



# Gene expression analysis during the induction and patterning of the conjunctival papillae in the chick embryonic eye



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## ABSTRACT

The induction and patterning of the conjunctival papillae (i.e. epithelial thickenings of the conjunctiva required for the induction of the underlying, neural crest-derived scleral ossicles) is complex. It takes place over a period of two days and follows a defined spatiotemporal sequence. In this study, we investigated the spatial and temporal expression pattern of four genes over seven morphological stages of development of these papillae. We show that  $\beta$ -catenin is expressed during the pre-patterning of the epithelium prior to papilla induction and second that  $\beta$ -catenin, *Ednrb* and *Inhba* are expressed during the induction and patterning of the conjunctival papillae. Furthermore, we identified two genes,  $\beta$ -catenin and *Prox1*, that may be involved in the induction of the underlying scleral bones. These data provide an excellent baseline for future studies, setting the stage for functional studies aimed at examining the role of these genes in the patterning of the scleral ossicle system. This study also outlines the similarities between the conjunctival papillae and other placodes and may provide insights into the evolution and development of the conjunctival papillae.

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## 1. Introduction

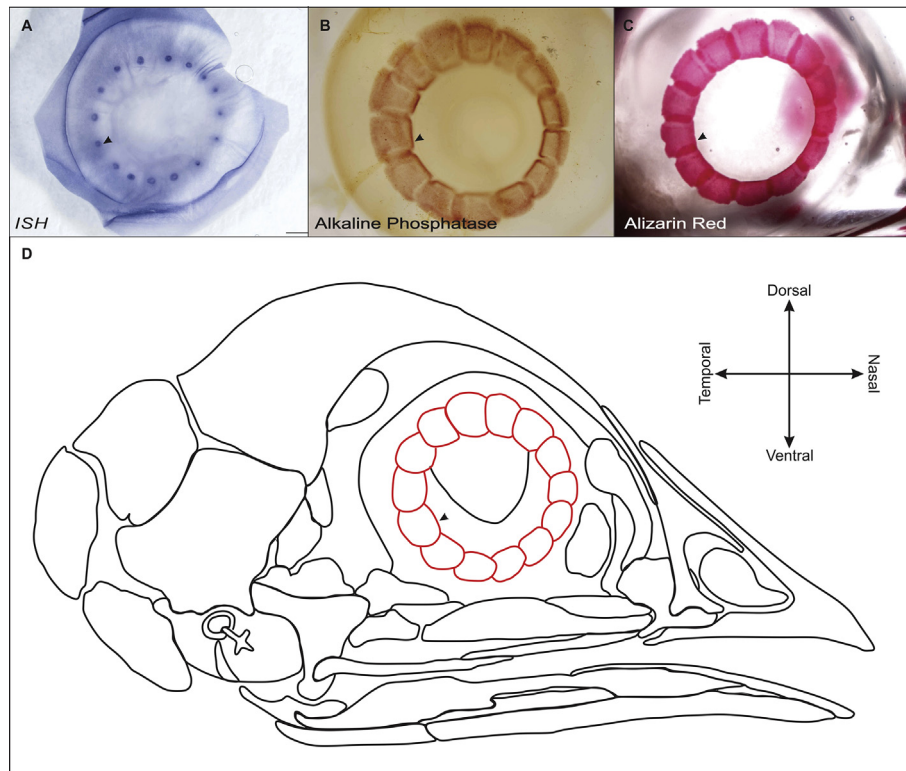
The conjunctival papillae are epithelial thickenings of the conjunctival epithelium that are required for the induction and patterning of the underlying neural crest-derived scleral ossicles (Fig. 1). The development of these conjunctival papillae begins at Hamburger and Hamilton (HH, 1951) stage 30 (approximately 7 days post fertilization [dpf]) with the induction of the first conjunctival papilla directly above the ciliary artery (Coulombre and Coulombre, 1962; Franz-Odenaal, 2008). Over the following two days, the rest of the conjunctival papillae are induced in a complex spatiotemporal pattern (Coulombre and Coulombre, 1962; Franz-Odenaal, 2008). This pattern consists of the induction of the temporal group, followed by the nasal group, the dorsal group, and finally the ventral group where the last conjunctival papilla is always induced directly above the choroid fissure at HH 34 (approximately 8 dpf) (Coulombre and Coulombre, 1962). This staggered spatiotemporal induction of the conjunctival papillae is

similar in the chicken *Gallus gallus* and in the snapping turtle *Chelydra serpentina* (Coulombre and Coulombre, 1962; Franz-Odenaal, 2006). Furthermore, Coulombre and Coulombre (1962) noted that the number of conjunctival papillae in each eye is variable based on the size of the eye, although it usually consists of 13–16 papillae per eye. The strain used in this study, however, has a modal number of 15 ossicles per eye and a fluctuating asymmetry with respect to the number of ossicles, which has been noted in 44% of individuals (Franz-Odenaal, 2008).

