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Sox9 is associated with two distinct patterning events during snake lung morphogenesis

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ABSTRACT

The evolutionary forces that allowed species adaptation to different terrestrial environments and led to great diversity in body shape and size required acquisition of innovative strategies of pattern formation during organogenesis. An extreme example is the formation of highly elongated viscera in snakes. What developmental patterning strategies allowed to overcome the space constraints of the snake's body to meet physiological demands? Here we show that the corn snake uses a Sox2-Sox9 developmental tool kit common to other species to generate and shape the lung in two phases. Initially Sox9 was found at low levels at the tip of the primary lung bud during outgrowth and elongation of the bronchial bud, without driving branching programs characteristic of mammalian lungs. Later, Sox9 induction is recapitulated in the formation of an extensive network of radial septae emerging along the elongated bronchial bud that generates the respiratory region. We propose that altogether these represent key patterning events for formation of both the respiratory faveolar and non-respiratory posterior compartments of the snake's lung.

1. Introduction

Adaptation for fitness and survival has been a driving force in the generation of the astonishing diversity in body size and shape in the animal kingdom. A prominent example is the appearance of a narrow, elongated body in multiple lineages of reptiles and amphibians (Caldwell, 2003; Cohn and Tickle, 1999; Woltering et al., 2009). This morphological oddity has reached to extremes in the serpentine body of the snake and their ancestors. This conferred adaptive advantages that underpinned their highly successful adaptive radiation and allowed these species to hunt in underground tunnels, swim in water or climb trees (Wallach, 1998).

Morphological adaptations in the body shape, such as those that evolved in snakes are established through changes in developmental mechanisms. The elongated body and high vertebral count is already generated early in the embryo by the rapid production of a strikingly large number of somites (Gomez et al., 2008). The evolution of a narrow, elongated body is accompanied by corresponding changes in the size and morphology of internal organs (Atwood, 1916; Bellairs and

Underwood, 1951; Hopkinson and Pancoast, 1837), which required designing appropriate strategies of pattern formation during organogenesis.

The lung represents a key evolutionary advancement, enabling vertebrate land colonization with efficient exploitation of aerial oxygen. The primitive vertebrate lung is exemplified in the Dipnoi (lungfish) as a simple sac-like structure with respiratory epithelium lining its lumen (Hsia et al., 2013). As the lung evolved, septation of these sac-like structures optimized aerial respiration efficiency by increasing the surface area for gas exchange to meet metabolic demands (Maina, 2002). An additional evolutionary innovation exemplified in mammals and archosaurs (crocodilians and birds) was the emergence of a branched airway component. This provided a tree-like system of air-conducting tubules ending in respiratory saccules, to clean and distribute the air homogeneously to all parts of the lung (Duncker, 1978; Weibel et al., 2005). In principle, these two basic designs could be achieved during development by: a) activating a gene network for specification of a respiratory compartment distal to the trachea and generate a saccular lung, as in the *Xenopus* (Chang et al., 2013; Rankin et al., 2011) or, b) by

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activating a network for specification of a proximal-distal axis to give rise to two distinct compartments, a proximal branched airway system ending in multiple distal respiratory units, as in the mammalian lung.

While serving as a developmental framework to explain the evolutionary changes in the respiratory system for many vertebrate taxa, the proximal-distal two-compartment regulatory gene network has been unable to explain how proper pattern formation is achieved in species with unusual body shape, like snakes. Their highly elongated, and relatively narrow, body cavity imposes physiological constraints on mechanical respiration that requires both conducting and gas-exchange

compartments to be accommodated along a main body axis that in some species, such as *Python reticulatus* Schneider 1801, can stretch over 10 m (Benedict et al., 2022). Moreover, the positioning of a gas exchange compartment distal to a system of conducting airways is incompatible with what is seen in most snakes, in which the distal (posterior, caudal) end of the lung is devoid of functional respiratory structures (Wallach, 1998). These features open relevant questions about the ontogeny of the lung in snakes. What developmental strategies underlie these morphological phenotypes? How did the structural and cellular changes in morphology evolve from the primitive sac-like to the complex

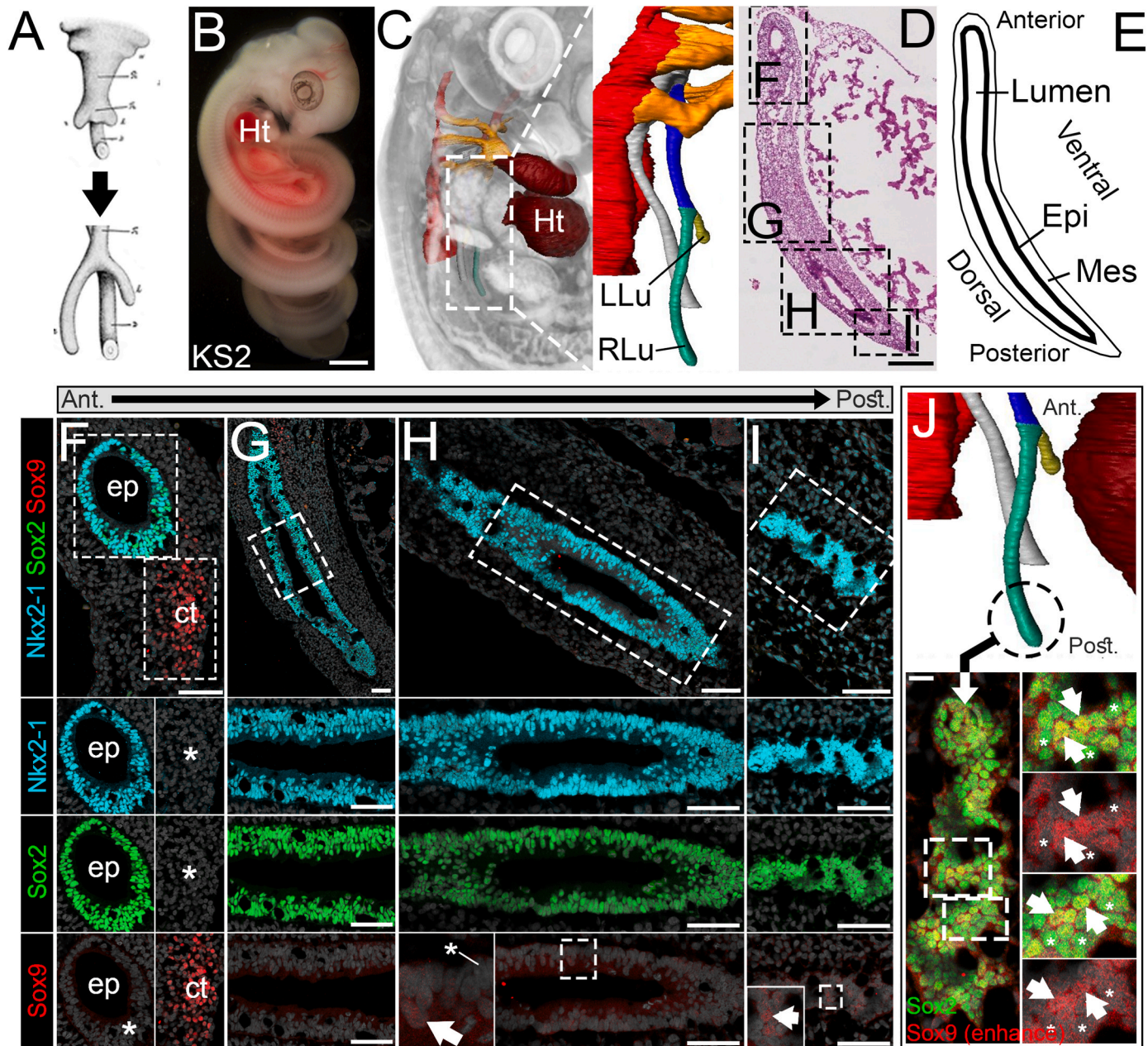


Fig. 1. The early corn snake lung is a Sox2⁺ epithelial tube with a mosaic Sox9⁺Sox2⁺ at the distal tip.

