Developmental Biology 365 (2012) 251–258

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

BMP and Hedgehog signaling during the development of scleral ossicles

Kellie Duench, Tamara A. Franz-Odendaal *

Department of Biology, Mount Saint Vincent University, 166 Bedford Highway, Halifax, Nova Scotia, B3M 2J6, Canada

ARTICLE INFO

Article history:
Received for publication 31 January 2011
Revised 9 February 2012
Accepted 10 February 2012
Available online 17 February 2012

Keywords:
Skeletal development
Intramembranous bone
Neural crest cells
Induction
Compensation
Gallus gallus
Scleral papillae
Ex ovo culturing
Condensations
Dermal bone conjunctival papillae

ABSTRACT

Bone development is a complex process, involving multiple tissues and hierarchical inductive interactions. The study of skeletal development has largely focused on endochondral bones while intramembranous bones, such as the scleral ossicles within the avian eye, have received less attention. Our previous research directly demonstrated the involvement of *sonic hedgehog* and suggested the involvement of *bmp2* and 4 during the development of scleral ossicles. The bones of the sclerotic ring are induced by overlying conjunctival papillae at HH 35 and 36. Here, we examine the spatial and temporal expression patterns of *ptc1*, *ihh*, *bmp2*, *bmp4* and *bmp7*. We show that the cells of conjunctival papillae express *ptc1*, *ihh* and *bmp2* at these stages; coincident with *shh* expression previously described. Interestingly, both *ihh* and *ptc1* are also expressed in the mesenchyme underlying the papillae unlike *shh* and *bmp2*. *Bmp4* and *bmp7* are not expressed in these regions at any stages examined. Furthermore, using Noggin soaked beads implanted adjacent to papillae, we provide direct evidence that the BMP family of genes are important factors in the development of scleral ossicles. Localized inhibition of BMPs in this way causes a reduced expression of *ihh* in the surrounding tissue demonstrating that the BMP and Hedgehog pathways interact. Our data also demonstrates that the sclerotic ring has an intrinsic ability to compensate for missing elements. The scleral ossicle system provides a unique opportunity to investigate the epithelial–mesenchymal induction of intramembranous bones of the vertebrate skull.

Introduction

Skeletal development occurs via two main types of ossification; endochondral and intramembranous. Previous studies have largely focused on the development of endochondral bones (particularly in the limb) (reviewed in Johnson and Tabin, 1997). In comparison, far less is known regarding the development of intramembranous bones. The study of calvariae has produced the majority of the information regarding molecular pathways involved during intramembranous bone development (Abzhanov, et al., 2007; Cho et al., 2006; Elola et al., 2007; Hornik et al., 2004; Kang-Young et al., 2005; Kim et al., 1998; Opperman, 2000). However, the developmental pathways for other intramembranous bones, such as the scleral ossicles of the eye, remain unknown.

In birds, the scleral ossicles form a sclerotic ring, which plays a role during accommodation to achieve visual acuity and is responsible for maintaining eye shape (Franz-Odendaal and Vickaryous, 2006; Walls, 1942). These scleral ossicles are neural crest derived intramembranous bones that develop through interactions with conjunctival papillae (Couly et al., 2002; Franz-Odendaal and Vickaryous, 2006). These papillae are small, transient clusters of epithelial cells that form outgrowths of the conjunctival epithelium (Coulombre and Coulombre, 1962). Despite the fact that these papillae form from the conjunctiva, they are commonly referred to as 'scleral' papillae (Hamburger and Hamilton, 1951). These papillae can be found in direct correlation (1:1 ratio) with the number and pattern of scleral ossicles (Fig. 1) and there is a unique pattern to their development (first investigated and described by Coulombre and Coulombre, 1962). More recently this pattern was described in *Gallus gallus* (Franz-Odendaal, 2008) showing that the number of papillae present can range from 13 to 16 per eye. First a small group of papillae (3–4) will form over the ciliary artery, followed by a second group of papillae directly across from the first group (Fig. 1). Temporal then nasal groups of papillae form until there is a complete ring. Conjunctival papillae begin to develop at HH 30, by HH 37 the papillae have completely degenerated. The number of papillae (and therefore scleral ossicles) is often asymmetric from right to left eye in the same embryo, however there is rarely a difference of more than one papilla/ossicle per eye (Franz-Odendaal, 2008). Several studies demonstrate that the conjunctival papillae are inducing the underlying ectomesenchyme to form skeletal condensations. i) Removal of a single scleral (epithelial) papillae was shown to prevent the formation of the underlying ectomesenchymal scleral ossicle (Coulombre and Coulombre, 1962); ii) recombination experiments involving conjunctival epithelium and the mandibular or maxillary ectomesenchyme, induces bone in the mesenchyme (Hall, 1981); and iii) A diffusible factor signals between the scleral papillae and the underlying mesenchyme (Pinto and Hall, 1991). Despite this evidence, very little is