After their induction, these conjunctival papillae undergo a complex morphological development prior to inducing the underlying skeletogenic condensations (Fig. 1B). This morphological development, known as the M-stages of papilla development (Murray, 1943), is characterized by distinct changes in morphology of the conjunctival papillae between HH 30 and HH 37 (7–11 dpf). The papillae are inductively active at M-stages 4 and 5 (corresponding to HH 35 and HH 36) (Coulombre and Coulombre, 1962; Hall, 1981). Two gene families have so far been investigated, Hedgehog (*Hh*) and bone morphogenetic proteins (Franz-Odenaal, 2008; Duench and Franz-Odenaal, 2012) in this system. Using *in situ* hybridization it was determined that *Shh*, *Ihh*, *Ptc*, and *Bmp2* are expressed in the conjunctival papillae and/or the subjacent mesenchyme at HH 35 (Franz-Odenaal, 2008; Duench and Franz-Odenaal, 2012). Furthermore, when either the *Hh* or *Bmp* gene

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**Fig. 1.** A summary of the development of the sclerotic ring. **A)** Development of the sclerotic ring begins with the conjunctival papillae (arrow). **B)** The conjunctival papillae then induce scleral condensations in a 1:1 ratio in the underlying scleral mesenchyme (arrow). **C)** The scleral condensations then increase in size until they overlap and begin to ossify, forming the scleral ossicles (arrow). **D)** This schematic of the chicken head; adapted from (Gross and Hanken, 2008); shows the position of the sclerotic ring (red), made up of the overlapping scleral ossicles, in relationship to the other bones in the head. The axis of this sclerotic ring is also indicated in the top right corner. In all images, the papilla/ossicle directly above the ciliary artery is indicated by the arrow.

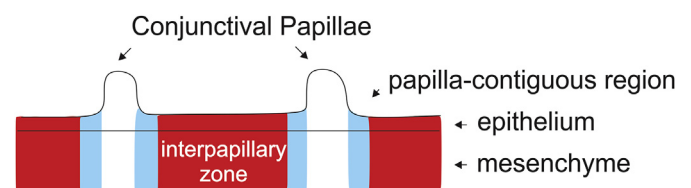
families were inhibited by the implantation of a bead soaked with an inhibitor (either cyclopamine or noggin, respectively) adjacent to a conjunctival papilla, the underlying condensation does not form (Franz-Odenaal, 2008; Duench and Franz-Odenaal, 2012). Therefore, both *Hh* and *Bmp* gene families are required for the development of the underlying scleral condensations, these genes also interact with one another, however, the manner in which they induce the underlying condensation is currently unknown (Franz-Odenaal, 2008; Duench and Franz-Odenaal, 2012). After their induction, the scleral condensations increase in size and begin to ossify at HH 38 (12 dpf); becoming the scleral ossicles (Fig. 1C) (Coulombre and Coulombre, 1962; Franz-Odenaal, 2008). These scleral ossicles then continue to increase in size and overlap, forming the sclerotic ring (Coulombre and Coulombre, 1962; Franz-Odenaal, 2008). This sclerotic ring does not articulate with the rest of the skull (Fig. 1D) and likely serves a role in visual accommodation in non-mammalian vertebrates by preventing distortion of the eyeball during acute vision (Slonaker, 1918, 1921; Curtis and Miller, 1938; Walls, 1942).

We identified four genes ( $\beta$ -catenin, *Prox1*, *Ednrb*, and *Inhba*) that are involved in the induction and patterning of other epithelial thickenings, such as the cranial and cutaneous placodes. Here we examine these molecular factors in order to determine whether they are expressed during the stages of conjunctival papillae development. We show that one of these genes is expressed just prior to the induction and patterning of the conjunctival papillae (HH 30), that three genes are expressed in the conjunctival papillae during their induction and patterning (HH 31 to HH 34), and that two of these genes may be required for the induction and patterning of the underlying scleral condensations. These results also demonstrated that the development of the conjunctival papillae has three stages: i) the

pre-patterning of the epithelium, ii) induction of the papilla itself, and iii) induction of the subsequent structure; which is similar to other placodal systems. This study provides a number of new insights into the induction and patterning of the conjunctival papillae and will serve as a key foundational study for the development and patterning of the sclerotic ring. Moreover, as the development of the sclerotic ring is similar to that of other neural crest-derived, intramembranous bones, this study may also provide key insights into the molecular factors required for the induction and patterning of other intramembranous bones.

## 2. Results

The temporal and spatial gene expression pattern of four genes is described below. They are expressed within the conjunctival papillae themselves, within the papillae contiguous region (i.e. the area adjacent to the conjunctival papilla that extends to the interpapillary zone; Fig. 2) and/or within the underlying mesenchyme



**Fig. 2.** Schematic illustrating the different papilla regions. The papilla region (white), the papilla-contiguous region (light blue), and the interpapillary region (red). This schematic introduces the papilla-contiguous region and will be useful for future characterization of gene expression in the conjunctival papillae and underlying mesenchyme in later studies.

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