(A) Summary of earliest stages of corn snake lung development as depicted by Schmalhausen (1905). Wholemount (B) and MicroCT-scanned (C) KS2 embryo with the anterior thoracic region 3D-reconstructed in Amira, including heart (dark red), pharyngeal arches (orange), dorsal aortae (bright red), tracheo-esophageal complex (grey/blue) and left (yellow) and right lung buds (green). (D) H&E-stained section of right lung, and accompanying schematic (E). (F–I) Sox2/Sox9/Nkx2-1 IF stains of regions highlighted in (D). Arrow in (H) highlights rare low Sox9-expressing cells (compare with signals in cartilage primordia, ct, in F). (J) Sox2/Sox9 IF image of the distal tip of the lung with MicroCT reconstruction depicting the approximate location of stained region. Merged and single-channel images with enlarged panels depicting mosaic Sox9 signals (arrows) and non-expressing cells (asterisks). Amira visualizations are from van Soldt et al. (2015). Asterisk depicts absence of signals in areas indicated. Ant, anterior; Post, posterior; ep and Epi, lung epithelium; Mes, lung mesenchyme; LLu, left lung; RLu, right lung; Ht, heart. DAPI is grey in all IF panels. Scale bar denotes 2 mm (B); 50 μ m (D–I); or 10 μ m (J).

mammalian and archosaur lung morphology?

In addition, valuable insights can be provided by comparative analysis of lung development in sister groups to the archosaurs and mammalian species, both of which feature a branched airway tree. However, in the mammalian lineage no extant representatives of these species are known (Kemp, 2011). In this context the reptiles constitute an attractive alternative system to explore as their sac-like lung structure bear similarities to the ancestral amphibian lung, while also showing airway features of more derived lung morphologies (archosaurs).

Here we show that, during lung morphogenesis of the corn snake (*Pantherophis guttata*) the epithelial compartment expresses key conserved canonical distal (Sox9) and proximal (Sox2) cell fate markers of the developing mammalian lung (Danopoulos et al., 2017; Nikolić et al., 2017; Yang et al., 2018). However, the developing snake uses these signals reiteratively in two distinct patterning events. First in an event in which the primary lung bud elongates widely, consistent with the high elongation of the main body, expressing Sox9 weakly at the bud tip but never forming the distinct distal respiratory compartment typical of mammalian lungs. Then later, in a second event, Sox9 re-emerges at the tips of a vast network of newly-formed radial buds that arise from the elongated main bronchus to generate the gas-exchange (faveolar) lung.

Thus, the corn snake uses a developmental tool kit common to other species (*Xenopus*, mouse, chicken) for patterning of the developing lung. However, the components are deployed differently in the developing snake's lung to meet the morphological and functional demands of the disproportionate elongated body of these species.

2. Results

2.1. Lung bud outgrowth is associated with the induction of a Sox2-Sox9 double-labeled population of tip epithelial cells

Little is known about the patterning events that give rise to the respiratory system in the snakes. Currently there is no report on lung organogenesis to address this gap of knowledge. Thus, here we conducted an analysis of the lung development in the corn snake embryo (*Pantherophis guttata* Linnaeus, 1766) to identify key patterning events associated with the formation of these distinct compartments. Morphological descriptions of the emergence of the lung and tracheal primordia in the embryonic snake have been reported as early as the beginning of 20th century. These identify primordial respiratory structures arising from the ventral-lateral aspect of the foregut with subsequent caudal elongation of the right lung bud and truncation of the left lung, (Fig. 1A) (Harrison and Denning, 1929; Schmalhausen, 1905; van Soldt et al., 2015). However, no further information is available about subsequent patterning events that lead to formation of distinct compartments in this lung. Thus, we used MicroCT imaging, histological, immunofluorescence (IF) and confocal microscopy to systematically examine the morphological changes and molecular markers that characterize the key events in the development of the corn snake lung.

At Khannoon Stage 2 (KS2 (Khannoon and Evans, 2014)) the tracheal primordium and primary lung buds have already emerged but they still occupy a very small portion of the snake embryo relative to the whole body, which then has considerably elongated (Fig. 1B). MicroCT scanning and 3D reconstruction revealed distinct left (truncated) and right (elongated) lung buds and clear separation of the trachea and esophagus (Fig. 1C). Hematoxylin & Eosin (H&E) staining shows the trachea and lung bud lined by a pseudostratified columnar epithelium throughout the anterior-posterior axis (Fig. 1D). IF shows strong labelling for Nkx2-1 confirming the respiratory identity of the epithelium (Fig. 1F–I).

Interestingly, at this stage the bronchial bud consisted essentially of a population of Sox2-expressing epithelial cells with only a small tip domain populated by Sox2/Sox9 double-positive cells (Fig. 1I–J, Figs. S2A–C). Unlike in the mouse lung bud tips, there are no Sox9+, Sox2-cells forming a distinct compartment. Sox9 expression was weak and mosaic-like, intermingled with Sox2 in a relatively small number of

cells at the tip of the bronchial bud compared to the predominant Sox2 domain (Fig. 1F–J). The weak Sox9 epithelial signals contrasted with the stronger expression in mesenchymal condensations that will later form the cartilage rings in the trachea (Park et al., 2010; Turcatel et al., 2013) (Fig. S1B, Figs. S2A–B). Disruption of epithelial expression of Sox9 in the developing mouse lung interferes with the balance of progenitor cell self-renewal and differentiation and inhibits branching morphogenesis (Chang et al., 2013; Rockich et al., 2013). Whether the low Sox9 levels in these double-labeled tip cells is part of a mechanism that controls expansion and differentiation during elongation of this bronchial bud, is unclear.

2.2. Dorsal-ventral patterning and luminal expansion establish a regionally distinct compartment in the developing snake lung

By stage KS3 the developing lung has extended caudally beyond the axial level of the heart (compare Fig. 2A–C with Fig. 1C–D). Then, a series of morphogenetic events takes place in the anterior region of the bronchial bud resulting in expansion of the lumen and the appearance of marked dorsal-ventral differences in the cytoarchitecture of the epithelium.

Ventrally, the epithelium remained tall columnar pseudostratified epithelium surrounded by a loosely-organized mesenchyme periodically containing scattered areas of mesenchymal condensations characteristic of cartilage primordia. By contrast, on the dorsal side, the bronchial epithelium changed from pseudostratified to simple cuboidal and associated with luminal bulging in its dorsal-lateral aspect. Notably, while Sox2 continued to be expressed throughout the bronchial epithelium, its levels became much reduced dorsally compared to the ventral side (Fig. 2D). In addition, on the dorsal aspect of the developing bronchus, the lumen appeared more bulged, and numerous small protrusions were observed at the dorsal side of the bronchial wall suggesting that the cuboidal epithelium was undergoing a buckling process (Fig. 2C–D). At this stage the protrusions were not associated with obvious changes in organization of the underlying mesenchyme locally. None of these features were observed in the trachea or the caudal end of the elongating bronchial bud, which remained pseudostratified and homogeneously labeled with Sox2 as in the previous KS2 stage (Fig. 2E–F).

Thus, the events in KS3 established morphological differences in the developing snake lung that set the stage for the appearance of a regionally distinct functional compartment. These morphogenetic events occurred as the bronchial bud continued to elongate in line with the cranial-caudal growth of the body.

2.3. Epithelial buckling generates an orthogonal network of ridges with distinct Sox2 and Sox9 compartments

We reasoned that the epithelial buckling initiated at KS3 could represent the initiation of a process of ridges/septae formation to ultimately generate a larger surface area to be later patterned into the gas exchange region of the snake lung.

Analysis of KS4 embryos showed clear ridges circumferentially oriented along the dorsal epithelium of the bronchial bud (Fig. 3D). Notably, the appearance of these ridges was accompanied by marked differences in Sox2-Sox9 expression along the luminal-basal axis of the epithelium. Sox2 became strongly expressed in the apical (luminal) epithelium decreasing toward the basal aspect of these ridges. Conversely, Sox9 signals were found in the epithelial cells at the base of these ridges, but not in the luminal epithelium (Fig. 3G). During subsequent stages (KS5, KS6) the space between these ridges widened and then deepened, from a combination of events, presumably including regional differences in cell proliferation (Fig. 3D–F). Preliminary analysis of 5-Ethynyl-2'-deoxyuridine (EdU) labeling at KS6 suggested that although only 20% of the epithelial cells were proliferating, the majority were Sox9+ rather than Sox2+ (Figs. S2D–E).