* Corresponding author. Fax: +1 902 4576455.
E-mail address: Tamara.Franz-Odendaal@msvu.ca (T.A. Franz-Odendaal).

0012-1606/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2012.02.016

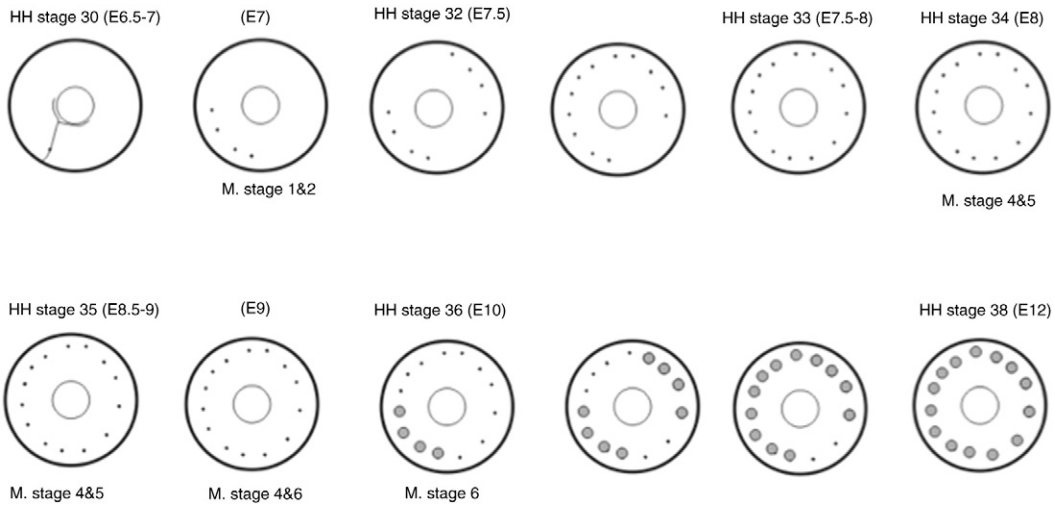

Fig. 1. Schematic showing the sequence of scleral papillae development and ossicle formation. Hamburger and Hamilton (HH) stages of embryonic development are given. Scleral papillae M stages are from Murray (1943). Solid black circles represent papillae, gray circles represent either ossicle condensation or scleral ossicles. Ciliary artery is shown in HH 30 to demonstrate the location of the first papillae and omitted from other schematics. Anterior is to the right.

known about which signaling molecules are involved in the developmental pathway of scleral ossicles.

Recently, we identified the involvement of a Hedgehog family in scleral ossicle development (Franz-Odendaal, 2008). When exogenous cyclopamine, an inhibitor of Hedgehog proteins, was applied next to a papilla at stage HH 35 and 36, the formation of the underlying ossicle was inhibited. Furthermore, *sonic hedgehog* expression was found in the conjunctival papillae at these stages indicating that it was a possible candidate for the epithelial mesenchymal induction of scleral ossicles. This study suggested, for the first time, a role for the Hedgehog family in the development of scleral ossicles, however, other members of the Hedgehog family, namely *Indian hedgehog* and *desert hedgehog*, were not ruled out at the time. *Desert hedgehog* is not found in the genome of chicken, zebrafinch or in the lizard genus, *Anolis*, and may be absent from all reptiles. *Indian hedgehog* however, is involved in osteoblast differentiation during endochondral ossification in the limb (e.g. Chung et al., 2001; Hu et al., 2005; Karp et al., 2000; Lai and Mitchell, 2005; Pathi et al., 1999) and more recently shown to be involved in the development of intramembranous neural crest derived bones of the avian and murine skull (Abzhanov et al., 2007). *Ihh* therefore could not be ruled out as a player in the induction of scleral ossicles and warrants further investigation.

