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Mechanisms of Larynx and Vocal Fold Development and Pathogenesis

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Abstract

The larynx and vocal folds sit at the crossroad between digestive and respiratory tracts and fulfill multiple functions related to breathing, protection and phonation. They develop at the head and trunk interface through a sequence of morphogenetic events that require precise temporo-spatial coordination. We are beginning to understand some of the molecular and cellular mechanisms that underlie critical processes such as specification of the laryngeal field, epithelial lamina formation and recanalization as well as the development and differentiation of mesenchymal cell populations. Nevertheless, many gaps remain in our knowledge, the filling of which is essential for understanding congenital laryngeal disorders and the evaluation and treatment approaches in human patients. This review highlights recent advances in our understanding of the laryngeal embryogenesis. Proposed genes and signaling pathways that are critical for the laryngeal development have a potential to be harnessed in the field of regenerative medicine.

Keywords

laryngeal; vocal cords; congenital; embryology

1. INTRODUCTION

Voice problems occur in between 3% and 9% of the population in the United States (Roy *et al.*, 2005). Although a majority of these problems arise due to vocal fold (VF) structural and neurologic abnormalities, phonotrauma and/ or laryngeal inflammation (Van Houtte *et al.*, 2010), there is a clear segment of the population in which voice disorders develop secondary to defects that occur during intrauterine development (Bhattacharyya, 2015). These congenital voice disorders can be caused by a wide variety of abnormalities along the aerodigestive tract, including larynx and vocal folds (VF). Molecular and cellular mechanisms that control formation of laryngeal structures are beginning to be elucidated,

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but we are still far behind the research that has been done in upper airways or lungs (Morrisey and Hogan, 2010; Herriges and Morrissey, 2014). Frequently used animal models, such as felines, non-human primates, canines, pigs, rabbits or rats allow for the examination of laryngeal biology and testing of broad arrays of medical, surgical and behavioral interventions (Lüthe, Häusler and Jürgens, 2000; Andreatta *et al.*, 2002; Connor *et al.*, 2002; Bless *et al.*, 2004; Rousseau *et al.*, 2004; Goding, Al-Sharif and McLoon, 2005; Bergrin *et al.*, 2006; Thomas *et al.*, 2009), however, these animal models have limitations for studying epithelio-mesenchymal interactions in genetically linked diseases related to laryngeal development and/or regeneration. The recent introduction and use of transgenic mice in voice research should significantly improve our understanding of normal laryngeal cell biology and genetically linked disease processes.

Basic research into the mechanisms controlling laryngeal morphogenesis is important for a number of reasons. First, congenital laryngeal defects are potentially life threatening at birth, as they can be accompanied with airway obstruction, and can cause problems in voicing across the lifespan (Wyatt and Hartley, 2005; Ahmad and Soliman, 2007). A better understanding of how these early developmental processes are regulated is necessary for understanding and evaluating functional prognosis in treatment approaches. Second, abnormalities in formation of laryngeal structures can seriously affect development and maturation of other respiratory structures, such as lungs. Failure in VF recanalization in laryngeal webs/atresia results in polyhydramniotic lungs (Liberty *et al.*, 2013), while congenital laryngeal clefts may lead to chronic interstitial lung damage related to aspiration and gastroesophageal reflux (Neilan *et al.*, 2012). Lastly, identifying genes and signaling molecules that drive VF morphogenesis could be utilized in VF tissue engineering to improve postnatal VF tissue repair (Li *et al.*, 2016; Ban *et al.*, 2017; Kumai, 2019) and/or to create developmentally derived human VF mucosa for clinical and pharmacological applications (Lungova *et al.*, 2019).

Here, we address current understanding of molecular and cellular mechanisms that regulate different stages of the laryngeal morphogenesis, from specification of the laryngeal field to complete VF separation using transgenic mouse models and correlate these findings with human data from patients with congenital laryngeal malformations. We also point to events which are underexplored and that deserve further attention. Proposed genes and signaling pathways that are critical for development of the larynx and VF have a potential to be harnessed in the field of VF regenerative medicine.

2. STRUCTURE AND FUNCTION OF THE LARYNX AND VOCAL FOLDS

The mammalian larynx fulfills multiple functions related to breathing, protection and phonation starting with the first breath of a newborn. During breathing, the larynx maintains a widely open air channel with abducted VF to control inspiratory and expiratory airflow. Nevertheless, during swallowing VF approximate, the epiglottis closes the entrance into the laryngeal cavity and the larynx acts as a sphincter to avoid aspiration. The VF are housed in the larynx and besides swallowing, they are involved in voice production in humans (Miri, 2014). Whether the VF participate in audible and ultrasonic vocalizations in mice is still debatable (Mahrt *et al.*, 2016; Riede, Borgard and Pasch, 2017).

Current descriptive anatomic studies have revealed similarities in the structure and function between human and murine larynges and VF (Marques *et al.*, 2007; Thomas *et al.*, 2008, 2009) (Fig. 1A–C). In both species, the laryngeal framework is composed of four main cartilages interconnected by ligaments and membranes, and moved by extrinsic and intrinsic laryngeal muscles. These cartilages include the epiglottis (Epi), thyroid cartilage (TC), cricoid cartilage (CC) and a pair of arytenoid cartilages (AC) which rotate and rock on the CC to facilitate VF movement. Specific to humans are pairs of cuneiform and corniculate cartilages. The corniculate cartilages are minor cartilaginous structures, which articulate with the apices of the AC and prolong them backward and medial. The cuneiform cartilages are located within the aryepiglottic folds and act to strengthen the VF. Specific to rodents is an alar cartilage (Ar) that is situated superior to the VF ventral attachment and is involved in ultrasonic vocalization (Thomas *et al.*, 2009; Mahrt *et al.*, 2016; Riede, Borgard and Pasch, 2017).

The intrinsic laryngeal muscles are skeletal voluntary muscles and are innervated by cranial nerves. The intrinsic laryngeal muscles common to both species include the posterior cricoarytenoid muscles (PCA) located in the tracheoesophageal septum, lateral cricoarytenoid (LCA) and cricothyroid muscles (CT), located on the lateral aspect of the larynx. LCA controls VF adduction for sound production and/or swallowing, while PCA controls VF abduction for breathing. The CT muscle works as a VF tensor. Specific to humans are transverse and oblique arytenoid muscles located in the tracheoesophageal septum. They approximate the VF along with LCA. Specific to rodents is a thin pair of muscles called the superior cricoarytenoid (SCA). These muscles connect the dorsal surface of the AC with the CC midline prominence. They assist in the VF adduction and likely serve the same role as the transverse and oblique interarytenoid muscles in humans (Inagi, Schultz and Ford, 1998; Thomas *et al.*, 2008).

