig.6). Targeted deletion of *Dlx5* in mice leads to defects of the lower jaw. Meckel's cartilage is shortened and dysmorphic in particular⁴¹. Interestingly, *Dlx5/6*^{-/-} double mutant mice exhibit an absence of Meckel's cartilage, and interestingly, vibrissae (mouse whiskers) are distributed symmetrically on the upper and lower jaw. These phenotypes reflect a transformation of the lower jaw to upper jaw^{42, 43} (table 5). Consistent with this idea, the shape of the maxillary and mandibular prominences are identical during embryogenesis. This suggests that upper jaw fate may represent a default state and that lower jaw fate is controlled by *Dlx5/6* activity.

Little is known concerning *Dlx3/4* genes and their involvement in PA patterning. *Dlx3*^{-/-} mutants die at E10.5 due to placental failure, however no PA phenotype has been described⁴⁴. A *Dlx4* knock out has not yet been reported.

Interestingly, endothelin (*Edn*) signaling also regulates PA patterning and is an upstream regulator of *Dlx* gene activity. In mouse, chick and zebrafish, *Edn1* is expressed in the ectoderm, core paraxial mesoderm and endodermal pouches, whereas its receptor *Ednra* is expressed in all NCC^{45, 46}. Both *Edn1* and *Ednra* mutant mice each exhibit a phenotype that is similar to *Dlx5/6*^{-/-} mutants, in the form of a small mandible, and agenesis of Meckel's cartilage and the hyoid bone, reflecting a homeotic transformation of the lower jaw to an upper jaw identity^{47, 48}. Inactivation of endothelin signaling in avian embryos also elicits a similar phenotype⁴⁹. Conversely, ectopically overexpressing *Edn1* in the maxillary prominence induces an ectopic dentary bone (mandible) in the maxillary region, demonstrating that the upper jaw adopted a lower jaw identity⁵⁰ (table 5). *Edn1* and its receptor *Ednra* regulate *Dlx5/6*, which in turn induces downstream targets specific to distinct proximodistal regions with the 1st PA. *Hand2* for example, is expressed in the mediodistal region of the mandibular prominence⁵¹ and targeted deletion of a specific enhancer of *Hand2* in NCC of the 1st and 2nd PA leads to mandibular hypoplasia,⁵² mimicking the *Dlx5/6* mutant homeotic transformation phenotype. Interestingly, *Hand2* also acts in a feedback loop that downregulates *Dlx5/6* in the most distal part of the mandibular region⁵³. Therefore, 1st PA patterning and consequently, upper and lower jaw formation, are regulated primarily via an *Edn/Dlx/Hand* signaling axis. This signaling mechanism has been validated in mouse, avian and aquatic models as well as in humans with auriculocondylar syndrome⁵⁴.

A *Hox* code for Anterior-Posterior Patterning

In insects and vertebrates, *Hox* genes play a key role in body plan organization along the anterior-posterior axis⁵⁵. The *Hox* genes are organized in clusters, and through a process known as co-linearity, the spatio-temporal expression of these genes controls segmentation and PA identity in the head. *Hox* genes are not expressed in the 1st PA, however, they are expressed in the NCC of 2nd to 6th PAs. In general, each PA is under the control of one *Hox* group, such that the *Hox2* group governs 2nd PA development, the *Hox3* group regulates 3rd PA patterning, and the *Hox4* group patterns the 4th PA^{56, 57} (fig.6).

Mice mutant for *Hoxa2* die perinatally and exhibit duplications of 1st PA derived elements in the place of 2nd PA derived structures^{58, 59}. Conversely, overexpression of *Hoxa2* in the 1st PA of avian and frog embryos leads to transformation of first arch skeletal elements such as Meckel's cartilage into second arch structures^{60 61}. Collectively, this suggests that *Hoxa2* is the primary determinant of 2nd PA identity. Interestingly, simultaneous inactivation of the entire *Hoxa* cluster in all pharyngeal arch NCC leads to the presence of supernumerary derivatives of the 1st PA, such as incus, malleus and Meckel's cartilage throughout the entire pharyngeal region⁶². Thus all of the PAs may have the potency to form 1st PA derivatives, however, *Hox* genes are incompatible with jaw formation as they inhibit the development of 1st PA characteristics. This implies that the identity of the 1st PA represents a *Dlx* dependent/ *Hox* independent default state. Furthermore, perhaps evolution the pharyngeal

arches commenced with a uniform default 1st PA identity, with different groups of *Hox* genes subsequently providing identity to each of the more posterior regions of the head and neck.

