Determining the role of SOX9 in avian beak development and morphogenesis

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75 YEARS UC DAVIS VETERINARY MEDICINE

INTRODUCTION

Vertebrate-specific neural crest (NC) stem cells differentiate into multiple tissue types during embryogenesis. These cells form in the developing central nervous system and undergo an epithelial to mesenchymal transition (EMT). NCCs migrate to distant sites and form many tissues, including the craniofacial bone and cartilage which becomes the jaw in mammals and beak structures in birds (**Fig. 1**). Avian craniofacial development is conserved with mammalian development, but there are gaps in knowledge about the specific molecular mechanisms and proteins that guide formation of NC derivatives across species. NC cells express the SRY-Box Transcription Factor-9 (SOX9), which drives specification, EMT, and differentiation at early developmental stages. The Rogers' Lab identified that Sox9 gene and protein expression is heterochronous between chicken and quail embryos during NC development[1-6]. To understand the functional timeline for SOX9 necessity, and the downstream morphological changes that occur in its absence, we performed immunohistochemistry (IHC) and *in situ* hybridization chain reaction (HCR) followed by fluorescence imaging in various stages of chicken embryos. We next performed knockdown experiments to define the necessity of SOX9 for craniofacial morphogenesis. We *hypothesize* that the loss of SOX9 prior to NC EMT will inhibit NC cell migration and differentiation, leading to abnormal beak morphogenesis. Understanding the role of SOX9 in craniofacial development may help to define molecular changes in specific congenital abnormalities such as cleft palate and micrognathia.

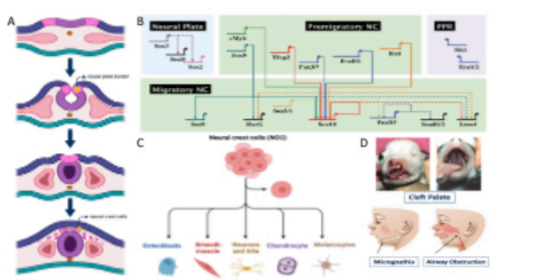

Figure 1. Neural crest formation, regulation, and proliferation. (A) NC cells arise in the dorsal neural tube and will migrate after EMT. **(B)** A map of the genes and proteins that drive both formation of NCCs and EMT[7]. **(C)** NCCs differentiate into multiple adult cell/tissue types. **(D)** Craniofacial abnormalities are common neurocristopathies[8,11].

METHODS

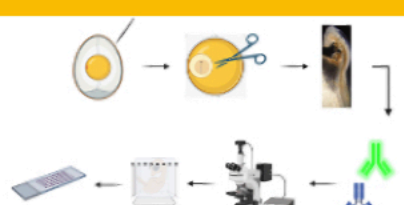

Figure 2. Methods used in study. Chick embryos were dissected at various stages of development including Hamburger Hamilton (HH) stage 4, 8-9, 26-27. HCR and IHC were performed, and embryos were imaged using fluorescence microscopy for wholemount , and subsequently sectioned and imaged again.

RESULTS

Table 1. Overview of IHC markers used.

Marker	Associated Cell Type/ Structure
SOX9, SOX10, PAX7	Neural crest
RUNX2	Developing lateral line and osteogenic NCCs
Olig2	Oligodendrocyte precursors and developing motor neurons
BMP4	Cell lineage regulation and inducing osteogenic differentiation
TUBB3	Protein component of microtubules in neurons
GFAP	Intermediate filament protein associated with astrocytes and ependymal cells of the CNS
CAD11	Calcium dependent cell adhesion protein
3G2/4E2	Schwann cell associated antigen and neuromuscular junctions
Col2a	Extracellular matrix protein secreted by chondrocytes during cartilage formation and endochondral ossification
Collagen Type IX	Fibril associated and distinct component of cartilage
FGF	Cell signaling proteins that regulate cell proliferation and differentiation
P0	Schwann cell progenitors

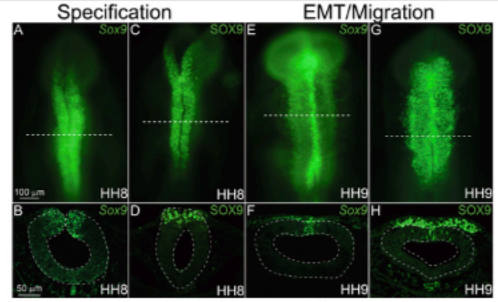

Figure 3. SOX9 is expressed at the onset of NC specification and during EMT. (A, B, E, F) HCR performed in chick embryos shows that the premigratory NC cells strongly express *Sox9* transcripts at both specification (HH8) and EMT (HH9). **(C, D, G, H)** IHC shows that SOX9 protein is also expressed strongly in the premigratory and migratory NC cells (adapted from Monroy et al., 2022).