The VF stretch horizontally across the larynx; they attach to the TC ventrally and AC dorsally. Alike in humans, the murine VF are composed of three distinct tissue types (Fig. 1D–H). The outer layer is squamous non-keratinizing stratified epithelium (EP), which transitions into pseudostratified cranially, in the laryngeal vestibule, and caudally, in the subglottic region. Squamous epithelial cells are adapted to withstand constant abrasions they experience during collisions and form a protective barrier against injury and environmental or systemic-based irritants (Levendoski, Leydon and Thibeault, 2014). Below the epithelium is the lamina propria (LP) with fibroblasts and extracellular matrix. This layer responds to the mechanical force applied by the air passing from the lungs. In humans, the LP gradually changes its density from the jelly-like superficial layer to the stiffer layers containing elastic and collagenous fibers and the muscle. The change in density from the superficial layer to the muscle allows the creation of the mucosal wave that occurs during vibration. The presence or absence of the mucosal wave determines VF vibratory behavior and thus influences the voice quality (Miri, 2014). The thyroarytenoid (TA) muscle forms the deepest VF portion in humans and mice (Hirano, 1974; Lungova *et al.*, 2015; Tabler *et al.*, 2017). The function of this muscle is to shorten and relax the VF, which modifies VF elasticity and tension. While rodents use a different mechanism to generate ultrasonic and audible sound than humans (Mahrt *et al.*, 2016), their precise control of laryngeal activity and properties of

the VF are similar to humans and other mammals (Riede, 2011; Watts, Marler and Rousseau, 2011; Riede, Borgard and Pasch, 2017).

Mice vocalizations can have a fundamental frequency range between 100 and 120,000 Hz, whereas the range for human vocalizations is much reduced in comparison, between 100 and 3000 Hz (Mahrt *et al.*, 2016; Titze, Riede and Mau, 2016). In humans, fundamental frequency is determined by mass and tension of the VF and air pressure during vibration. VF mass and tension are typically regulated via muscle contraction and relaxation of the various laryngeal muscles (TA, PCA, PCA, CT). Certainly, changes in the constituents of the lamina propria can also change VF mass. The supralaryngeal vocal tract, consisting of both the oral and nasal airways serve as a time-varying acoustic filter that suppresses the passage of sound energy at certain frequencies while allowing its passage at other frequencies. Formants are those frequencies at which local energy maxima are sustained by the supralaryngeal vocal tract and are determined, in part, by the overall shape, length and volume of the vocal tract (Zhang, 2016). Similar to humans, mice typically use their larynx to create sound, albeit differently, such that USV is possible. Theories into the mechanism(s) for the creation of ultrasonic vocalization (USV) has been various, with the most likely mechanism being the edge-tone model (Riede, Borgard and Pasch, 2017). In this model, a ventral pouch, which is positioned downstream of the VF and surrounded by cartilaginous structures, has part of its entrance edge supported by alar cartilage or “alar edge”. Glottal airflow passes over the alar edge of the ventral pouch. Contraction of the alar edge via contraction of the TA muscles results in a shorter distance between the edges of the ventral pouch and a smaller ventral pouch lumen. During voicing, undulating airflow interacts with the alar edge of the ventral pouch allowing for resonance within pouch, similar to a Helmholtz resonator. Fundamental frequency is dependent upon glottal airflow velocity and glottis to alar edge distance. Increases in fundamental frequency are perceived when airflow velocity increases and/or when the glottis to alar edge distance decreases, such that the volume in the ventral pouch decreases concomitantly. The TA muscle indirectly regulates airflow through the glottis via VF adduction and it has been hypothesized in its role in regulating glottis to alar edge distance and regulating ventral pouch size.

Overall, the larynx and VF in mice and humans are well suited to carry out their function. The finely orchestrated action of intrinsic laryngeal muscles attached to the laryngeal framework facilitates VF movement necessary for breathing, swallowing and vocalization. Moreover, the VF microanatomy is well adapted to withstand the high mechanical stresses and forces they experience during the vibratory cycle and collisions. Further looking into the developmental origin of laryngeal structures is important to understand the relationship between these structures, their unique properties and etiology of defects in laryngeal and VF development.

3. GENE FATE MAPPING OF THE LARYNGEAL AND VF STRUCTURES

Only recently comprehensive descriptions of developmental origin of mammalian laryngeal and VF tissue have become available using mouse transgenic models (Tabler *et al.*, 2017; Lungova *et al.*, 2018). These studies revised and extended previous contradictory investigations based on descriptive studies and lineage tracing experiments in birds (Noden,

1983, 1986; Huang *et al.*, 1997; Evans and Noden, 2006) or amphibians (Piekarski and Olsson, 2007).

Precursors for laryngeal and VF structures come from multiple sources in developing embryos (Fig. 2A). First, laryngeal structures develop in close proximity with the endodermal foregut tube, similar to other respiratory structures. Endodermal cells express sonic hedgehog (*Shh*) gene which is required for normal patterning of respiratory, digestive and genitourinary organs and enables to genetically label and follow the fate of endodermal cells (Harris-Johnson *et al.*, 2009). Second, the endodermal foregut tube is enveloped laterally and ventrally with the lateral mesoderm that expresses mesoderm posterior BHLH transcription factor 1 (*Mesp1*). *Mesp1* gene plays a key role in the rostro-caudal patterning of somitic mesoderm and labels mesoderm-derived tissues (Saga *et al.*, 1999). Third, the lateral mesoderm becomes partially displaced by secondary population of mesenchymal neural crest cells (NCC) that migrate to the site of laryngeal development from the hindbrain region. The NCC transiently express protooncogen wingless-type MMTV integration site family, member 1 (*Wnt-1*) gene, that is used to visualize descendant of the NCC (Chai *et al.*, 2000).

Lineage tracing experiments using *Shh*^{Cre+/-} *TdTomato* reporter mice (Lungova *et al.*, 2018) revealed that endodermal cells primarily give rise to the laryngeal and VF epithelium (Fig. 2B). These endodermal cells are formed during gastrulation (Garcia-Martinez, Alvarez and Schoenwolf, 1993) and are initially located ventral to the future postotic hindbrain region. Interestingly, the *Shh*^{Cre} lineage signal also extends into adjacent mesenchyme suggesting that some *Shh* expressing cells may delaminate into VF lamina propria. These cells intermix with *Sox9* expressing chondrocytes or surround *MF-20* expressing myoblasts (Lungova *et al.*, 2018). The function of these cells, as well as their contribution to differentiation of mesenchymal structures remains to be investigated.

