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# Ontogeny of the mouse vocal fold epithelium

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ABSTRACT

This investigation provides the first systematic determination of the cellular and molecular progression of vocal fold (VF) epithelium development in a murine model. We define five principal developmental events that constitute the progression from VF initiation in the embryonic anterior foregut tube to fully differentiated and functional adult tissue. These developmental events include (1) the initiation of the larynx and vocal folds with apposition of the lateral walls of the primitive laryngopharynx (embryonic (E) day 10.5); (2) the establishment of the epithelial lamina with fusion of the lateral walls of the primitive laryngopharynx (E11.5); (3) the epithelial lamina recanalization and separation of VFs (E13.5–18.5); (4) the stratification of the vocal folds (E13.5–18.5); and (5) the maturation of vocal fold epithelium (postnatal stages). The illustration of these morphogenetic events is substantiated by dynamic changes in cell proliferation and apoptosis, as well as the expression pattern of key transcription factors, FOXA2, SOX2 and NKX2-1 that specify and pattern the foregut endoderm. Furthermore, we documented the gradual conversion of VF epithelial cells from simple precursors expressing cytokeratins 8 and 18 in the embryo into mature stratified epithelial cells also expressing cytokeratins 5 and 14 in the adult. Interestingly, in the adult, cytokeratins 5 and 14 appear to be expressed in all cell layers in the VF, in contrast to their preferential localization to the basal cell layer in surrounding epithelium. To begin investigating the role of signaling molecules in vocal fold development, we characterized the expression pattern of SHH pathway genes, and how loss of *Shh* affects vocal fold development in the mutant. This study defines the cellular and molecular context and serves as the necessary foundation for future functional investigations of VF formation.



## Introduction

Guided derivation of stem cells into various cell lineages represents a promising and valuable approach to generate functional units for tissue repair (Jiang et al., 2007; Wong et al., 2012; D'Amour et al., 2005). However, to successfully replicate the process of cell derivation in vitro, it is necessary to precisely define the dynamic cellular and molecular mechanisms underlying normal development in vivo in the mammalian embryo. Recently published derivation of vocal fold (VF) epithelial cells from induced pluripotent stem cells (Leydon et al., 2013) has reminded us that little is known about the development and differentiation of the VF epithelial cells as compared to those of other foregut epithelial surfaces including the esophagus (Yu et al., 2005; Jacobs et al., 2012), stomach (Kim et al., 2005, 2011), trachea and lung (Domyan and Sun, 2011; Morrisey and Hogan, 2010). Although we have shown that induced pluripotent stem cell-derived epithelial cells do stratify, they produce markers that are universal and are not tissue specific, thereby limiting their therapeutic applications for VF wound healing and regeneration (Leydon et al., 2013).

Existing studies describing the development of the larynx and VF in humans or other mammals such as mice consist of either morphological investigations based on conventional H&E staining (Hopp, 1955; Walander, 1955; Zaw-Tun and Burdi, 1985; Sanudo and Domenech-Mateu, 1990), or clinical investigations comparing normal versus pathological morphologies (Benjamin and Inglis, 1989; Kakodkar et al., 2012). These studies documented the basic steps of laryngeal cavity and VF development (Henick, 1993). The laryngeal cavity is tubular in shape and lined with mucosa. The superior aspect of the cavity (laryngeal vestibule) opens into the pharynx. The inferior aspect of the cavity is continuous with the lumen of the trachea. VFs are located in the inferior larynx at the top of the trachea. They open during inhalation and come together to close the glottis during swallowing and phonation. The luminal surface of the VF is covered by a protective layer of stratified squamous epithelium. On the surfaces of the epithelial cells are microridges and microvilli that help to spread and maintain a mucous coat (Hirano et al., 1983). Beneath the VF epithelium are lamina propria and vocalis muscle (thyroarytenoid

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muscle) that forms the body of VFs (Hirano and Bless, 1993). Stratified squamous epithelium of the VF transitions to a ciliated pseudostratified columnar epithelium at the supraglottis (rostral to VF), and infraglottis (caudal to VF). Recent studies comparing mature mouse and human VFs have documented similar laryngeal framework, epithelial and muscular organization, VF aging characteristics, as well as similar distribution of elastic and collagen fibers in the lamina propria (Watts et al., 2007, 2011). Proper patterning of these fibers is essential for effective vibration of VF during voice production in humans or during ultrasonic acoustic signal production in rodents (Johnson et al., 2010).

The laryngeal cavity emerges as primitive laryngopharynx and was a part of the foregut endoderm, which expresses *Foxa2*. This transcription factor plays an important role in initiating liver specification (Li et al., 2009) and in the differentiation of pancreatic α-cells (Lee et al., 2005). It also participates in the development of the normal bronchus and lung epithelium (Basseres et al., 2012). It is known that the epithelial lining of the trachea and lung arises from the ventral portion of the foregut endoderm that expresses *Nkx2-1* (Que et al., 2007, 2009; Harris-Johnson et al., 2009; Domyan and Sun, 2011; Ferri et al., 2013). In contrast, the epithelial lining of the esophagus arises from the dorsal portion of the foregut endoderm that expresses *Sox2* (Miller et al., 2012; Woo et al., 2011; Jacobs et al., 2012). In addition to being markers, *Nkx2-1* and *Sox2* are each essential for establishing the initial dorsal–ventral patterning of the foregut tube and proper development of the trachea versus esophagus (Que et al., 2007). At later stages in more distal tissue *Nkx2-1* is required for early lung branching morphogenesis, while *Sox2* is required for differentiation of airway and esophagus cell types (Kelly et al., 1996; Que et al., 2007, 2009). SOX2 interacts with another master transcription factor p63, which is best known for its requirement in the maintenance of the basal cell progenitor state (Daniely et al., 2004). Additional genes such as *Wnt2*, *Wnt2b* and *Barx1* are expressed in the mesenchyme underlying the foregut epithelium and also play a role in trachea/esophagus development. While little is known in terms of nascent VF gene expression, based on its proximity to the trachea and esophagus, we hypothesize that some of the previously mentioned transcription factor genes may also be expressed in the VFs and may impact VF formation and VF epithelium differentiation.