By the KS6 stage regional differences in Sox9 and Sox2 expression

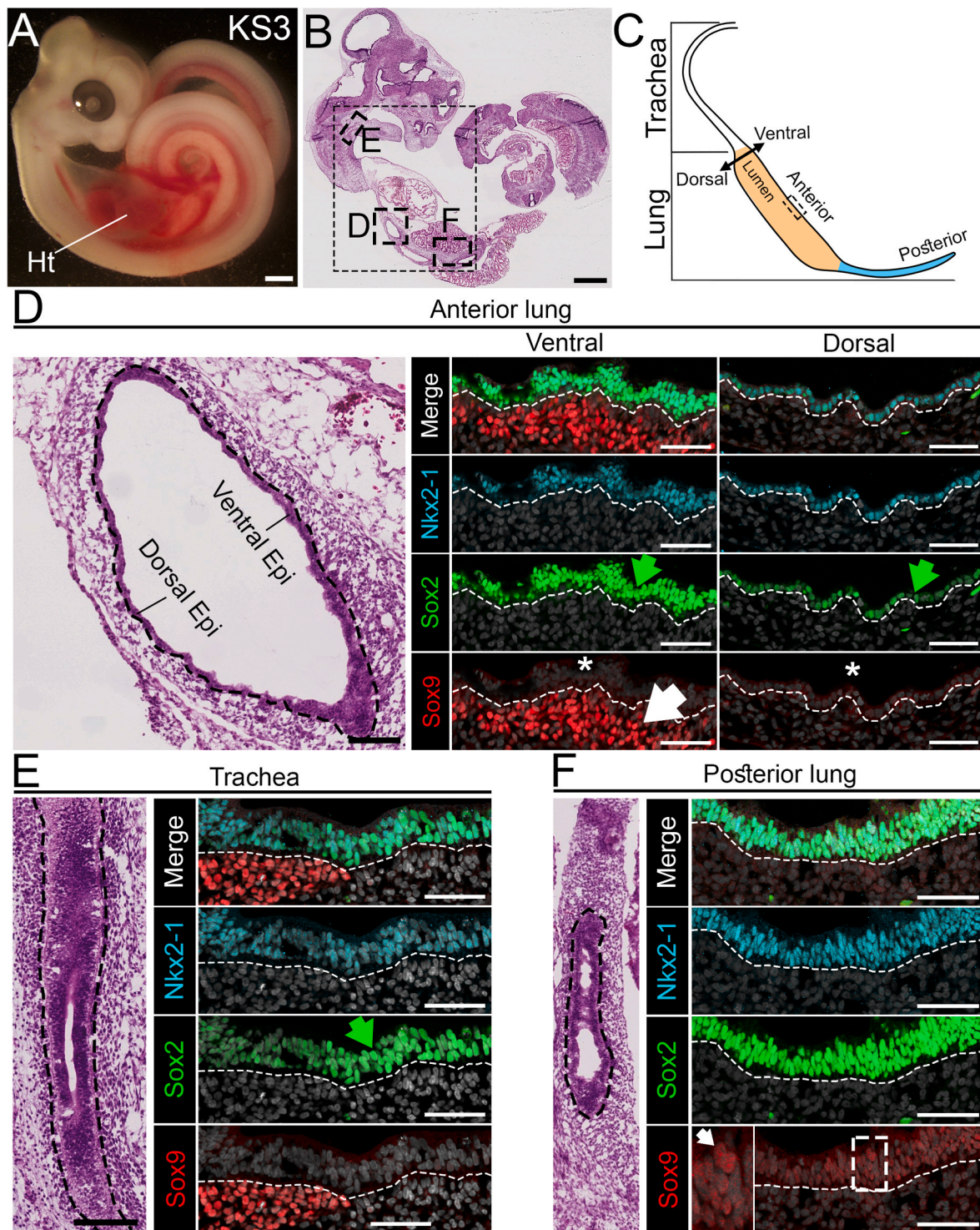


Fig. 2. Regional dilation followed by epithelial buckling establish morphologically distinct compartments at the dorsolateral aspects of the anterior lung. Wholemount (A) and sectioned (B) KS3 stage corn snake embryo reveal regionalized cystic expansion of the lung (E–F), schematized in (C). The trachea remains narrow but opens up into a cystically expanded anterior domain. The posterior domain remains narrow. (D–F) H&E and Sox2/Sox9/Nkx2-1 IF stains in the anterior lung (D), trachea (E) and posterior lung (F). Arrows and asterisks denote expression or absence of relevant marker. DAPI is grey in all IF panels. Scale bars denote 1 mm (A, B) or 50 μ m (D–F). Ht, heart.

along the luminal-basal axis of the ridges were accentuated with the appearance of a strong Sox9⁺ domain at the base of the ridges (Fig. 3G–I, white arrows in Sox9 single channel panels). This differed considerably from the weak Sox9 (Sox2-double labeled) previously observed in the tip epithelial cells during primary bronchial bud outgrowth (Fig. 1J). The ridge elongation and the appearance of a

dedicated Sox9⁺ compartment suggested that septal structures were emerging in the dorsal-lateral epithelium to initiate primordial faveoli formation (Fig. 3D–F).

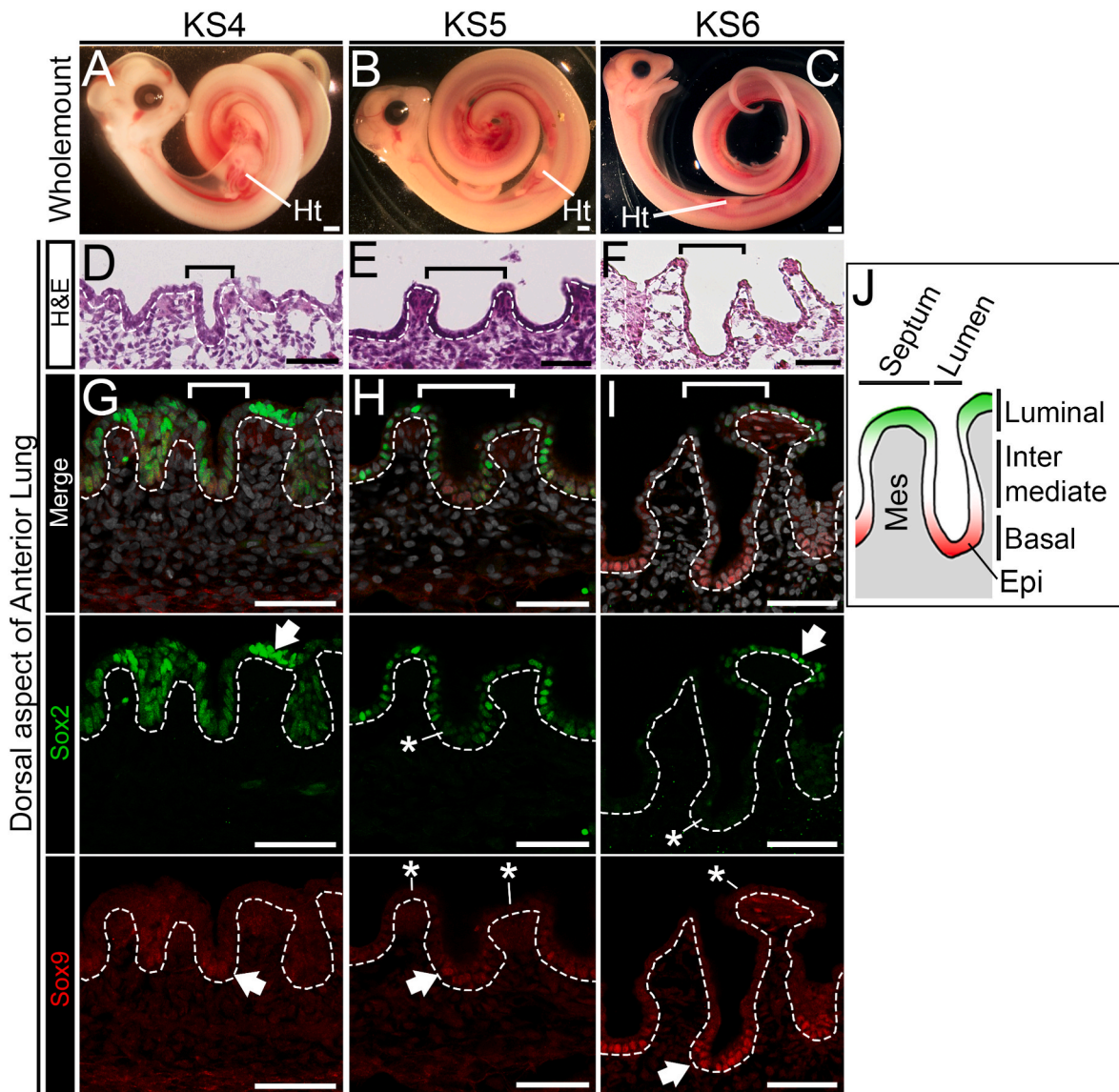


Fig. 3. Epithelial buckling establishes septae that are patterned along their luminal-basal axis into Sox9+ basal and Sox2+ luminal compartments. (A–C) Wholemount images of KS4 (A), KS5 (B) and KS6-stage (C) embryos. (D–F) H&E closeups of representative areas of the dorsal epithelium of the anterior lung at KS4 (D), KS5 (E) and KS6 (F). (G–I) Sox2/Sox9 IF stains of equivalent regions to (D–F). Sox2 is initially expressed throughout the epithelium, with Sox9 upregulated basally (G). By KS6, Sox2 and Sox9 expression patterns are restricted to distinct compartments (I). Arrows emphasize localized expression of Sox2 or Sox9. Asterisks denote absence of either Sox2 or Sox9 expression. Dashed lines in high-mag insets denote basal aspect of the epithelial lining. Scale bars denote 1 mm (A–C) or 50 μm (D–I). Ht, heart.