Lateral mesoderm is a source of the AC and CC, and intrinsic laryngeal muscles including TA muscle as determined by *Mesp1*^{Cre+/-} *TdTomato* reporter lineage tracing (Tabler *et al.*, 2017) (Fig. 2C). Further investigation of the origin of intrinsic laryngeal muscles have revealed that mammalian intrinsic laryngeal muscles originate from the cranial (unsegmented) mesoderm based on the robust *Isl1* expression, similar to the head muscles, and not from the somitic (segmented) *Pax3* positive mesoderm as classic skeletal limb muscles do (Tabler *et al.*, 2017). A close relationship between intrinsic laryngeal and extraocular head muscles has been confirmed by independent experiments using animal models and human tissues. They demonstrate that intrinsic laryngeal muscles share many structural features with extraocular muscles and stand apart from typical limb skeletal muscles in terms of their muscles fiber size, myosin heavy chain isomorphs, innervation patterns, regenerative capacity and response to diseases (Shiotani, Westra and Flint, 1999; Périé *et al.*, 2000; Goding, Al-Sharif and McLoon, 2005; Shinnars, Goding and McLoon, 2006; Marques *et al.*, 2007; Thomas *et al.*, 2008). Myogenic cells enter the branchial arches and migrate into the site of laryngeal development along with NCC, which is reflected in their innervation by the cranial nerves (superior and inferior branches of the vagal nerve) (Noden *et al.*, 1999; Frisdal and Trainor, 2014). Myogenic precursors are committed to the myogenic lineage prior to leaving the somites, as evidenced by their expression of the

skeletal muscle-specific genes, such as *Myf5* (Noden *et al.*, 1999). Mechanisms that specify and guide migration of myogenic precursors into the laryngeal region as well as their further differentiation into intrinsic laryngeal muscles have not yet been fully elucidated.

Mapping of descendants of NCC using *Wnt1^{Cre+/-}mGFP* reporter mice (Tabler *et al.*, 2017) has shown the neural crest cells are a pluripotent population of cells that represent precursors for connective and nerve tissues (Fig. 2D). NCC contribute to the formation of the loose connective tissue in the ventral LP and the TC, except for the medial caudal-most portion of the TC (Tabler *et al.*, 2017). *Wnt1^{Cre+/-}mGFP* positive cells have also been found within laryngeal muscles and were identified either as neurons by acetyltubulin immunofluorescent staining or fascia separating the muscles (Tabler *et al.*, 2017). Similar to myogenic precursors, NCC move *en masse* along branchial arches into the target site and mix with the mesoderm-derived structures during craniofacial morphogenesis (Noden and Trainor, 2005). Recent experiments using *Fuz^{-/-}* mutants have shown that laryngeal NCC become specified prior to leaving the hindbrain region. Whole animal deletion of *Fuz* gene results in excessive accumulation of NCC in the developing larynx which leads to severe malformations of laryngeal cartilages and muscles and the absence of glottis. However, conditional deletion of the *Fuz* gene in *Wnt1^{Cre+/-}Fuz^{Flox/-}* mutants does not affect laryngeal morphogenesis (Tabler *et al.*, 2017). Further experiments are needed to elucidate the specification and migratory pattern of laryngeal NCC as well as their integration into mesoderm-derived tissues, such as laryngeal cartilages, or nerve tissue.

In summary, lineage-tracing data has revealed two important aspects of laryngeal morphogenesis that deserve further investigations. First, the intrinsic laryngeal muscles originate from different mesodermal cell populations and develop via distinct genetic programs than typical skeletal limb muscles. This supports the notion that intrinsic laryngeal muscles represent specialized muscle groups closely related to the extraocular and head muscles that significantly differ from typical skeletal muscle structure and function (Marques *et al.*, 2007). This could have an impact on voice therapy, as several voice therapy approaches suggested in the literature are based on principles of exercise physiology in classic skeletal muscles, as discussed elsewhere (Thomas *et al.*, 2008). Second, the larynx develops at the “head (neural crest-derived) - trunk (mesoderm-derived)” interface and cells from both sides of this interface move into the site where the larynx develops. This requires precise temporo-spatial coordination to achieve an integrated outcome. The complexity of development, therefore, renders the laryngeal apparatus susceptible to perturbation and subsequently the pathogenesis of congenital anomalies. In the next part of this review, we will focus on developmental events that are critical for understanding the laryngeal and VF morphogenesis and can lead to laryngeal congenital malformations.

4. OVERVIEW OF LARYNGEAL AND VOCAL FOLD MORPHOGENESIS

The first signs of laryngeal morphogenesis appear during early embryonic stages, embryonic (E) day 9.5 in mice, which corresponds to week 4 (Carnegie stage 11) in humans, with a formation of a sagittal slit at the level of the fourth pharyngeal pouch, labelled as the laryngotracheal groove (LG) (Zaw-Tun and Burdi, 1985; Sanudo and Domenech-Mateu, 1990; Henick, 1993) (Fig. 3A–C). There has been much controversy over the interpretation

of this anatomic location. In the past, some authors believed that the LG represented the primordium of the upper subglottic (or infraglottic) cavity (Sanudo and Domenech-Mateu, 1990). The most recent re-examination of the early development of the mammalian larynx using computer generated three-dimensional (3D) reconstruction supports the notion that this opening represents the primordium of the supraglottic cavity, called the primitive laryngopharynx (LPh), which develops as the RD expands and elongates (Henick, 1993). As an anatomic landmark, the primitive LPh appears at E10.5 in mouse models and Carnegie stage 13 in humans (Zaw-Tun and Burdi, 1985; Henick, 1993; Lungova *et al.*, 2015). It is located between the fourth pharyngeal pouch, as its cranial border, and the cephalic portion of the respiratory diverticulum, as its caudal border. The VFs originate from the caudal LPh segment; the cranial part of the LPh becomes the laryngeal vestibule (LV) (supraglottis), while the segment below the prospective VFs, including the cephalic portion of the respiratory diverticulum (RD), becomes the subglottis (SG).