Consistent with this idea, *Hoxa3* appears to be important for 3rd PA identity⁶³. *Hoxa3* null mutant mice fail to form the buds of thymus and parathyroid and develop an impaired carotid artery system⁶⁴. The thyroid defects seen in *Hoxa3* single mutants are exacerbated in double mutants with either *Hoxb3* or *Hoxd3* paralogs. The removal of one functional copy of *Hoxa3* from the *Hoxb3*, *Hoxd3* double mutants (*Hoxa3* +/-, *Hoxb3* -/-, *Hoxd3* -/-) results in the failure of the thymus and parathyroid glands to migrate to their normal positions in the throat. Thus the *Hox* group 3 genes have overlapping functions in development of the pharyngeal organ primordia, structures that are derived from the 3rd and 4th PA pouches⁶⁵.

RA is a well-known regulator of *Hox* gene activity and mechanistically this is achieved through the presence of retinoic acid response elements (RARE) in the promoter regions of many *Hox* genes⁶⁶. In the presence of RA, the retinoid receptors, RARs and RXRs form heterodimers that bind RAREs to regulate *Hox* gene activity⁶⁷. Interestingly, RA also influences *Dlx* gene activity as well. E8.5 mouse embryos treated transiently with exogenous RA, phenocopy the lower jaw to upper jaw transformations observed in *Edn1* -/- and *Dlx5/6* -/- mutant mice. RA does not directly affect the migration or survival of NCC⁶⁸, rather exogenous RA represses Endothelin signaling in the 1st PA, which as described above is a direct regulator of *Dlx* gene activity⁶⁹. Thus RA signaling is a critical mediator of pharyngeal segmentation and patterning through its roles in endoderm development and in the regulation of *Dlx/Hox* gene activity.

Conclusion

The pharyngeal apparatus is essential for the dual functions of respiration and feeding. It is formed from a reiterated series of outgrowths on the side of the embryo termed pharyngeal arches (PAs), whose anatomical composition is highly conserved throughout evolution. Each PA is composed of two epithelia, the endoderm and ectoderm that together line a mesenchymal core composed solely of mesoderm in invertebrate embryos, or mesoderm and NCC in vertebrate embryos. These three germ layers, endoderm, mesoderm and ectoderm, as well as the NCC, sometimes called the 4th germ layer, are responsible for establishment of the skeleton, musculature nervous system and vasculature of the vertebrate face, neck and upper thorax.

The endoderm plays a critical role in PA segmentation and patterning. Through migration and a process call out-pocketing, the endoderm evaginates to contact the ectoderm and in doing so forms the pharyngeal pouches which separate the individual PAs. Thus the pharyngeal endoderm is essential for pharyngeal segmentation and this process is regulated primarily by Fgf signaling. Furthermore, the endoderm is a source of signals that direct the differentiation of PA neural crest cells into cartilage and this includes Fgf, RA and Tbx1 signaling.

The number of PAs varies between species and the aquatic to land dwelling lifestyle changes that were accompanied by gill to lung transitions may be associated with the general decrease in PA number in reptiles and mammals compared to fish. However, the molecular regulation of anterioposterior and dorsoventral PA identity and polarity has been largely conserved. The 1st PA, is regulated by *Dlx* gene activity, with *Dlx5/6* playing a primary role in distinguishing mandibular identity (lower jaw) from a potentially default maxillary identity (upper jaw). Interestingly, *Hox* genes which are not expressed in the 1st PA, are actually incompatible with jaw formation. Instead distinct groups of *Hox* genes govern the identity and patterning of the more caudal 2nd to 6th PAs, with *Hoxa2* being the primary determinant of 2nd PA. Therefore a combinatorial code of *Dlx* and *Hox* genes is critical for establishing the identity and subsequent development of individual PAs during formation of the pharyngeal apparatus.