2.4. Septal morphogenesis occurs with the stepwise establishment of an extensive network of smooth muscle bundles

The systematic formation of ridges that we observed at KS4–6 was reminiscent of that described in the generation of the villus in the chick intestinal epithelium (Shyer et al., 2013). In the developing intestine, epithelial buckling results from sheaths of smooth muscle that envelop and confine the proliferating intestinal epithelium, restricting local epithelial expansion (Shyer et al., 2013). We examined how the distribution of smooth muscle in the developing snake lung changed during ridge formation in the snake lung.

Immunofluorescence staining for alpha-smooth muscle actin (α SMA, Acta2) identified a smooth muscle layer surrounding the esophageal epithelium as early as the KS2 stage, but no signals were present in the mesenchyme around the Sox2+ bronchial bud (Fig. 4A). By KS3, α SMA+ cell clusters were found in loosely-organized bundles underneath the bronchial epithelium (Fig. 4B, asterisk). The epithelial ridges described

above were still too indistinct to be detected by MicroCT, but became readily apparent at KS4, at which point they formed rings that encircled the bronchial bud (Fig. 4B–C, bottom panels). In 2D sections, the α SMA+ clusters, now seen as large bundles, were localized in close apposition to the apical (luminal) region of these ridges (Fig. 4C, arrow). During intestinal villi formation, a circumferential smooth muscle layer serves as a stiff mechanical barrier to drive luminal epithelial folding and ridge formation (Shyer et al., 2013). At KS4, however, we found α SMA+ clusters forming a circumferential arrangement of smooth muscle rings rather than a continuous sheet, suggesting that the mechanism by which smooth muscle drives elongation of these ridges to form septa in the snake differs from that of chick intestinal villus formation.

At KS5–KS6 MicroCT revealed the emergence of a complex septal network with smaller septa running longitudinally in-between the existing ones, consistent with the formation of second and third generation of septa (Fig. 4D, E, Figs. S3A–B) (Maina, 2002; Wallach, 1998). The complexity of this network was not apparent in 2D sections, but was

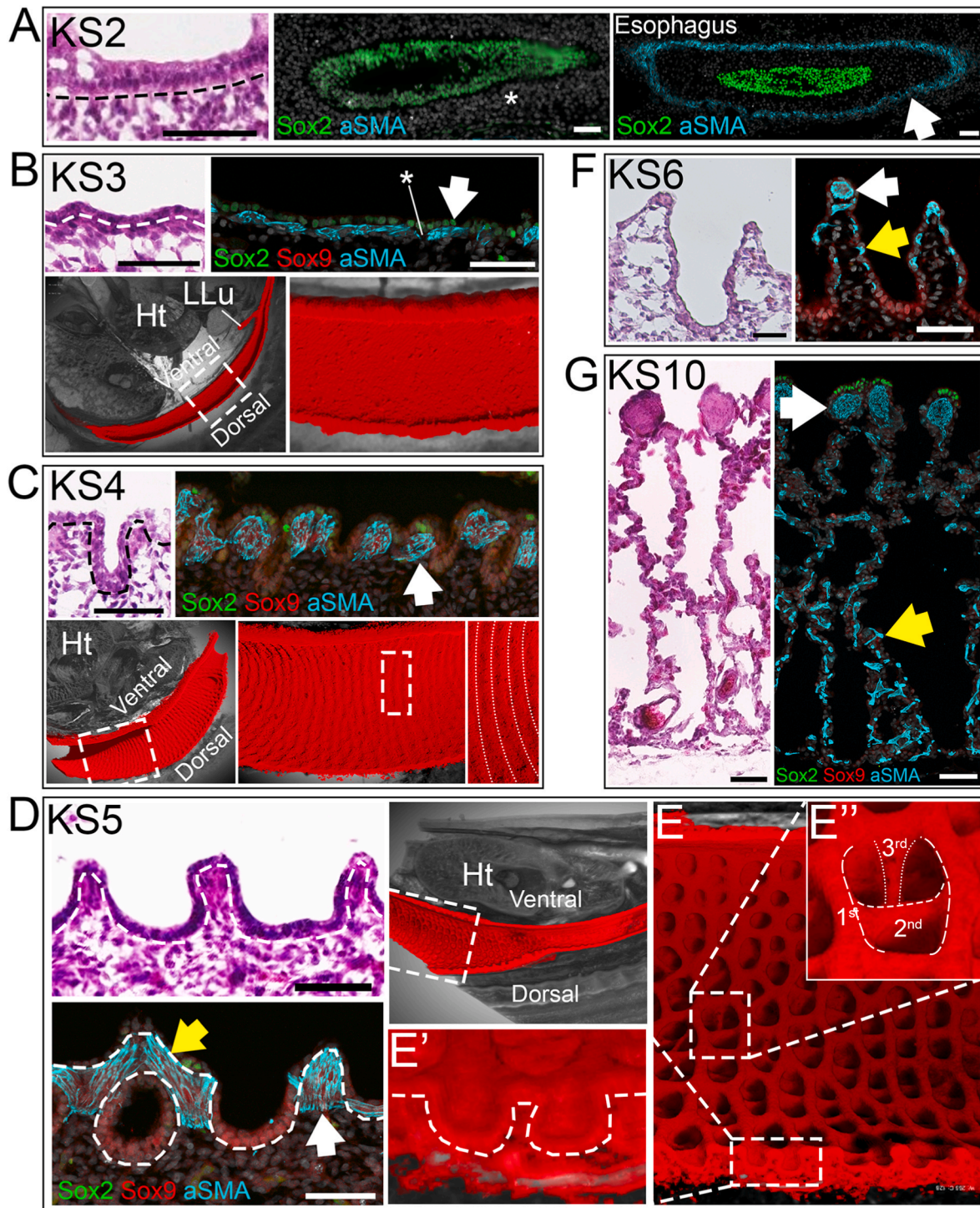


Fig. 4. aSMA⁺ smooth muscle clusters localize to the luminal region of the septa. (A–G) Matching H&E and aSMA/Sox2/Sox9 IF stains depicting progressive stages of faveologensis from KS2 (A) to KS10 (G) during corn snake lung development. Stains of select developmental stages are complemented by high-resolution MicroCT scans and 3D reconstructions to show the structure of the lungs (B–D). A Sox2⁺ pseudostratified epithelium, devoid of αSMA at KS2 (A), develops distinct αSMA⁺ clusters by KS3 (B), after which the epithelium folds, with αSMA⁺ clusters localizing to the luminal domains of the folds by KS4 (C). Folds appear as distinct ridges in 3D MicroCT reconstructions, which form networks by KS5 (D) with at least three septal generations (E, E''). Then, septa increase in length and αSMA⁺ loci appear in intermediate mesenchymal domains of the septa (F, G). White and yellow arrows denote luminal αSMA clusters and intermediate αSMA loci, respectively. Asterisks denote absence of αSMA⁺ clusters. Dashed lines in H&E stains and IF stain in (D) denote basal aspect of epithelial lining. Dashed and dotted lines in (E'') denote three septal generations, indicated by '1st', '2nd' and '3rd'. DAPI is grey in all IF panels. Scale bars denote 50 μm. Ht, heart; LLu, left lung.

revealed by slicing through the 3D reconstructed lung (compare Fig. 4D with E'). αSMA could be seen arranged as thick bundles underneath the tall septal epithelium or as bands or thin layers underlying the epithelium of the shallower septae. We interpreted these different

arrangements to be morphologically characteristic of the first, second or third-generation septa that will form the snake's faveolar lung (Fig. 4D, Fig. S3C),

Further elongation of the septa toward the lumen was accompanied

by the appearance of small clusters of α SMA+ cells throughout the septal wall, becoming more numerous by KS10 (Fig. 4F–G). By then, these α SMA clusters also appeared as bands that spanned the width of the septal wall (Fig. 4G, yellow arrow). Given the interconnected nature of the septa at KS5, we envision that the KS10 smooth muscle network is particularly intricate, spanning the septa in all three dimensions.

Thus, a radial network of ridges and septa emerges orthogonal to the main axis of the developing snake lung. We found this to be associated with two intimately related patterning events, the induction of a Sox9 epithelial compartment and the differentiation of smooth muscle defining different septal generations.