In the course of development (Fig. 3), the primitive LPh becomes bilaterally compressed due to the approximation of the lateral walls toward the center of the lumen (at E10.5 in mice and Carnegie stage 13 in humans). Soon after, the closely juxtaposed lateral walls fuse together and form the epithelial lamina (EL) which temporarily obliterates the ventral LPh lumen (at E11.5 in mice and Carnegie stages 14–16 in humans). The EL is first reorganized to form the laryngeal part of the tracheoesophageal septum (E13.5), and then completely disintegrates to open the laryngotracheal tube (E13.5–18.5 in mice; Carnegie stages 19–23 up to week 12 in humans). The process of EL recanalization initiates with the formation of cavities such as the laryngeal cecum (ventrally) and pharyngoglottic duct (dorsally) that extend into the primitive LPh from cranial and caudal directions, respectively and exert pressure on the epithelial walls. By the gradual expansion of these cavities, the laryngeal cecum unites with the pharyngoglottic duct and VF move apart (Lungova *et al.*, 2015, 2018). In both species, mice and humans, the process of EL disintegration is concurrent with the laryngeal cartilage chondrification, intrinsic laryngeal muscle formation and initiation of stratification in the VF epithelium (Lungova *et al.*, 2018). Laryngeal cartilages and muscles complete their development prior to VF separation. Soon after VF separation (E17.5–18.5 in mice and week 12 in humans), the intrinsic laryngeal muscles approximate the VF to close the glottis or pull them apart to enable bidirectional flow of amniotic fluid (Liberty *et al.*, 2013). After delivery, amniotic fluid is replaced with air as a newborn takes its first breath. In humans, the presence of laryngeal cartilages as well as detection of the VF motion serve as orientation points for sonographic examination of the developing human fetal larynx as early as 11 weeks' gestation to evaluate obstructive pathological lesions (Liberty *et al.*, 2013). Inability to identify these anatomical elements along with the marked polyhydramnios and echogenic lungs indicate congenital upper airway anomalies such as laryngeal atresia or tracheoesophageal fistula (Liberty *et al.*, 2013).

In summary, the major morphogenetic events of the laryngeal and VF embryogenesis include the apposition of lateral walls of the primitive LPh and the EL formation that bring VF primordia together. Then it is the separation of the larynx from the esophagus and the EL disintegration once supporting mesenchymal structures develop and firmly anchor growing VF. The process of the EL recanalization prevents airway obstruction and is critical for further development of other parts of the respiratory system, particularly the lungs.

Reciprocal epithelial-mesenchymal interactions that regulate these morphogenetic events are poorly understood as laryngeal development has received little attention by experimental biologists.

5. EARLY STAGES OF LARYNGEAL MORPHOGENESIS - SPECIFICATION OF THE LARYNGEAL AND VOCAL FOLD PROGENITORS

5.1. Signaling pathways that control early LPh remodeling and specification of VF epithelial progenitors

Early stages of laryngeal morphogenesis are associated with development of the respiratory diverticulum (RD) in the ventral foregut endoderm. Thus, signaling pathways that drive respiratory progenitor identity are likely to act on specification of the laryngeal cell fate as well. RD embryogenesis begins with a separation of the trachea from the foregut; the lung bud appearing as an out pouching of the RD at E9.5 in mice and approximately 28 days in humans (Morrissey and Hogan, 2010; Herriges and Morrissey, 2014). This early histogenic RD remodeling requires a localized domain of *Nkx2-1* expression in the ventral wall of the anterior foregut endoderm and the presence of *Shh* (Arsi *et al.*, 2003). Reducing levels of *Nkx2-1* and/or *Shh* or disabling the ability of adjacent mesodermal cells to properly respond results in defects of tracheoesophageal separation and tracheal chondrogenesis leading to tracheoesophageal atresia/fistula (Litingtung *et al.*, 1998). Recent studies have shown that *Shh* and *Gli1*, *Gli2* and *Gli3* are strongly expressed in the primitive LPh endoderm and surrounding mesenchyme, respectively (Lungova *et al.*, 2015; Tabler *et al.*, 2017) (Fig. 4A). Deletion of *Shh* in *Shh* null mutants affects the apposition of the lateral walls; they do not approach each other and the primitive LPh remains open. This is consistent with the fact that lack of *Shh* signaling may downregulate gene expression and proliferation of the adjacent mesenchymal target cells. Moreover, *Shh* deletion also alters *Nkx2-1* and *Sox2* expression in the primitive LPh endoderm, which may also disrupt approximation of lateral walls.

Besides *Shh* and a localized expression of *Nkx2-1*, the developing RD responds to *Wnt* signals emanating from adjacent mesoderm. Combined loss of *Wnt2/2b* in the ventral anterior foregut mesoderm leads to a complete loss of *Nkx2-1* lung endoderm and a failure to form the trachea and branching lungs (Goss *et al.*, 2009). This phenotype is recapitulated upon loss of *beta-Catenin* in the anterior foregut endoderm (Harris-Johnson *et al.*, 2009). Specific to laryngeal development, high levels of *Wnt* signaling, as indicated by *Axin2 in situ* hybridization, has been reported in the primitive LPh endoderm and in adjacent mesenchyme during EL formation at E11.5 in mice (Lungova *et al.*, 2018) (Fig. 4B). Conditional deletion of *beta-Catenin* in the LPh endoderm disrupts the timing of EL formation. Nevertheless, the approximation of lateral walls appear normal suggesting that *Wnt* signaling was not required for the juxtaposition of the lateral walls, but for their fusion and specification of VF basal epithelial progenitors. Investigation of other signaling pathways, such as *Bmp* or *Fgf* signaling may help to understand epithelio-mesenchymal interactions that control these early morphogenetic events.

5.2. Signaling pathways that control migratory cell populations and differentiation of the mesenchymal progenitors

Early stages of LPh remodeling are also associated with intense migration of NCC and cranial myoblasts (Frisdal and Trainor, 2014) and initial differentiation of mesenchymal progenitors in the LP (Fig. 4A). Excessive or insufficient cell migration as well as inability of migratory cells to respond to positional clues may disrupt development of laryngeal cartilages, muscles and laryngeal innervation. Signaling pathways known to date that control migration and/or incorporation of laryngeal NCC and myoblasts in the LP include 1) *Shh* signaling via *Gli* proteins expressed in the LP mesenchyme and 2) signaling pathways that control pharyngeal segmentation, as the migratory cell populations enter the laryngeal region via the fourth and sixth pharyngeal (branchial) arches.

Recent studies have shown that *Shh/Gli* signaling is likely involved in regulation of migration and/or integration of laryngeal NCC in the LP. Deletion of *Gli3* in *Gli3*^{-/-} mutant mice results in an excessive accumulation of NCC in the ventral glottic compartment. This influences the attachment of the vocal ligaments and muscles as well as vocalization (Tabler *et al.*, 2017). *Gli3* mutant mice can also display a more severe phenotype with a wide range of developmental abnormalities resembling Pallister-Hall syndrome (Bose, 2002). Similar to human patients with Pallister-Hall syndrome, *Gli3* mutant mice showed an absence of the epiglottis and a laryngeal cleft resulting in an abnormal connection between the larynx and esophagus (Ondrey F, Griffith A, Van Waes C, Rudy S, Peters K, McCullagh L, 2000; Bose, 2002). In human patients, voice and laryngeal defects are commonly associated with defects in *Shh/Gli* signaling pathway (França *et al.*, 2010). Apart from defects described in Pallister-Hall syndrome, bifid epiglottis and laryngeal clefts are found in patients with Oral-Facial-Digital syndromes and Bardet-Biedl syndrome (Silengo *et al.*, 1987; Stevens and Ledbetter, 2005; Hayes *et al.*, 2008; Franco and Thauvin-Robinet, 2016).