The present study is a continuation of our investigation into scleral ossicle induction and development. First, we investigate the temporal and spatial expression pattern of the hedgehog receptor *ptc1* during scleral ossicle development. Patched1 (ptc1) is a transmembrane ligand receptor for multiple Hh proteins and is associated with a G-protein coupled transmembrane receptor molecule smoothened (smo) (Carpenter et al., 1998). Ptc1 normally inhibits the function of smo in the absence of any Hedgehog signal. However, when a Hedgehog protein binds to the ligand receptor ptc1, smo is no longer inhibited and a transmembrane transduction reaction occurs activating the downstream Hedgehog target. Ptc1 is also a downstream target of Hh signaling and therefore the location of the *ptc1* receptor is often used as an indicator of Hh activity (Harfe et al., 2004; Marigo et al., 1999; Traiffort et al., 1998). Next, we investigated the expression pattern of *Indian* hedgehog during ossicle development for the reasons described earlier.

We also wanted to determine whether the BMP family was involved in ossicle induction and/or development. The Bone Morphogenetic Protein (BMP) family of genes are a family of secreted proteins known to be important in bone growth and development. The role of BMPs includes the recruitment of mesenchymal cells into skeletogenic condensations (Hall and Miyake, 2000), the commitment of neural crest derived mesenchymal cells to skeletogenic lineages (Abzhanov et al., 2007), and epithelial–mesenchymal interactions (e.g. tooth development) (Thesleff, 2003). Our research previously demonstrated via real-time PCR that there is an increase in expression of the genes *bmp2* and *bmp4*, at HH stage 36 (the induction stage) relative to HH stage 33 (prior to induction). Here, we investigate the spatial and temporal expression pattern of *bmp2, bmp4,* and *bmp*7 during scleral ossicle induction and demonstrate that only bmp2 is expressed between HH stages 34.5 and 36. Furthermore, we demonstrate that scleral ossicles can be locally inhibited by exogenous Noggin, a secreted protein which binds to BMP proteins (predominately bmp2, bmp4 and bmp7) rendering them inactive (Zimmerman et al., 1996).

Our findings suggest that *ihh* is likely an important signaling molecule during the development of scleral ossicles since its expression is most similar to the expression of its downstream target, ptc1. *Shh* is likely involved only in the maintenance and/or proliferation of the papillae. Furthermore, we show that BMPs play critical role in the development of scleral ossicles, and that *bmp2* signaling interacts with *ihh* signaling in this system. Finally, our findings suggest that scleral ossicles have an intrinsic ability to compensate for missing elements within the sclerotic ring. Overall, these results provide a significant contribution to unravelling the molecular pathway underlying the development of scleral ossicles, and shed light on the development of other intramembranous bones within the vertebrate skull.

Materials and methods

Chicken embryos

Fertilized chicken eggs of the strain *Gallus gallus* were obtained from Cox Brothers Ltd, Truro, Nova Scotia, Canada. Eggs were incubated at 37 °C with approximately 40% humidity and turned once daily. Chicken embryos were staged using the Hamburger and Hamilton (1951) staging chart. Embryos were staged at HH stage 19 at the onset of *ex ovo* culturing and again prior to bead implantation at HH stage 35.

Ex ovo culturing

An *ex ovo* culturing method was used instead of windowing the eggs to ensure that there was no restriction in accessing the embryo during bead implantation at advanced stages of embryonic development. The *ex ovo* method was used as described in Franz-Odendaal