2.5. Sox2 and Sox9 maintain their expression in the differentiated airway and respiratory cell components of the mature faveolar lung

Around hatching (P0), a network of septa occupied much of the anterior-dorsal region of the snake lung configuring the structure of the faveolar lung (Fig. 5A–D, blue annotations). These septa continued to elongate from late gestation to the neonatal period and to adulthood (Fig. 5E–G) showing that lung development continues post-hatching, as in mouse and human (Burri, 1984; Zeltner et al., 1987; Zeltner and Burri, 1987). Thick α SMA bundles appear in adulthood at the apical-most part of the septae (Fig. 5H–K, arrows). At the ventral side, the bronchial tube becomes more evident as the cartilage forms well-defined rings

(Fig. 5A–D, yellow annotations).

In the developing mouse lung, the Sox9 and Sox2 epithelial compartments undergo a program of differentiation that will give rise to the various cell types in the alveoli and airways, respectively (Rawlins et al., 2009; Treutlein et al., 2014). We used a panel of established lung epithelial markers in IF assays to identify the differentiated cellular phenotypes populating the snake lung. IF analysis of Sox2 and Sox9 in sections of the faveolar lung showed persistent and distinct expression in the luminal epithelium, reminiscent of the distribution in the earlier KS4–6 stages, including co-labeling with Krt8 (Fig. 6A–C). Mucociliary differentiation typical of the amniote airways, as confirmed by acetylated alpha-tubulin (aTub) labeled multiciliated cells and Alcian blue-stained mucous cells (Fig. 6D–F), was identified.

Previous reports describe the faveolar septum as the gas-exchange domain of the snake, consisting of a thin respiratory epithelium overlying dense capillary networks (Lucht and Kardong, 1981; Maina, 1989; Maina et al., 1999; Okada et al., 1962). While we did not succeed in staining for capillaries using antibodies against endothelial markers (CD31, Isolectin B4, VE-Cadherin), we had evidence of nucleated blood cells, accumulated in narrow spaces at the luminal edge of the septal domain in structures morphologically consistent with septal capillaries by H&E staining (Fig. 6G–I). This was further reinforced by our finding that Krt8 labels uniformly the snake faveolar epithelium, including the thin-walled presumptive pneumocyte 1 (Fig. 6H and I). The close

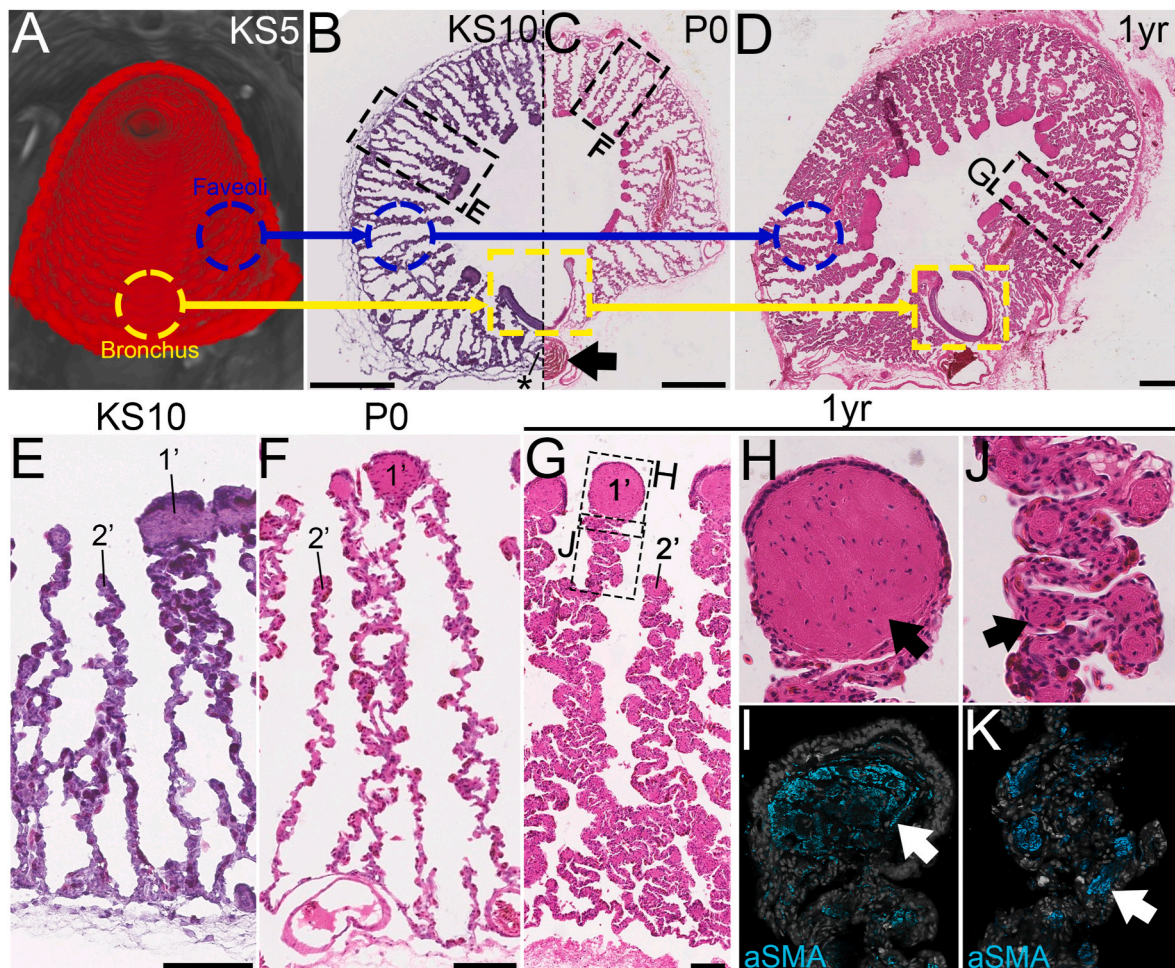


Fig. 5. Septa increased in length post-hatching to become intricately folded. (A) MicroCT reconstruction of KS5 lung compared to (B, C, D) H&E-stained transverse section of the faveolar (vascular) snake lung at different stages (KS10, P0, 1 year-old). Regionally equivalent domains are highlighted diagrammatically in yellow (intrapulmonary bronchus) or blue (faveolar septum). (E–G) are high magnification areas indicated by boxes in B–D. (H–J) are enlarged areas indicated by boxes in G showing morphologically distinct smooth muscle distribution (arrow) in the luminal and the intricately folded septum, confirmed by α SMA staining (I–K). 1' and 2' highlight primary and secondary faveolar septae. DAPI is grey in all IF panels. Scale bars denote 500 μ m (B–D); 100 μ m (G) or 50 μ m (E–F).