The pharyngeal arches and their segmental arrangement represent another source of signaling for successful migration and incorporation of NCC and myoblasts. Signaling pathways that control caudal patterning of the pharyngeal apparatus, include - retinoic acid (RA), *Tbx1*, *Hox* genes, and *Fgf* signaling (Fig. 4A). RA is a morphogen that is important for the caudal pharyngeal pouch segmentation and formation of the 3rd to 6th pharyngeal arches (Vermot *et al.*, 2003). Vitamin A-deficient rat embryos demonstrate laryngotracheal cartilage malformation, incomplete separation of the glottis, and/or laryngeal clefts (Tateya *et al.*, 2007). Similarly, mouse mutants for *Raldh2* gene, the key enzyme responsible for the conversion of vitamin A (retinol) into retinoic acid, exhibit defects in 3rd to 6th pharyngeal arches formation, which also leads to laryngeal cartilage malformations (Niederreither *et al.*, 2003; Vermot *et al.*, 2003).

Besides a direct effect of RA on pharyngeal segmentation, RA also mediates transcription of other genes such as *Tbx1* or *Hox* genes. Recent studies have shown that, in a mouse model, decreased embryonic levels of RA downregulate the expression of *Tbx1*, which results in pharyngeal apparatus malformations and defects in cardiovascular and craniofacial development resembling a DiGeorge syndrome phenotype (Roberts *et al.*, 2005). The similar phenotype could be achieved by the mesenchymal inactivation of *beta-Catenin* which also suppresses the expression *Tbx1* and its downstream signaling pathways (Huh and Ornitz,

2010). These data confirmed that mesenchymal *Tbx1* is necessary for pharyngeal arch development (Zhang, Huynh and Baldini, 2006) and mutation in *Tbx1* gene is closely associated with the etiology of DiGeorge syndrome. In patients, DiGeorge syndrome, or velo-cardio-facial syndrome, is linked with the 22q11.2 microdeletion (Wong *et al.*, 2019), which alters *Tbx1* gene expression (Lipson *et al.*, 1991; Chieffo *et al.*, 1997; Morrow *et al.*, 2018). Besides congenital heart defects and craniofacial malformations, these patients exhibit a wide range of laryngeal abnormalities including laryngeal webs (the most frequent anomaly), subglottic stenosis, laryngomalacia and/or atrophic VF (Fokstuen S, Bottani A, Medeiros PF, Antonarakis SE, Stoll C, 1997; Ebert *et al.*, 2018).

In addition to regulation of *Tbx1* expression, RA is a well-known regulator of *Hox* gene activity through the presence of RA response elements in the promoter region of many *Hox* genes (Marshall H, Morrison A, Studer M, Pöpperl H, 1996; Frisdal and Trainor, 2014). Analyses of *Hox* gene loss-of-function in transgenic mice identified two *Hox* gene groups, *Hoxa-3* and *Hoxa-5*, which are involved in laryngeal and tracheal cartilage development. Deletion of *Hoxa-3* in the caudal pharyngeal endoderm results in multiple skeletal defects, including dysmorphogenesis of the CC and TC (Manley and Capecchi, 1997). Similarly, loss-of-function in *Hoxa-5* gene in the mesenchyme surrounding the RD reveal a hyperplastic cricoid, disorganization of the tracheal rings and hypoplasia of the pulmonary tree (Aubin *et al.*, 1997).

Lastly, *Fgf* signaling also plays a key role in pharyngeal pouch/arch formation and segmentation. Signals from the pharyngeal endoderm mediated via *Fgf3* and *Fgf8* interact with the *Fgf* receptors expressed in the NCC to facilitate the development of NCC-derived cartilages (Frisdal and Trainor, 2014). The role of *Fgf* signaling in the laryngeal morphogenesis has not been investigated yet.

6. EPITHELIAL LAMINA FORMATION AND RECANALIZATION

The next critical period of the laryngeal development that deserves further attention is related to the establishment of the EL and its recanalization. In a mouse model, the lateral walls of the primitive LPh are brought together and fuse to form the EL at E11.5 (Fig. 4B). The movement of the lateral walls towards the midline is likely caused by rapidly proliferating epithelial and mesenchymal cells, which simultaneously initiate differentiation into VF basal progenitors (epithelial cells) and cartilages (mesenchymal cells) at the sites of swellings (Zaw-Tun and Burdi, 1985; Sanudo and Domenech-Mateu, 1990; Lungova *et al.*, 2015). The decrease in cell proliferation and/or failure in differentiation of epithelial or mesenchymal progenitors may disrupt EL formation and its consequent recanalization. If EL recanalization does not occur until E18.5 in mice and the week 12 in humans, laryngeal webs will result (Zaw-Tun and Burdi, 1985; Sanudo and Domenech-Mateu, 1990; Henick, 1993; Lungova *et al.*, 2018).

Laryngeal webs are congenital defects associated with different degrees of failure of EL resorption during intrauterine development. They can cause acute, life-threatening respiratory complications in newborns, and chronic, lifelong problems with voicing (Hartnick and Cotton, 2000; Wyatt and Hartley, 2005; Ahmad and Soliman, 2007).

Molecular mechanisms that underline the establishment of the EL and its recanalization are beginning to be elucidated. To date the proper timing of the EL formation and recanalization has been linked to *beta-Catenin* function (Huh and Ornitz, 2010; Lungova *et al.*, 2018). Conditional deletion of *beta-Catenin* in the LPh endoderm delays the fusion of the lateral walls, which leads to the absence of the tracheoesophageal septum and defective VF separation resembling milder to severe cases of laryngeal webs (Lungova *et al.*, 2018). The delay in EL formation is more than likely caused by a decrease in cell proliferation in epithelial and mesenchymal cells mediated by the interaction between *Cyclin D1*, a downstream target of *beta-Catenin*, and cell cycle inhibitor *p27^{kip}* and by the defective differentiation of VF basal progenitors into functional p63+ cytokeratin (K)8- basal cells capable of stratification. Moreover, downregulation of *beta-Catenin* in the LPh endoderm had a secondary effect on differentiation of mesenchymal cells in the LP, which could also act on EL recanalization (Lungova *et al.*, 2018). Involvement of mesenchymal structures in EL recanalization has been confirmed in other experiments. In mice with a DiGeorge syndrome phenotype, manipulation of *beta-Catenin* - *Tbx1* signaling in the mesenchyme led to mesenchymal defects and problems with the EL recanalization (Huh and Ornitz, 2010) suggesting that the EL disintegration depends on reciprocal interactions between epithelial and mesenchymal cells in the LP.