In this study, we built upon existing knowledge and carried out a systematic determination of the cellular and molecular progression of VF epithelium development in mice. We examined the patterns of transcription factors, SOX2, NKX2-1 and FOXA2, and the patterns of cell proliferation and apoptosis during key morphogenetic events including apposition of the lateral walls of the primitive laryngopharynx, the formation of the epithelial lamina, and its recanalization. To trace the differentiation of VF epithelial cells, we investigated the temporo-spatial localization of simple epithelial markers, keratins (K) 8 and K18, versus stratified epithelial markers, K5 and K14, during the differentiation of VF epithelial cells. Based on our findings, we define, for the first time, five landmark events of VF development. We hope that this work provides the foundation for future elucidation of the mechanisms that drive the formation of a functional VF.

## Materials and methods

### *Mouse matings and tissue collection*

Wild-type Swiss Webster males and females were mated, and noon of the day when vaginal plugs were found was designated as Embryonic day (E) 0.5. Pregnant females were sacrificed at E10.5, E11.5, E13.5, E15.5, E16.5 and postnatal (P) stages, P0 and adult (6–8 weeks), following regulations of protocols approved by the University of Wisconsin Animal Care and Use Committee. Mouse larynges were dissected and immediately fixed in 4% paraformaldehyde in phospho-buffered saline at 4 °C/overnight, dehydrated in a gradient series of ethanol, treated with xylene and embedded in paraffin. Paraffin blocks were cut into serial sections (5 μm), dewaxed and rehydrated, heated to boiling in 10 mM citrate buffer pH=6 for antigen retrieval and then stained using standard IHC or IF protocols. *Shh* homozygous null mutants were generated by mating $Shh^{cre/+}$ heterozygotes (Harfe et al., 2004).

### *Immunohistochemistry staining*

Primary antibodies used were mouse anti-NKX2-1 antibody diluted at 1:100 (LS Bio; Seattle, WA, USA); rabbit anti-SOX2 antibody diluted at 1:500 (Novus Biologicals; Littleton, CO, USA); rabbit anti-K5 antibody diluted at 1:250 (Abcam; Cambridge, UK); rabbit anti-K8 antibody diluted at 1:250 (LS Bio; Seattle, WA, USA); rabbit anti-K18 antibody diluted at 1:250 (LS Bio; Seattle, WA, USA); rabbit anti-K14 antibody diluted at 1:250 (ProteinTech; Chicago, IL, USA); rabbit anti-FOXA2 antibody diluted at 1:2000 (Abcam; Cambridge, UK). Sections were incubated with the primary antibodies at 4 °C overnight. Secondary antibodies used were anti-mouse and anti-rabbit Ig ImmPress reagent kit peroxidase (Vector Laboratories; Peterborough, UK). Antigen detection was performed using a DAB kit (Vector Laboratories; Peterborough, UK). Slides were counterstained using hematoxylin.

### *Immunofluorescent staining*

Primary antibodies used were mouse anti-NKX2-1 antibody diluted at 1:100 (LS Bio; Seattle, WA, USA), rabbit anti-SOX2 antibody diluted at 1:500 (Novus Biologicals; Littleton, CO, USA), rabbit anti-K8 antibody (LS Bio; Seattle, WA, USA) diluted at 1:250; mouse anti-p63 antibody (Santa Cruz Biotechnology; Dallas, Texas, USA) diluted at 1:200. Sections were incubated at 4 °C overnight. Secondary antibodies used were Cy3-conjugated anti-mouse antibodies (Jackson ImmunoResearch; West Grove, PA, USA) diluted at 1:200; FITC-conjugated anti-rabbit antibodies (Jackson ImmunoResearch; West Grove, PA, USA), diluted at 1:100 and applied 1 h at room temperature (RT). Slides were mounted using Vectashield (Vector Laboratories; Peterborough, UK). In case of whole mount IF staining, dissected respiratory structures were treated with rabbit anti-K8 antibody (LS Bio; Seattle, WA, USA) diluted at 1:250 overnight at RT and by secondary antibody Cy3-conjugated anti-rabbit antibody (Jackson ImmunoResearch; West Grove, PA, USA), diluted at 1:200, applied 4 °C/overnight. Slides were mounted using Vectashield (Vector Laboratories; Peterborough, UK).

### *TUNEL assay*

TdT-mediated dUTP-biotin nick end labeling (TUNEL, Chemicon-Millipore; Billerica, USA) was employed to detect apoptosis. After rehydration, sectioned samples were treated with proteinase K at 20 μg/ml at room temperature for 15 min (Chemicon-Millipore; Billerica, MA, USA). After equilibration buffer, the reaction mixture was prepared as per manufacturer instructions and applied at 37 °C for 50 min. After anti-digoxigenin-peroxidase reaction 30 min/RT, positive cells were visualized by chromogen substrate diaminobenzidine (DAB kit, Vector Laboratories, Peterborough, UK) and slides were counterstained with hematoxylin.

### *Cell proliferation assay*

Pregnant females received an intraperitoneal injection of 100 μg EdU (Sigma Aldrich; St. Louis, MO, USA) per gram