Analysis of the FGFR spatiotemporal expression pattern within the chicken scleral ossicle system

Shruti Kumar, Tamara A. Franz-Odendaal

ABSTRACT

Fibroblast Growth Factors (FGFs) play several important roles during organ morphogenesis and act as multifunctional growth factors that bind to their membrane bound receptors (FGFRs) and activate further downstream signalling pathways. Several studies have investigated the function and expression of FGF/FGFRs in endochondral bone development, however, we know little about their role in the development of neural crest derived intramembranous bones. Here, we investigate the expression of ‘b’ and ‘c’ isoforms of FGFRs 1–3 during the development of the scleral ossicles, a ring of neural crest derived intramembranous bones in the chicken eye. These bones are induced by conjunctival papillae. We identified the expression of both ‘b’ and ‘c’ isoforms of FGFRs1-3 during phase 1 of ossicle development when conjunctival papillae development takes place. In contrast, during phase 2, when skeletal condensations are induced in the mesenchyme, all isoforms were down-regulated. This data shows for the first time the presence of FGFRs in the chicken sclera, thus implicating FGFs as a signalling pathway potentially involved in scleral ossicle development.

1. Introduction

The scleral ossicles are overlapping, intramembranous, trapezoid shaped bones (Coulombre and Coulombre, 1973; Couly et al., 1993; Franz-Odendaal and Vickaryous, 2006) that are induced by epithelial-mesenchymal induction (Pinto and Hall, 1991; Franz-Odendaal, 2008). This developmental process begins with the development of a series of epithelial structures called conjunctival papillae, which form in a ring at the corneal-scleral limbus of the eye (Murray, 1943; Couly et al., 1962). After these papillae develop, they induce skeletogenic condensations below, in the neural crest derived ectomesenchyme, in a 1-to-1 manner, ultimately leading to the formation of a series of bone plates in the eye, the scleral ossicles (Coulombre and Coulombre, 1973; Fyfe and Hall, 1983). The domestic chicken Gallus gallus has 14–16 conjunctival papillae per eye (Franz-Odendaal, 2008). The first papilla is visible at approximately 6.5 days of embryonic development at Hamburger and Hamilton stage 30 (HH30) over the ciliary artery (Hamburger and Hamilton, 1951). Subsequent papillae appear neighbouring the first papilla in the temporal region (group 1) at HH30.5 followed by three to four papillae in the nasal region (group 2) at HH31, three to four papillae in the dorsal region (group 3) at HH32 and finally two papillae in the ventral (group 4) region at HH33 (Fig. 1A). The last papilla can be observed at HH34 (8 days of development) over the choroid fissure (Hamburger and Hamilton, 1951; Coulombre and Coulombre et al., 1962). After inducing the underlying scleral ossicles to form, the papillae then begin to degenerate. Skeletogenic condensations can be observed in the unstained eye at HH37 after the removal of the eyelid and nictitating membrane, however small condensations of osteoblasts are present starting at HH34 as evidenced by alkaline phosphatase staining (Andrews and Franz-Odendaal, in press). These skeletogenic condensations increase in size via cell migration (Jabalee et al., 2013) and begin mineralizing around HH38 (12 days of development). At this stage, all the papillae have disappeared, and a thin boney ring of scleral ossicles can be observed (Franz-Odendaal, 2008). As the scleral ossicles enlarge, they begin to overlap each other and are held together by dense connective tissue (Franz-Odendaal, 2008). In summary, the process of scleral ossicle formation involves the formation of the conjunctival papillae followed by a phase of scleral ossicle induction and growth.