7. DIFFERENTIATION OF MESENCHYMAL CELLS IN THE LAMINA PROPRIA

EL recanalization is accompanied with simultaneous differentiation of mesenchyme into cartilages; muscles, fibroblasts and neurofilaments that are major structural and functional components of the larynx and VF (Fig. 4B, C). The inability of mesenchyme to properly differentiate may lead to anatomic abnormalities of laryngeal cartilages and muscles and/or defective neuromuscular control that can affect the lumen patency and/or VF mobility.

7.1. Differentiation of supportive cartilages

In a mouse model, differentiation of laryngeal cartilages initiates with the formation of the EL, when an early marker of cartilaginous structures SOX9 (Elluru and Whitsett, 2014), appears at the site of arytenoid swellings (prospective AC) and ventrally close to the tip of the EL (Lungova *et al.*, 2018). This ventral region of SOX9+ cells gives rise to the intermediate lamina of the TC. The lateral laminae of the TC develop in a close proximity to the thyroid gland and gradually fuse with the intermediate lamina to form the TC. The CC likely initiates its development later along with the establishment of the tracheoesophageal septum (at E13.5 in mice). Failure in septum formation in *beta-Catenin* conditional mutants leads to the absence of the CC (Lungova *et al.*, 2018). The subepithelial location of SOX9+ cells, next to the EL expressing *Shh*, is likely important for *Sox9* gene activation. As previously reported, in the somitic mesoderm, *Shh* signals from the notochord promote *Bmp* expression in the adjacent mesenchyme to induce chondrogenesis and expression of cartilage specific genes such as *Sox9* (Zeng *et al.*, 2002). Similarly, *Sox9* expression could be promoted by *Fgf* signaling. Explants of airway structures including the larynx cultured in the exogenous FGF18, demonstrated hyperplastic growth of laryngeal and tracheal cartilages and increased expression of *Sox9* (Elluru, Thompson and Reece, 2009).

Sox9 has been identified as an early marker and important regulator of cartilage differentiation in both mice and humans (Bell *et al.*, 1997; Akiyama *et al.*, 2002; Mori-Akiyama *et al.*, 2003). Humans and transgenic mice with haplosufficiency of *Sox9* expression have, among other skeletal defects, upper airway defects, secondary hypoplasia of laryngeal and tracheal cartilages (Elluru and Whitsett, 2014). Similarly, in mice, conditional deletion of *Sox9* using *Wnt1^{Cre+/-}* results in specific loss of the TC (Mori-Akiyama *et al.*, 2003). Interestingly, migration and localization of *Wnt1^{Cre+/-} Sox9^{Flox/-}* NCC demonstrate that NCC that successfully reach their laryngeal destination, lose their chondrogenic potential. During chondrogenesis, *Sox9* is co-expressed with *Col2a1*, the gene encoding type-II collagen, a major cartilage matrix protein, which is a direct regulatory target of *Sox9* (Bell *et al.*, 1997). In humans and mice, a total loss of type-II collagen function in null mutants results in severe skeletal and cartilaginous abnormalities but individuals heterozygous for *Col2a1* are relatively mildly affected (Li and Darwin, 1995; Ritvaniemi *et al.*, 1995).

Other signaling pathways that have been shown to regulate the development and differentiation of laryngeal cartilages include *Wnt* via *R-spondin 2* and *Eph/EphrinB2* signaling. Whole animal loss-of-function *R-spondin 2* leads to laryngo-tracheal malformations (Bell *et al.*, 2008). In these animals, arytenoids fused to the ventral part of the cricoid, and the dorsal aspect of the cricoid was absent. Defective *Eph/Ephrin B2* signaling affects the midline fusion of the CC in the dorsal laryngotracheal wall producing posterior laryngeal clefts (Neilan *et al.*, 2012). Patients with laryngeal clefts suffer from dysphagia, with possible aspiration, and often have associated neurological disorders (Neilan *et al.*, 2012; Chiang *et al.*, 2014), which corresponds with the role of *Ephrins* in the septation events (Dravis and Henkemeyer, 2011) as well as in the axon guidance in the central and peripheral nervous system (Dickson, 2002; Neilan *et al.*, 2012).

Immaturity of neuromuscular control along with possible defects in chondrification may also contribute to laryngomalacia (Kusak *et al.*, 2017). Laryngomalacia is the most common laryngeal anomaly in infants (Bedwell and Zalzal, 2016), which can occur as an isolated anomaly or as a part of malformation complexes. Patients with laryngomalacia exhibit omega shaped epiglottis, redundant bulky arytenoids and inward collapse of the arytenoid folds, causing inspiratory stridor and airway obstruction (Thompson, 2007). Molecular data are available for syndromic type of laryngomalacia, in DiGeorge or Bardet-Biedl syndromes, as these syndromes are associated with malacic bifid epiglottis (Chieffo *et al.*, 1997; Stevens and Ledbetter, 2005; Tsurumi *et al.*, 2010; Ebert *et al.*, 2018).

7.2. Differentiation of muscles and nerves

Along with laryngeal cartilages, mesenchymal cells in the LP differentiate into intrinsic laryngeal muscles. These muscle cells do not show overt signs of muscle organization, such as alignment of primary myocytes, until adjacent cartilaginous structures have begun to condense (Lungova *et al.*, 2018). There are two theories to explain the genesis of laryngeal muscles (Som and Curtin, 2014). The sphincter theory states that the larynx is surrounded throughout its initial stages of development by two mesenchymal zones or sphincters, one outside and one inside the larynx. The outer zone gives rise to inferior pharyngeal

constrictor muscles, the CT and the TC, while the inner zone gives rise to the TA, lateral and dorsal intrinsic laryngeal muscles, and remaining cartilages. The alternate explanation is that laryngeal muscles evolve from individual mesenchymal masses. In a mouse model, double staining for distribution of SOX9+ chondrocytes and MF-20+ myoblasts in developing LP supports the second theory of individual mesenchymal masses. The TA muscle initiates its differentiation during the EL establishment at E11.5 along with the differentiation of AC and the TC, while dorsal intrinsic laryngeal muscles develop two days later along with the tracheoesophageal septum and CC (Lungova *et al.*, 2018).