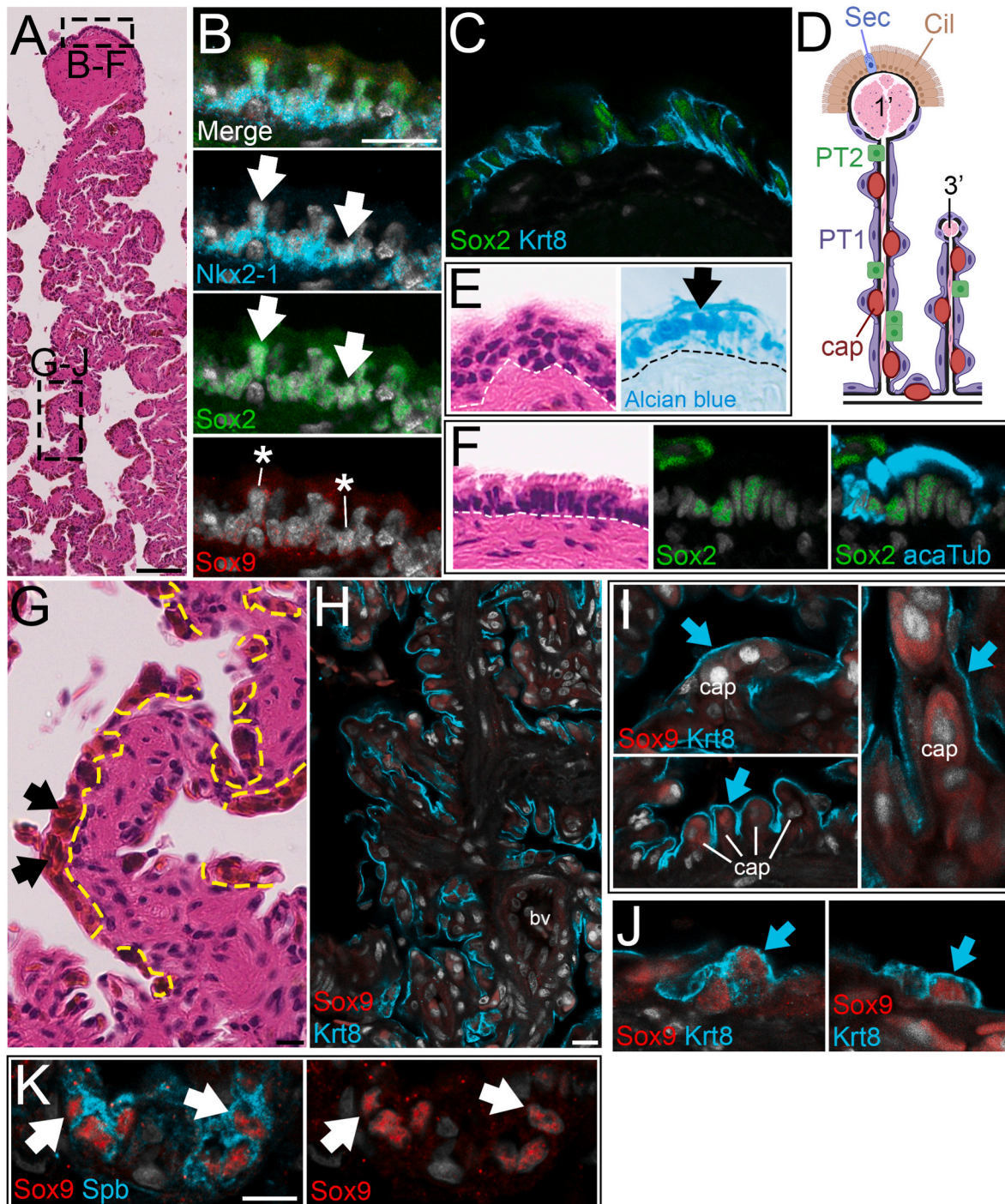


Fig. 6. Sox2 and Sox9 mark airway and respiratory epithelia populated with compartment-specific cell types.

(A) H&E staining showing the boxed areas of the faveolar septum examined in B–K. (B–C) IF showing that the Nkx2-1+Sox2+Krt8+ luminal epithelium is Sox9-. (D) Schematic of the corn snake faveolus (1' and 3' septae represented), depicting ciliated (cil), secretory cells (sec), type 1 and 2 pneumocytes (PT1, PT2), and septal capillaries (cap). (E–F) Secretory (Alcian blue) and ciliated (acetylated a-tubulin) cell differentiation in luminal epithelium (Sox2+, H&E; B–F boxed region from A). (G–J) H&E and IF for Krt8 and Sox9 of the faveolar septum (area boxed in A). (G) A capillary (cap) network (outlined in yellow), containing nucleated blood cells (black arrows) is seen closely opposed to a thin-walled Krt8+ septal epithelium (H, I: blue arrow, presumptive pneumocyte 1). Krt8 stains uniformly the thin-walled and the cuboidal Sox2+ septal epithelium (blue arrows in I–J). Note non-specific Sox9 cytoplasmic staining in nucleated blood cells. Besides these superficially-located capillaries, interstitial blood vessels (bv) are also seen inside the faveolar septum (H). (K) cuboidal Sox9+ epithelial cell express surfactant protein B (Spb) suggesting they are pneumocyte type2. DAPI is grey in all IF panels. Scale bars represent 100 μ m (A); 20 μ m (B) and 10 μ m (G–H, K).

proximity of these nucleated blood cells to the thin Krt8+ epithelium was highly suggestive of the epithelial-apposed capillaries typical of an alveolar-capillary-like barrier. Although antibodies against established mammalian markers of alveolar type 1 cells, such as Pdpn and Hopx, failed to stain the thin-walled Krt8+ presumptive pneumocyte 1, we

found strong surfactant protein B (Spb) signals in the cuboidal-shaped faveolar Sox9+ cells consistent with labeling of pneumocyte 2 (PT2) (Fig. 6J–K). Also notable, deeper in the interstitial compartment of these faveolar septae, is the presence of vascular-like structures recognized by blood cells in their lumen (bv, Fig. 6H). Interestingly, Sox9 expression is

maintained in the mature Spb-expressing PT2 (Fig. 6K), in contrast to the mammalian lung, in which Sox9 is downregulated in the epithelium before it differentiates into an alveolar type 2 cell (Rockich et al., 2013).

This mucociliary differentiation profile seen at the luminal domains of the septa was also seen throughout the epithelia of the intrapulmonary main bronchus (ventral to the faveolar lung), and continued in the trachea, as part of a Sox2⁺, cuboidal epithelium (Fig. 7C–D, F, H–I). Further analysis identified p63-labeled basal cells in both the trachea and bronchial epithelium (Fig. 7E, J). These cells have been extensively characterized as multipotent progenitors that are essential components of the stem cell pool of the airway epithelium in mammals (Rock et al., 2010). Besides p63 and Sox2 expression, these cells are characterized by their basal location in the pseudostratified epithelium and absence of luminal markers of differentiation (Fig. 7J–K).

In summary, the data suggest that the tracheal and bronchial epithelia of the corn snake comprises p63⁺ progenitor and differentiated cell compartments.

2.6. Arrested development of the posterior lung, a potential evolutionary adaptation for the snake

An intriguing feature of the snake adult lung is that the faveolar region responsible for gas exchange (Fig. S1, RLU-vascular) does not extend to the caudal limit of the lung. Instead, this posterior most caudal region is lined by a non-respiratory epithelium with no resemblance to the abundantly vascularized faveoli (Fig. S1, RLU-membranous). The function of this distinct aseptate membranous (saccular) compartment is still poorly understood, serving presumably to store air and act as a potential bellows to maintain active or passive diffusion of air throughout the luminal spaces. Previous studies show that the length of the transition is species-dependent and occurs in a gradual fashion, such that the faveolar septae progressively decrease in length to ultimately flatten posteriorly (Kardong, 1972, 1980; Maina, 1989; Wallach, 1998). Our analysis of the mature corn snake lung confirmed these features (Fig. S1 and Figs. S4A–E).

Morphological and marker analysis in sections of adult lungs showed that the transition from the faveolar to the membranous region was also accompanied by a progressive change in the epithelial shape from cuboidal to a squamous-like epithelium at the posterior end