The pathways described in this review are conserved from mouse to jawless species such as lampreys. But the molecular mechanisms underlying the species-specific shape of bones of the face and neck are poorly understood. The signals emanating from the endoderm, ectoderm or mesoderm that control proliferation, differentiation, migration, morphology or survival of NCC remain to be fully elucidated. Approximately, one-third of congenital malformations affect the head region, and the identification of novel genes that intrinsically or extrinsically regulate NCC development, could further the development of therapeutic avenues for treating syndromes affecting the pharyngeal apparatus.

In summary, the development of the pharyngeal apparatus is a complex process that relies on precise tissue interactions and numerous distinct signaling pathways for its normal development. This complexity has facilitated vertebrate evolution, but is also prone to perturbation and the pathogenesis of congenital anomalies of the head and neck. Therefore it is important to continue to strengthen our understanding of the molecular mechanism and cellular processes that govern the establishment, segmentation and differentiation of the pharyngeal arches during formation of the pharyngeal apparatus.

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Figure 1. Structural Organization of the Pharyngeal Arches in a Human Embryo

Schematic illustration of the internal organization of the developing PAs. Each PA is numbered from 1 to 6. PAs are lined externally by ectoderm in pink and internally by endoderm in purple. Inside, each PA is a mesenchymal core (blue) composed of mesoderm cells surrounded by NCC cells alongside the nerve (yellow) and blood vessel (red) components.

Figure 2. Pharyngeal Arch Differentiation

Figure 2a: Schematic illustration of the pharyngeal region of a human embryo. Figure 2b: Skeletal derivatives of the NCC. Bones of the face are formed by two ossification processes. Endochondral bones are formed via a cartilage template and include the malleus (from Meckel's cartilage), and the incus (from maxillary cartilage) from the 1st PA (green). The stapes, the styloid process and lesser horn of hyoid (from Reichert's cartilage) come from the 2nd PA (red). Dermal bone formation consists of direct ossification of the NCC without a cartilage intermediate. Dermal elements include the squamous part of the temporal bone, the zygomatic, the maxillary bone and the mandible, which are derived from 1st PA (green). Figure 2c: Neural derivatives of the NCC. The trigeminal nerve (V) comprises 3 subdivisions: the ophthalmic nerve, the maxillary nerve and the mandibular nerve and develops in association with the 1st PA, (green). The facial nerve invades the 2nd PA, (red). The glossopharyngeal invades the 3rd PA, (purple) and the vagus nerve the 4th PA, (dark blue). Figure 2d: Muscular derivatives of the mesoderm. Muscles (green) of mastication are derived from mesoderm of the 1st PA. Muscles of facial expression (red) are derived from 2nd PA mesoderm. The stylopharyngeus (purple) is the only muscle that is derived from the 3rd PA mesoderm. The external muscles of the pharynx (blue) are derived from 4th PA mesoderm.

Figure 3. Establishment of the musculature, vasculature, and PNS of the face in mouse embryo
 a. LacZ staining marking NCC. b. *tbx1* *in situ* hybridization showing the myogenic core of each PA. c. Pecam immunostaining marking endothelial cells of the developing vasculature. d. Tuj1 immunostaining marking NCC and placode-derived neurons, e. *Eya2* *in situ* hybridization, delimiting the cranial placodes.

Figure 4. Pharyngeal Arch Vascular Patterning

The maxillary artery (green) is derived from the 1st PAA. The stapedial (red) is derived from the 2nd PAA. The carotid system (purple) derives from the 3rd PAA. The left 4th PAA gives rise to the aortic arch while the right PAA gives rise to the subclavian artery and contributes to the pulmonary arteries (light blue). Finally, the 6th PAA give rise to the ductus arteriosus (dark blue) and the pulmonary arteries (light blue).

Figure 5. Evolutionary Cladogram

Evolutionary tree showing the classification of species from deuterostomes to mammals. NCCs appear during the transition to vertebrates.

Figure 6. *Dlx* and *Hox* gene control of pharyngeal patterning

Dapi staining of E10.5 mouse embryo. Expression domain of *Dlx1* and *Dlx2* (yellow) in the 1st and 2nd PA. Nested expression of *Dlx5* and *Dlx6* (orange) within the *Dlx1* and *Dlx2* domains of expression. *Hox2* group genes are expressed in the 2nd PA (pink) and more posterior Pas, while *Hox* group 3 (blue) and *Hox* group 4 (green) genes are expressed in the 3rd and 4th PAs respectively.