Simultaneous with differentiation of laryngeal cartilages and muscles, axons invade the mesenchyme of the LP (Pitman *et al.*, 2013). In a rat model of laryngeal muscle innervation, staining for neuronal class III β -tubulin confirmed the presence of axons in the LP during the establishment of the EL, which correlates with the onset of myogenesis in the VF. Nerve growth cones enter the larynx from the dorso-lateral region in a time and muscle specific manner (Pitman *et al.*, 2013). After complete EL recanalization (E21 in a rat, which correlates with E17.5 – 18.5 in a mouse) neuromuscular junctions are present with a single axon innervating multiple motor end plates arranged in a central band within the muscle. In vertebrate embryogenesis, axon guidance is highly conserved and controlled via extracellular chemoattractants such as netrins, Slits, semaphorins, ephrins (Dickson, 2002) and nerve growth factors (NGF) (Vega-Cordova *et al.*, 2010). Delay or errors in the development of the neurological control of the laryngeal musculature may lead to the collapse of laryngeal tissues as described in laryngomalacia (Kusak *et al.*, 2017) or defects in VF mobility, known as VF paralysis (Hsu *et al.*, 2015). This type of disorder can be either unilateral or bilateral and is associated with neonatal stridor and airway obstruction. Approximately half of all cases of bilateral VF paralysis are congenital followed by birth trauma or idiopathic (Daya *et al.*, 2000). In humans, novel chromosomal translocation between chromosomes 5 and 14 has been identified in familial congenital bilateral VF paralysis (Hsu *et al.*, 2015). There are currently no molecular data in animal models that elucidate the temporo-spatial relationship between myogenic progenitors and ingrowing peripheral recurrent nerve to validate human data and propose other mechanisms responsible for defects in laryngeal myogenesis and neuromuscular control.

7.3. Differentiation of fibroblasts in the LP and extracellular matrix deposition

Considerably less is known about the development and differentiation of the fibroblasts in the LP and proteins they secrete into the extracellular matrix (ECM), such as collagen and elastin. Abnormalities in production of these proteins can negatively influence VF biomechanics and voice production (Watts, Awan and Marler, 2008; Watts, Marler and Rousseau, 2011). In humans, haploinsufficiency in the elastin gene expression is associated with Supravalvular aortic stenosis and Williams syndrome. These patients have abnormal, rough, and hoarse voices as compared to normals, and have greater instability of fundamental frequency during phonation (Watts, Awan and Marler, 2008). Molecular data of patients with Williams syndrome revealed deletion of the critical region at the chromosome 7q11.23 which includes the elastin gene suggesting that abnormal VF elastin production may account for hoarse voice (Vaux *et al.*, 2003). Future research regarding the molecular

mechanisms of ECM stratification and remodeling is necessary to understand VF tissue biomechanics in order to generate strategies for its restoration.

All genes and signaling pathways known so far that participate in the larynx and VF morphogenesis are summarized in the Table 1.

8. SUMMARY AND FUTURE DIRECTIONS

Despite recent investigations, we are only beginning to understand the cellular and molecular mechanisms of the larynx and VF development. As we have seen, the larynx is composed of many closely related interdependent tissues and cell types - epithelial cells, fibroblasts, and different kinds of ECM, cartilages, muscle tissue, and nerves. In order to move further in our understanding of the mechanisms of the laryngeal embryogenesis we need to know much more about the transcription factors and signaling pathways that mediate the reciprocal interactions between these progenitors. So far, the focus has been on the specification of the VF epithelium and the early stages of the primitive LPh remodeling. Particularly, because these early morphogenetic events occur during periods of organogenesis are closely associated with respiratory, cardiac or craniofacial development. The unique morphogenetic event, which seems to be critical for the VF development, is the establishment of the EL. Why the lateral walls of the primitive LPh temporarily fuse and obliterate the ventral lumen is still a mystery. At the moment we can only guess that EL formation may be required for symmetric development of the VF and supportive mesenchymal structures in the LP to ensure that the VF meet midline and close the glottis after they fully separate. EL formation likely starts with signals from the epithelium, but how these signals are translated into mesenchymal cells is not known. Similarly, involvement of mesenchymal structures in EL formation and recanalization remains to be investigated.

Determination of the contribution of other cell types in laryngeal and VF embryogenesis is also very important. These cell types include: 1) VF fibroblasts in the LP for studying molecular basis of ECM compartmentalization with different biomechanical properties, 2) invading axons for investigation of mechanisms of recurrent nerve innervation and 3) endothelial cells for studying the development of VF vasculature. Besides supplying tissues with nutrients and oxygen, blood brings immune cells into the VF region. Further research is needed to examine the contribution of immune cells to laryngeal and VF morphogenesis.

From the clinical perspective, further investigations into candidate genes and signaling molecules responsible for defective phenotypes described here may allow for improved genetic testing, better prenatal counseling, and potentially better insight into disease pathophysiology and mechanisms of congenital laryngeal disorders. Similarly, candidate genes and signaling pathways active during VF development may allow for improved therapies for postnatal VF repair and regeneration for both the LP and epithelium.

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Figure 1: Morphology of the murine larynx and vocal folds.

(A-F) Schematic illustrations of the murine laryngeal cartilages and muscles. Sagittal section through the murine larynx showing the position of the VF (in yellow) and epiglottis, thyroid cartilage, alar cartilage, arytenoid cartilages and cricoid cartilage (A). Frontal view of the murine larynx showing the attachment of the cricothyroid muscle to the thyroid and cricoid cartilages (B). Sagittal section through the murine larynx showing the attachment of the thyroarytenoid muscle, lateral cricoarytenoid muscle, superior and posterior cricoarytenoid muscles (C). Transverse sections of the murine VF, the cranial (anterior) section (D) and caudal (posterior) section (E). Bracketed region in the panel of E shows the detailed morphology of the right vocal fold (F). (G, H) Hematoxylin-eosin staining showing morphology of the murine vocal folds in coronal (G) and transversal section (H). Bracketed regions in the panel of G and H show detailed morphology of the right vocal fold in coronal and transversal sections, respectively. Scale bar = 500µm. Abbreviations: A, anterior; Ar,

alar cartilage; AC, arytenoid cartilage; CC, cricoid cartilage; CT, cricothyroid muscle; D, dorsal; Ep, epithelium; Epi, epiglottis; LP, lamina propria; P, posterior; PCA, posterior cricoarytenoid muscle; SCA, superior cricoarytenoid muscle; TA, thyroarytenoid muscle; TC, thyroid cartilage; V, ventral; VF, vocal fold.

