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Vascular endothelial growth factor signaling affects both angiogenesis and osteogenesis during the development of scleral ossicles

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ABSTRACT

Intramembranous ossification is a complex multi-step process which relies on extensive interactions among bone cells and surrounding tissues. The embryonic vasculature is essential in regulating endochondral ossification; however, its role during intramembranous ossification remains poorly understood, and *in vivo* studies are lacking. Previous research from our lab on the development of the intramembranous scleral ossicles has demonstrated an intriguing pattern of vascular development in which the areas of future osteogenesis remain avascular until after bone induction has occurred. Such avascular zones are located directly beneath each of the conjunctival papillae, epithelial structures which provide osteogenic signals to the underlying mesenchyme. Here we provide a high-resolution map of the developing vasculature from the time of ossicle induction to mineralization using a novel technique. We show that *vegfa* is expressed by the papillae and nearby mesenchymal tissue throughout HH 34–37, when vascular growth is taking place, and is down-regulated thereafter. Localized inhibition of Vegf results in expansion of the avascular zone surrounding the implanted papilla and mispatterning of the scleral ossicles. These results demonstrate that Vegf signaling could provide important insights into the complex relationship between bone and vasculature during intramembranous bone development.

1. Introduction

Intramembranous ossification is the process by which bones are formed via direct deposition of bone within a connective tissue membrane. Unlike endochondral ossification, a cartilage template is not involved. Intramembranous ossification requires that mesenchymal precursor cells form a dense cluster, or condensation, differentiate into osteoblasts and secrete collagen-rich osteoid which then undergoes mineralization.

Extensive interactions with the surrounding vasculature are required for the formation, maintenance and repair of endochondral (Yin and Pacifici, 2001; Jacobsen et al., 2008; Eshkar-Oren et al., 2009; Duan et al., 2015; Wiszniak et al., 2015) and intramembranous (Thompson et al., 1989; Zelzer et al., 2002; Jacobsen et al., 2008) bones. During endochondral ossification of the limb skeleton, for instance, remodeling of the vasculature occurs in a number of predictable steps. First, although the early limb bud contains a nearly homogeneous vascular meshwork, vessels regress away from areas in which condensations will develop (Hurle, 1985; Eshkar-Oren et al., 2009). Once the cartilage element has formed, the surrounding vasculature is remodeled to serve the perichondrium but not the cartilage matrix or chondrocytes. Finally, vasculature invades the hypertrophic zone of the cartilage and carries with it the chondroclasts and osteoblasts required to replace the cartilage template with bone (Maes et al., 2010). Although osteogenic condensations also form in avascular zones (Thompson et al., 1989; Jourdeuil and Franz-Odendaal, 2012), the role of vasculature during the development of intramembranous bones is not well understood. Indeed, compared to endochondral bones, intramembranous bones have received little attention in the literature (Percival and Richtsmeier, 2013). In order to examine the complex relationship between vasculature and bone we studied an easily accessible, neural crest-derived ring of intramembranous bones in the chick skull, the sclerotic ring. This ring is made up of 13–16 individual scleral ossicles that provide structural support to the eye. Each ossicle is attached to its neighbors by dense connective tissue, and they together form the sclerotic ring (Franz-Odendaal and Vickaryous, 2006).

Scleral ossicles develop in the eyes of birds and other reptiles in a 1:1 relationship with overlying epithelial structures termed conjunctival papillae (Murray, 1941; Coulombre, et al., 1962; Franz-Odendaal and Vickaryous, 2006). The papillae develop in a conserved pattern as follows. The first papilla forms at Hamburger and Hamilton stage 30 (HH 30) directly over the ciliary artery, and is