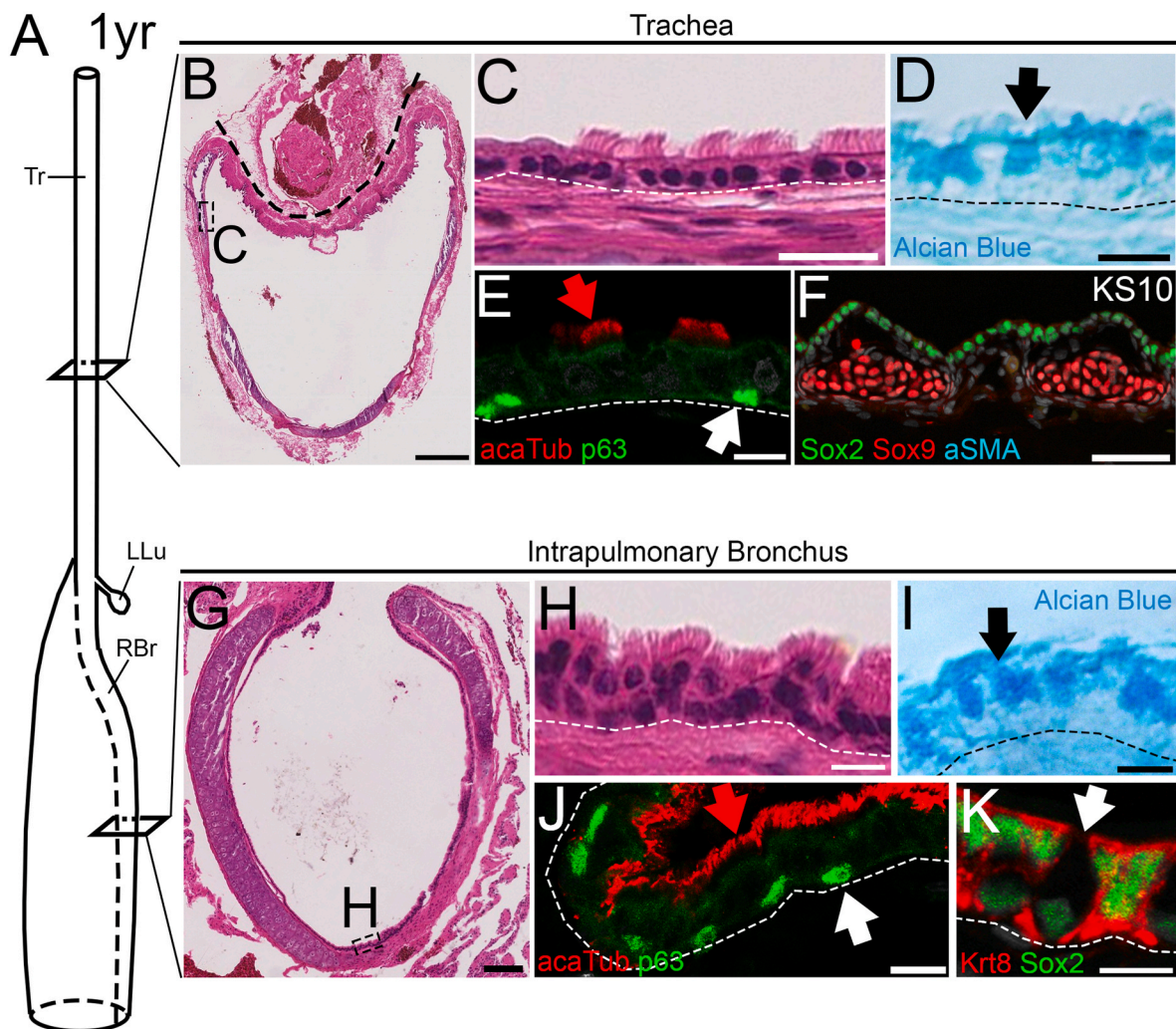


Fig. 7. The tracheal and bronchial airway epithelia comprise a basal cell compartment and luminal differentiated mucociliary epithelium. (A) diagram depicting approximate sampling locations in the trachea and intrapulmonary bronchus (squares). Transverse sections of H&E-stained trachea (B) and intrapulmonary bronchus (G) of the adult corn snake. High magnification panels showing markers and cellular structures consistent with acaTub⁺ ciliated cells (C, E and H, J, red arrow), Alcian blue⁺ mucous secretory cells (D, I), and p63⁺Sox2⁺Krt8⁺ basal cells (E–F and J–K, white arrow). Note clusters of non-epithelial cells expressing Sox9 signals in tracheal cartilage primordia. DAPI is grey in IF panels. Tr, trachea; LLu, left lung; RBr, right bronchus. Scale bars denote 500 µm (B); 100 µm (G); 50 µm (F); 20 µm (C) and 10 µm (D–E, H–K).

(Fig. S4F–M). Epithelial cells showed no evidence of cytodifferentiation but expressed Sox2 throughout the transition and in most of the membranous region of the lung. Interestingly, these cells also co-labeled with Sox9, however signals were largely cytoplasmic, except in a few scattered clusters in which double Sox9+/Sox2+ were distinctly detected in the nuclei (Fig. S4H–I, K–L). The significance of this is unclear and suggests that these cells remained immature in an undifferentiated state, presumably similar to their tip bud counterpart from the embryonic stages (Fig. 1F–J). At the caudal end of the membranous region epithelial cells that expressed Sox2 and Sox9 became very low or undetectable (Fig. S4M).

Notably, the transition from the faveolar to the membranous lung was associated with striking changes in the spatial organization of the smooth muscle layer (Fig. S4N). The thick smooth muscle bundles characteristic of the apical luminal compartment of the faveolar septa could be identified along the transition and the membranous region (Figs. S4F–I). However, the thin α SMA+ cell clusters of the faveolar septae were increasingly disorganized as the septae shortened along the transition region. They appeared to fuse into a thin layer and, together with the remaining thick apical α SMA+ bundles, were present throughout the posterior end of the lung (highlighted in Fig. S4N).

The relatively simple smooth muscle organization in this region was reminiscent of that seen at immature stages (e.g. KS3–4) before septa form (Fig. 4B). We speculate that the immature features we identified in both the epithelial and mesenchymal components of the adult membranous region could have resulted from early developmental patterning events, restricting Sox9 expression and preventing formation of a faveolar lung distal to the bronchus fostering formation of a membranous non-respiratory compartment of the snake lung.

3. Discussion

The morphogenetic events involved in the dramatic structural changes that took place in the respiratory system during evolution are poorly understood, particularly in species, such as snakes, which have an oddly elongated shape in which the respiratory tract runs, on average, through two-thirds of the adult body (Wallach, 1998). Here we show that the elongated morphology and spatial arrangement of the airway and gas exchange regions in the corn snake lung is achieved by the use of two distinct patterning strategies shaping the developing lung (Fig. 8). The first involves continued elongation of a primary bronchial bud with minimal Sox9 induction at the bud tip and no further development of a prospective respiratory compartment distal to this bud (KS2–3, Fig. 8). Then a second event takes place in the anterior (proximal) region of this bud which undergoes expansion and buckling of the dorso-lateral epithelium in areas of smooth muscle accumulation to form multiple dorsal ridges (KS4–5+, Fig. 8). These elongate to form a radial network of septa that is patterned into distinct Sox2+ (luminal) and Sox9+ (basal) epithelial compartments, which ultimately differentiate to give rise to the faveolar lung.

The process of ridge formation that we observed in the snake dorso-lateral lung epithelium seemed reminiscent of the domain branching reported in the developing mouse lung, during which epithelial buds emerge laterally from existing Sox2+ airway progenitors to become Sox9+ at their tips (Metzger et al., 2008). The similarity was surprising, given the lack of a branched airway morphology in the corn snake, and suggests that this type of morphogenetic program may have emerged in evolution before others, such as the orthogonal and bifurcating branching programs described as part of mouse lung development (Metzger et al., 2008). It was also notable that the faveologensis in the snake had key features in common with those reported in the *Xenopus* embryo and with alveolar secondary septation in the mouse lung (Burri, 1984; Rankin et al., 2015; Rose and James, 2013; Waterman, 1939). In mice, α SMA+ myofibroblasts form a fishnet-like network around pre-alveolar saccules and then either contract, forcing epithelial luminal evagination, or locally prevent saccular dilation (Boström et al., 1996;

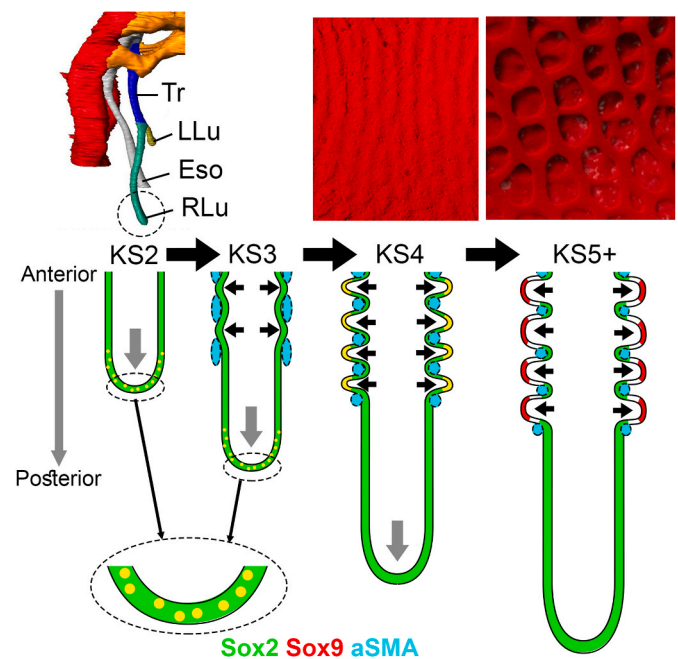


Fig. 8. Summary of patterning events that establish the airway and respiratory epithelial compartments in the corn snake lung. From left to right: Initial formation of trachea and primary lung buds (note left lung is truncated in this species). (KS2) During elongation of the primary right lung bud the epithelium is largely Sox2+ with weak, mosaic Sox9 expression at the tip (dotted circle). (KS3) Continued epithelial bud elongation with weak mosaic Sox9+Sox2 expression and buckling of the anterior region with formation of α SMA+ smooth muscle bundles leading to the appearance of ridges that protrude into the lumen as ring-like structures (MicroCT images at KS4 and KS5). (KS4) Concomitant appearance of a Sox9+ domain at the base of the elongating ridge, which first co-expresses Sox2. (KS5) Distinct apical luminal Sox2 domain which later differentiates into the respiratory region of the lung (faveolar). Diagram: Eso, esophagus; Tr, trachea; LLu, left lung; RLu, right lung.

Branchfield et al., 2016; Burri, 1984; Lindahl et al., 1997; McGowan et al., 2008; Mund et al., 2008; Prodhan and Bernard Kinane, 2002). In corn snake we identified a similar α SMA+ smooth muscle network enveloping the lung that has also been described in a variety of other reptiles and amphibians (Duncker, 1978; Kardong, 1972; Luchtel and Kardong, 1981; Maina, 1989; Perry and Duncker, 1978). Presumably, the alveolar secondary septation of the mammalian lung and the faveolar septation of the developing snake lung may represent a conserved morphogenetic strategy to increase the gas-exchange surface and meet the metabolic needs of postnatal life.