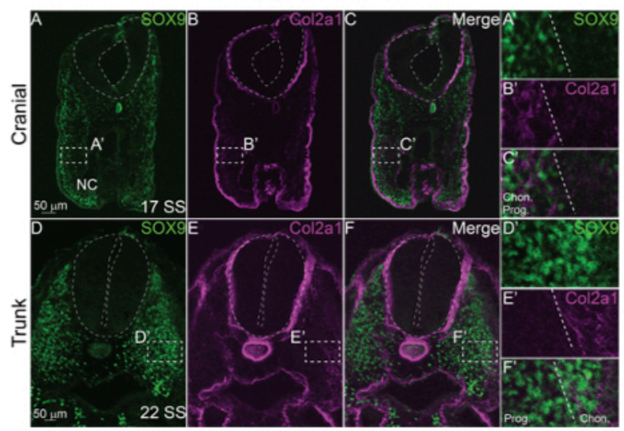

Figure 4. Lineage-specific SOX9-mediated chondrogenesis across axial levels. IHC for **(A- F)** SOX9 and Col2a1 in two stages of chick embryos. At 17 somite stage (SS), SOX9 is maintained in the dorsal neural tube and the ventral craniofacial region (brachial arches). **(A'- C')** Zoom in shows overlapping SOX9 and Col2a localization showing that all SOX9+ cells are chondrogenic in the ectodermally-derived craniofacial region. **(D- F, D'- F')** At 22SS in the trunk axial region, SOX9 and Col2a1 expression patterns differ from cranial. Within trunk sections, SOX9 is expressed throughout the mesodermally-derived sclerotome progenitors, but Col2a1 expression is limited to the distal region. Dashed lines outline the neural tube. Scale bars are as marked.

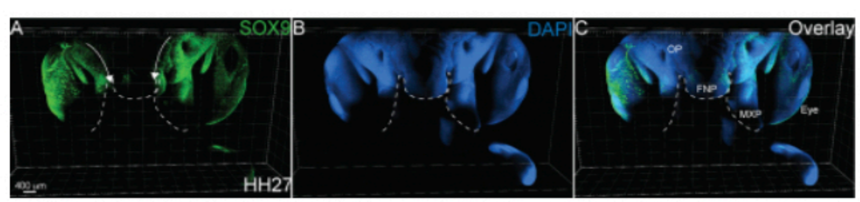

Figure 5. SOX9 protein expression is maintained in cells colonizing the chick craniofacial region, including the frontonasal process (FNP) and maxillary process (MXP). Light sheet fluorescent microscopy (LSFM) images of HH27 chick embryo. Dashed lines represent the facial prominences. **(A)** SOX9 protein expression, with arrows showing SOX9+ cells migrating into the developing face. **(B)** DAPI nuclear stain shows the three-dimensional structure of the developing chicken craniofacial structure. **(C)** Overlay of SOX9 protein expression and DAPI nuclear stain. OP= otic placode, FNP= frontonasal process, MXP- maxillary process.

DISCUSSION

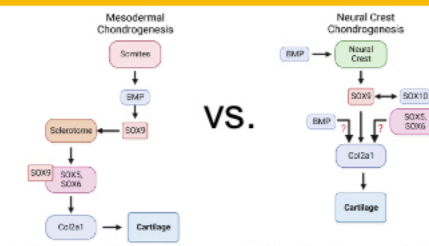

Figure 6. Putative relevant pathways driving chondrogenesis in mesoderm vs. NC-derived tissues[9].

The objective of this project is to:

1) define the role of SOX9 in beak formation in *Gallus gallus* (chicken) and
2) identify molecular changes in NC- derived tissues after loss of SOX9.

Here, we have:

- Identified differences in IHC (SOX9, Col2a1) expression across axial levels.
- Demonstrated that NC cells maintain SOX9 protein expression from the onset of formation to differentiation of craniofacial prominences.

Understanding the role of SOX9 in craniofacial chondrogenesis may illuminate similarities and differences of lineage-specific chondrogenesis.

FUTURE DIRECTIONS

- We will continue to characterize the colocalization of SOX9 and Col2a expression during craniofacial development.
- Future work will include analyses focused on the sufficiency and necessity of SOX9 to drive cartilage and bone formation in avians.

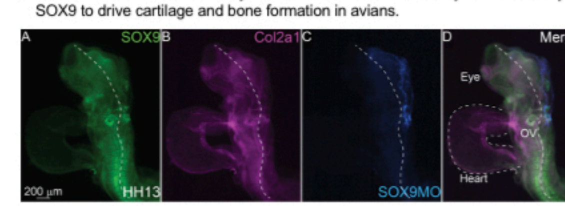

Figure 7. SOX9MO will be used for unilateral SOX9 knockdown. Chick embryos can be injected and electroporated *ex ovo* for unilateral perturbations[10]. IHC for **(A)** SOX9, **(B)** Col2a1, **(C)** SOX9MO and **(D)** Merge. Dashed lines show various anatomical features of the embryo at HH13.

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