Table 1
Skeletal derivatives of the PAs

PA	Endochondral bone (ossification of NCC-derived cartilaginous intermediate)	Dermal bones (direct ossification of NCC)
1	<p><u>From Meckel's cartilage:</u></p> <ul style="list-style-type: none"> • malleus <p><u>From maxillary cartilage:</u></p> <ul style="list-style-type: none"> • Incus • Alisphenoid 	<ul style="list-style-type: none"> • Maxilla • Zygomatic • Squamous portion of temporal bone • Mandible
2	<p><u>From Reichert's cartilage:</u></p> <ul style="list-style-type: none"> • Stapes • Styloid process • Upper part of the hyoid bone 	
3	-lower part of hyoid bone	
4	-Laryngeal cartilage	
6	-Laryngeal cartilage	

Table 2
Vascular derivatives of the PAs

Pharyngeal arch Arteries	Arteries in adult
1 st PA	Maxillary artery (small portion)
2 nd PA	Stapedial artery
3 rd PA	Common carotid artery First part of the internal carotid artery
4 th PA	<u>Left side</u> : segment of the aortic arch artery between the common carotid and the left subclavian artery <u>Right side</u> : proximal part of the right subclavian artery and the pulmonary arteries
6 th PA	<u>Right side</u> : disappears <u>Left side</u> : Ductus arteriosus

Table 3
Muscle derivatives of the PAs and their innervation

Pharyngeal arch	Muscle	Nerve
1 st PA	Mastication: <ul style="list-style-type: none"> • masseter • temporalis • medial and lateral pterygoid • anterior belly of digastric muscle 	Mandibular part of trigeminal (nerve V)
2 nd PA	<ul style="list-style-type: none"> • Buccinator • Expression muscles 	Facial (nerve VII)
3 rd PA	Stylopharyngeus	Glossopharyngeal (nerve IX)
4 th PA	Constrictors of pharynx, cricothyroid levator veli palatine	Vagus (nerve X)
6 th PA	Intrinsic muscle of larynx	Vagus (nerve X)

Table 4
Derivatives of pouches and clefts

Endodermal pouch	Anatomical adult structure derivative	Ectodermal cleft	Anatomical adult structure derivative
1 st pouch	Tubotympanic recess that leads to: <ul style="list-style-type: none"> • epithelium of tympanic cavity • eustachian tube 	1 st cleft	External auditory canal
2 nd pouch	Palatine tonsil epithelium	2 nd cleft	
3 rd pouch		3 rd cleft	
4 th pouch	Thyroid and thymus Ultimobranchial body	4 th cleft	
6 th pouch	Ultimobranchial body	6 th cleft	

Table 5
Mouse mutants and their phenotypes

genes	phenotype	references
<i>Dlx1</i> ^{-/-}	Defects of stapes and alisphenoid bones	Qiu <i>et al.</i> , 1997
<i>Dlx2</i> ^{-/-}	Defects in proximal elements of 1 st and 2 nd PAs	Qiu <i>et al.</i> , 1995
<i>Dlx1/2</i> ^{-/-}	Defects of proximal elements of 1 st and 2 nd PAs	Qiu <i>et al.</i> , 1997
<i>Dlx5</i> ^{-/-}	Lower jaw shortened	Depew <i>et al.</i> , 1999
<i>Dlx5/6</i> ^{-/-}	Lower jaw transformed into upper jaw	Beverdam <i>et al.</i> , 2002
<i>Edn1</i> ^{-/-}	Small lower jaw (absence of Meckel's cartilage)	Kurihara <i>et al.</i> , 1994
<i>Edn1</i> overexpression	Upper jaw transformed into lower jaw	Sato <i>et al.</i> , 2008
<i>Hand2</i> enhancer ^{-/-}	Mandibular hypoplasia	Yanagisawa <i>et al.</i> , 2003
<i>Hoxa2</i> ^{-/-}	2 nd PA elements transformed into 1 st PA elements	Rijli <i>et al.</i> , 1994
<i>Hoxa2</i> overexpression	1 st PA elements transformed into 2 nd PA elements	Grammatopoulos <i>et al.</i> , 2000
<i>Hoxa3</i> ^{-/-}	Absence of thymus and parathyroid and impaired carotid artery system	Manley <i>et al.</i> , 1998; Kameda <i>et al.</i> , 2009