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Figure 2: Developmental origin of laryngeal and vocal fold structures.

(A) Schematic illustration of major cell populations that contribute to the laryngeal and vocal fold structures including anterior foregut endoderm (in blue), lateral mesoderm (in red) and neural crest cells (in yellow). (B) Schematic illustration showing that anterior foregut endoderm-derived cells expressing *ShhCre^{+/-} TdTom Red* give rise primarily to the laryngeal and VF epithelium (in blue). (C) Schematic illustration showing that cells of the lateral mesoderm-derived cells expressing *Mesp1Cre^{+/-} TdTom Red* give rise to the cricoid cartilage, arytenoid cartilages, intrinsic laryngeal muscles, and the intermediate lamina of the thyroid cartilage in the caudal most region of the thyroid cartilage (in red). (D) Schematic illustration showing that neural crest-derived cells expressing *Wnt1Cre^{+/-} mGFP* contribute to formation of the loose connective tissue in the ventral lamina propria, nervous tissue and thyroid cartilage, except for its medial caudal-most portion (in yellow).

Abbreviations: AC, arytenoid cartilage; AFE, anterior foregut endoderm; CC, cricoid cartilage; D, dorsal; EP, vocal fold epithelium; LM, lateral mesoderm; NCC, neural crest cell; TA, thyroarytenoid muscle; TC, thyroid cartilage; V, ventral.

Figure 3: Schematic illustration of larynx and vocal fold development.

(A, B) Time scales of the laryngeal and VF development showing that the human larynx and VF (A) pass through similar developmental stages as the murine larynx and VF (B). These stages include the specification of the laryngeal field, apposition of the lateral walls, EL formation, EL recanalization and maturation of the VF epithelium and mesenchymal structures during the fetal period (in humans) and postnatally (in mice). Schematic illustrations demonstrate the murine larynx and VF development in more detail. The first image shows the position of the laryngotracheal groove at the level of the fourth pharyngeal pouch at stage E 9.5. At E10.5, the primitive LPh initiates its formation. It becomes bilaterally compressed due to the approximation of the lateral walls to the center of the lumen. At E11.5, the closely juxtaposed lateral walls fuse and create the EL. Differentiation of mesenchymal structures initiates. At E13.5, the tracheoesophageal septum is formed and

the EL begins its recanalization, due to the expansion of the laryngeal cecum and pharyngoglottic duct that exert pressure on the epithelial walls. During EL recanalization, laryngeal cartilages and muscles continue to differentiate. At E18.5, the VF are separated and mesenchymal structures are fully developed to anchor the growing VF. Abbreviations: EL, epithelial lamina; Es, esophagus; LB, lung bud; LC, laryngeal cecum; LG, laryngotracheal groove; LPh, primitive laryngopharynx; PA, pharyngeal arch; PD, pharyngoglottic duct; PP, pharyngeal pouch; RD, respiratory diverticulum; Tr, trachea; VF, vocal fold.

Figure 4: Genes and signaling molecules participating in larynx and vocal fold development.

(A) Genes and signaling molecules that control specification of the laryngeal field and early stages of primitive LPh remodeling. *Shh* is expressed in the LPh endoderm (red color) and likely controls the juxtaposition of *Nkx2-1* (green solid line) and *Sox2* (yellow solid line) expression domains in the LPh endoderm and cell proliferation. In the lamina propria, *Gli* genes along with genes involved in the pharynx segmentation, such as *RA*, *Tbx1*, *β-Cat*, *Hox* genes, *Fgf* signaling, contribute to initial differentiation of the mesenchymal cells and incorporation of migratory cell populations. (B) Genes and signaling molecules that control the fusion of lateral walls and simultaneous differentiation of supportive mesenchymal structures - cartilages (dark grey) and muscles (brown dashed lines). *β-Catenin* (blue color) expressed in the EL likely controls cell proliferation via *Cyclin D1* expression and simultaneous differentiation of basal *p63+* K8+ progenitors. In the lamina propria, laryngeal cartilages initiate differentiation into *Sox9*+ chondrocytes and *MF-20*+ myoblasts. Other genes and signaling molecules acting on differentiation of laryngeal cartilages and muscles

include *R-spondin2*, *Eph/EphrinB2* signaling. (C) Genes and signaling molecules that control EL recanalization and further differentiation of laryngeal cartilages (dark grey), muscle (brown dashed lines) and nerves (purple curvy lines). Proper stratification of VF basal progenitors has been linked to β -*Catenin* (blue color) which controls conversion of p63+ cytokeratin (K) 8+ basal progenitors into functional basal p63+, K8-cells capable of stratification. Abbreviations: AC, arytenoid cartilage; CC, cricoid cartilage; EL, epithelial lamina; LPh, primitive laryngopharynx; TC, thyroid cartilage.

Table 1:

Summary of genes and signaling pathways that are known to control murine larynx and vocal fold development

Stages	Duration	Characteristic events	Genes and signaling molecules
Specification of laryngeal and VF progenitors	E9.5 – E10.5	<ul style="list-style-type: none"> • Formation of the primitive LPh • Apposition of lateral walls • Migration of NCCs and myoblasts into the laryngeal region 	<i>Shh, Gli1, Gli2, Gli3, Nkx2-1, Sox2, FoxA2, Fuz, Myf5, RA, Tbx1, Hox genes, Fgf, Wnt/ beta-Catenin</i>
EL formation	E11.5 – E12.5	<ul style="list-style-type: none"> • Fusion of lateral walls • Differentiation of VF basal epithelial progenitors • Initiation of differentiation of cartilages and muscles 	<i>Wnt/beta-Catenin, Sox2, Nkx2-1, FoxA2, p63, K8, Cyclin D1, R-spondin 2, Bmp, Sox9, Fgf18, Col2A1, Ephrin B2/Eph, MF-20</i>
EL recanalization VF separation	E13.5 – E18.5	<ul style="list-style-type: none"> • Formation of the tracheoesophageal septum • Formation of the laryngeal cecum and pharyngoglottic duct • Initiation of stratification of VF epithelium • EL recanalization • Differentiation of laryngeal cartilages, intrinsic laryngeal muscles and neurons 	<i>Foxa2, Wnt/beta-Catenin, Sox2, Sox9, MF-20, Col2a1, p63, K8, K5, K14</i>
Maturation	Birth to postnatal stages	<ul style="list-style-type: none"> • Stratification of the VF epithelium • Differentiation and stratification of the lamina propria 	<i>p63, K5, K14, Sox2, Sox9, MF-20</i>