It is interesting that Sox9 and Sox2 have been reported as widely conserved signals in the development of the respiratory system of species with different lung morphologies and evolutionarily as distant as *Xenopus*, snake, mouse and humans. These signals, however, do not seem to be used at the same stage in all species. In the ancestral, unbranched saccular lung of *Xenopus* they appear only later in the faveolar septa. In contrast, both genes are expressed early in murine and human lungs, which have complex branching structures. Our analysis in the developing snake suggests that.

Sox9 is used reiteratively in two phases: first, at an early stage and at low levels, presumably contributing to outgrowth of the primary lung bud, but unable to drive the distal program of lung development characteristic of mammals. Later, Sox9 is re-expressed, now strongly to drive specification and differentiation of the respiratory region of the faveolar lung. It will be informative to investigate this developmental program in other squamates to find whether the patterning events we found in the corn snake are common to other snakes.

4. Materials and methods

4.1. Animal samples

See Table 1 for a complete list of samples with staging. Two adult *Pantherophis guttatus* samples used for gross analysis of external lung morphology, and three embryonic samples used for MicroCT imaging, were from the collection of the Institute of Biology, University of Leiden. Adult specimens were donated by Dutch pet owners as carcasses after the animals had died from natural causes. The acquisition of these specimens predates the Nagoya protocol and was done in accord with local regulations. Specimens were fixed in 4% paraformaldehyde (PFA) and stored in 70% ethanol upon donation. One adult, one six-month-old, one hatchling and all other embryonic corn snake samples were obtained from Dr. Neil Ford at the University of Texas at Tyler, Tyler, TX (UT Tyler). Fertilized eggs were either incubated at UT Tyler, or shipped with sufficient vermiculate to maintain adequate temperature and humidity and to protect the eggs during travel, and then incubated at 28 °C with normal room air. Hatchlings, younglings (6-month-old and adult (1 year old) samples were euthanized at UT Tyler by an overdose of isoflurane in an airtight chamber and death was ascertained by ensuring lack of breathing and muscle tone. Samples were then refrigerated and shipped overnight with cold packs to minimize tissue decomposition.

Because there is no published staging table for corn snake, Khannoon staging for Egyptian cobra (*Naja haje haje*) was used (Khannoon and Evans, 2014), since the exterior anatomy of the embryos through progressive stages of development was found to be similar to that of corn snake, and the incubation time to hatching was comparable (50–60 days). Stages were easily determined with reference to external characters (Khannoon and Evans, 2014). This staging information was used together with published information on lung development (van Soldt et al., 2015) to estimate the stage of lung development at the embryonic stages collected. Lack of perfect concordance between incubation time and developmental stage is presumably due to small variations in incubation temperatures and other factors.

4.2. Histology and fluorescent immunostaining

Corn snake whole embryos or dissected lungs and trachea were collected at the ages indicated (Table 1), rinsed in phosphate-buffered saline (PBS) and fixed in 4% PFA overnight at 4 °C. Samples were then processed for frozen (OCT) or paraffin embedding. Hematoxylin and eosin (H&E) staining was performed by the Columbia University Irving Medical Center, Herbert Irving Comprehensive Cancer Center

Molecular Pathology Core, and imaged with a Leica SCN400 Slide Scanner.

For immunofluorescent (IF) staining, sections (8 µm thickness) were concomitantly permeabilized and blocked in blocking buffer (5% donkey serum/0.3% Triton X-100 in PBS), and then incubated overnight at 4 °C with primary antibodies diluted in blocking buffer. The following day slides were washed in PBS, incubated for 2 h in 5% normal donkey serum with secondary antibodies and NucBlue Fixed Cell Stain ReadyProbes (DAPI) (Invitrogen, R37606), washed again in PBS, and finally mounted using Prolong Gold anti-fade reagent (Invitrogen, P36930). Images were taken on a Leica DMI-8 deconvolution microscope and a Zeiss LSM 710 confocal microscope system.

The following primary antibodies were used: rabbit anti-acetylated α-tubulin (Cell Signaling, 5335S), mouse anti-acetylated α-tubulin (Sigma, T7451), chicken anti-Krt5 (BioLegend, 905,901), rabbit anti-Krt8 (Abcam, ab53280), rabbit anti-Nkx2.1 (Seven Hills, WRAB-TTF1), rabbit anti-p63a (Cell Signaling, 13109S), rabbit anti-αSMA (Cell Signaling, 19245S), rabbit anti-SpB (Seven Hills, WRAB-48604), rat anti-Sox2 (ebioscience, 14-9811-82), goat anti-Sox9 (R&D systems, AF3075).

The following secondary antibodies were used: donkey anti-goat (conjugated with Alexa Fluor 568 or 647), donkey anti-mouse (conjugated with Alexa Fluor 488, 568, or 647), donkey anti-rabbit (conjugated with Alexa Fluor 488, 568, or 647), donkey anti-rat (conjugated with Alexa Fluor 488 or 647), donkey anti-chicken (conjugated with Alexa Fluor 647). All secondary antibodies were obtained from Jackson ImmunoResearch or Fisher Scientific.

4.3. MicroCT imaging

For x-ray contrast enhancement, embryos (Table 1) were stained for 1–4 d in 1% phosphotungstic acid (PTA) dissolved in 100% methanol [51, 52]. Samples were then brought back to fresh 100% methanol and mounted for scanning in plastic tubes or large pipette tips [53]. Attenuation-based microtomographic images were made using an Xradia MicroXCT system (Carl Zeiss X-Ray Microscopy, Pleasanton, CA), with a tungsten source (Hamamatsu L9421-02) set at 60 or 80 kV and 5W. Projection images were taken every 0.2° over a half rotation with 20–30 s exposure times. Images were acquired with pixel sizes of 4–10 µm, and tomographic reconstructions were made with the resident software (XMReconstructor) with final voxel sizes of 1.8–2.0 µm. Reconstructed images were exported as TIFF files and loaded into DragonFly (Object Research Systems (ORS) Object Research Systems Inc, 2020) for annotation.

4.4. EdU incorporation assay

To assess cell proliferation 1 mg EdU (5 mg/mL stock solution in PBS) was injected into a fertilized, incubated corn snake egg 23 days after oviposition (dao)(KS6-stage), and harvested 24 h later for OCT embedding and sectioning. EdU was detected by immunostaining using the Click-iT EdU Alexa Fluor 647 Imaging kit (Thermo Fisher Scientific, C10340) following heat-based antigen retrieval as described above for immunofluorescent staining and imaging. Sections were subsequently co-stained for Sox2 and Sox9 as described above. Proliferation was assessed as percent of EdU labeling in the single Sox2+ or Sox9+ or double Sox2+Sox9+ epithelial cells.

4.5. Alcian blue staining

Alcian blue staining was performed by first incubating sections in 3% acetic acid for 2 min, followed by Alcian Blue incubation for 5 min at room temperature, as previously reported (Yang et al., 2018).

Table 1

List of embryonic corn snake samples with ages (days after oviposition, dao) and staging according to Khannoon (Khannoon and Evans, 2014).

Sample ID #	Incubation age	Khannoon Stage (KS)	Usage
PG1-8	9dao	KS2	IF, H&E
PG10-11	51dao	KS10	IF, H&E
PG12	1 year old		IF, H&E
PG13	6 months old		IF, H&E
PG14	Hatchling (P0)		IF, H&E
PG15	11dao	KS3	IF, H&E
PG16	11dao	KS2	IF, H&E
PG17	11dao	KS2	IF, H&E
PG18	13dao	KS3	IF, H&E
FV211	9dao	KS3	MicroCT
PG19	13dao	KS2	IF, H&E
PG20-21	15dao	KS4	IF, H&E
FV117	15dao	KS4	MicroCT
PG22	17dao	KS4	IF, H&E
PG23-24	19dao	KS5	IF, H&E
FV115	12dao	KS5/6	MicroCT
PG25	24dao	KS6	IF, H&E, EdU labeling
PK17	Adult		Gross histology
PK21	Adult		Gross histology

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Author contributions

Conceptualization: BS, WVC. Methodology: BS, BDM, WVC. Investigation: BS, BDM, WVC. Visualization: BS, BDM, WVC. Supervision: WVC. Writing—original draft: BJS, WVC, BDM, MKR. Writing—review & editing: BJS, WVC, BDM, MKR.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ydbio.2023.11.006>.

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