During embryogenesis, transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP), hedgehog (HH), fibroblast growth factor (FGF) and wingless (Wnt) families are key signalling pathways that play several important roles during tissue morphogenesis. The induction of scleral ossicles requires diffusible signals from the mesenchyme to the epithelium (during phase 1) and then from the epithelium to the mesenchyme (phase 2, Pinto and Hall, 1991). BMP2, Sonic Hedgehog (SHH)
and Indian hedgehog (IHH) are expressed at HH35 and HH36 in the conjunctival papillae coinciding with the time when ossicle condensations form. Localised inhibition of the HH and BMP pathway at HH35 results in the absence of the ossicle beneath the implanted papilla (Franz-Odendaal, 2008; Duench and Franz-Odendaal, 2012). More recently, several other genes were recently identified in the system that could possibly be involved in the development of the conjunctival papillae and/or in the induction of scleral condensations. These include β-catennin, Ednrb, Inhba, Prox1 (Jourdeuil and Franz-Odendaal, 2016) and VEGFα (Jabalee and Franz-Odendaal, 2015). Some of these genes are expressed in the papillae themselves, some in the contiguous region surrounding the papillae (Fig. 1B) and some in the mesenchyme below the papillae. The potential role of FGF signalling in this system (i.e. during the conjunctival papillae development, phase 1, and during scleral ossicle induction and growth, phase 2) has not yet been explored.

In Gallus gallus, four FGFRs (FGFR1-4) have been identified, along with 13 FGFs (FGF1-4, 8-10, 12, 13, 16, 18-20) (This and This, 2005). FGFRs are tyrosine kinase receptors that contain an extracellular ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. The extracellular domain is composed of three immunoglobulin-like domains (I, II and III). FGFRs1-3 can undergo alternative splicing at its carboxy-terminal in the immunoglobulin-like domain III, forming either ‘b’ or ‘c’ isoforms (Miki et al., 1992; Eswarakumar et al., 2005). These isoforms are often specific to either the epithelium (b) or the mesenchyme (c; reviewed in Ornitz and Itoh, 2001). The binding of FGF along with their cofactors heparan sulfate proteoglycans (HSPG) (Mohammadi et al., 2005) results in dimerization and auto-transphosphorylation of the receptor and activates further downstream intracellular signalling pathways (Itoh and Ornitz, 2004; Sarabipour and Hristova, 2016).

In this study, we investigated the expression pattern of the ‘b’ and ‘c’ isoforms of FGFRs1-3 during conjunctival papillae development (phase 1, HH30-33, 6.5–8 days of embryonic development) and during scleral ossicle development (phase 2, HH34-37, 8–11 days of embryonic development). We show that the majority of the FGFRs are expressed prior to the morphological appearance of the papillae and that there is a dynamic spatio-temporal expression pattern within and surrounding the conjunctival papillae prior to complete downregulation by HH37. We also show that FGFR isoform tissue specificity is not stringent in the scleral ossicle system as in other systems. Results from this study can be used to identify the FGFs that may be involved in the development of the scleral ossicle system and possibly other intramembranous bones. Furthermore, the identification of FGFRs during conjunctival papillae development provides important knowledge on the development of non-neurogenic placodes, which share many similarities with conjunctival papillae.

2. Materials and methods

2.1. Chicken embryos

Fertilized chicken eggs were obtained from Nova Scotia Agricultural College (Truro, NS) and kept at 4 °C until incubation. The eggs were incubated at 37 ± 1 °C in 40% humidity and turned 180° once daily. At approximately 3 days post fertilization (HH20/21), eggs were removed from the incubator for ex-ovo shell-less culturing as previously described (Cloney and Franz-Odendaal, 2015). Embryos were collected at embryonic days 6.5, 7, 7.5, 8, 9, 10 and 11 (HH30-37) and fixed in 4% paraformaldehyde.

2.2. Whole mount in situ hybridization (WMISH)

Plasmids containing FGFRs1b, 1c, 2b, 2c, 3b and 3c were a kind gift from Dr. Gary Schoenwolf (Department of Neurology and Anatomy, University of Utah School of Medicine, USA). A Whole mount in situ hybridization protocol was adapted from Nieto et al. (1996) and Franz-Odendaal (2008). For each gene, two controls (a no probe control and a no antibody control) were also processed to ensure that the expression pattern observed was valid. All probes were made from previously