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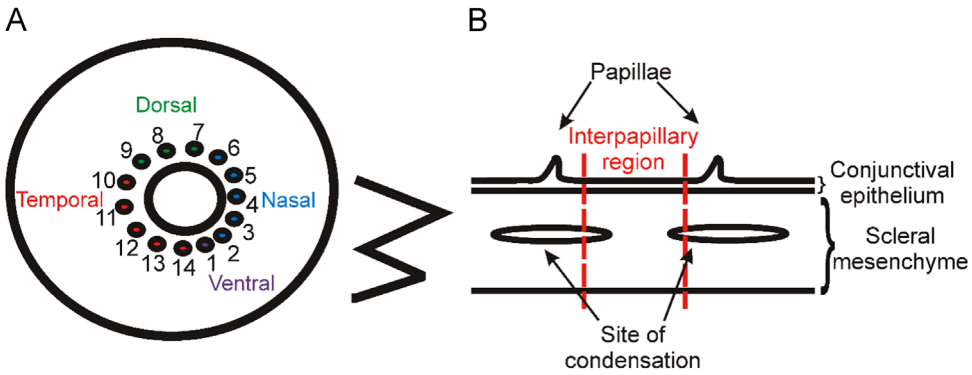

Fig. 1. (A) Schematic showing the numbering convention of the conjunctival papillae and scleral ossicles. Papilla #12 forms directly over the ciliary artery (not shown) and all other papillae are numbered according to their position relative to #12. Anterior is to the right. (B) Cross section through two conjunctival papillae. The papillae are epithelial structures which overlie the scleral mesenchyme. The space between neighboring papillae is termed the interpapillary region. The scleral ossicle condensations form in the mesenchyme directly below each papilla.

designated papilla #12. This is followed by the remainder of the temporal group (#10-14), the nasal group (#2-6), the dorsal group (#7-9) and finally a ventral papilla (#1) forms directly over the choroid fissure (Coulombre et al., 1962; Franz-Odendaal, 2008; Fig. 1A). The ring of papillae is completely formed by HH 34 (Franz-Odendaal, 2008). The papillae are separated from one another by an interpapillary zone (Fig. 1B). The conjunctival papillae induce the formation of the ossicles in the underlying scleral mesenchyme beginning at HH 35 (Fig. 1B); the papillae then slowly degenerate over the next 1.5–2 days (Coulombre et al., 1962; Van de Kamp, 1968; Franz-Odendaal, 2008). Since the papillae develop at different times around the eye, the induction of the underlying scleral ossicles is similarly staggered. The skeletogenic condensations are first visible in the mesenchyme in histological section by HH 36.5 (Jabalee et al., 2013). These condensed cells differentiate into osteoblasts and deposit osteoid until approximately HH 38, when mineralization begins (Franz-Odendaal, 2008; Zhang et al., 2012).

Recently we used a combination of camera lucida drawings and erythrocyte autofluorescence to demonstrate that vasculogenesis begins in the sclera of the chick eye at HH 34, the same time at which the complete papilla ring has formed (Jourdeuil and Franz-Odendaal, 2012). These small, open-ended vessels continue to branch and connect with one another, forming a vessel meshwork at HH 35. Interestingly, distinct avascular zones are seen below the base of the papillae in the temporal region, much like the avascular zones which precede limb cartilage formation (Eshkar-Oren et al., 2009; Jourdeuil and Franz-Odendaal, 2012). In the limb, vascular regression results in localized areas of hypoxia (low oxygen tension) thought to be necessary for chondrocyte differentiation (Amarilio et al., 2007; Provot et al., 2007). That avascular zones precede the formation intramembranous bones during development is especially odd given the apparent inverse correlation between hypoxia and osteoblast differentiation demonstrated in cell culture (Salim et al., 2004; D'Ippolito et al., 2006).

This study furthers our recent exploration into the role of vasculature in scleral ossicle development. First, we re-visit vascular growth in the sclera using a novel high-resolution technique (Takase et al., 2013) and extend our analysis to older stages not previously examined. This technique only detects blood vessels that have connected with the main vascular system of the embryo and exhibits much higher resolution than erythrocyte autofluorescence used previously (Jourdeuil and Franz-Odendaal, 2012), particularly at advanced stages of development. We then analyzed the expression of vascular endothelial growth factor A (*vegfa*) using *in situ* hybridization from HH 34–38.5. Vegf is a well-known regulator of angiogenesis and plays a key role in coordinating blood vessel formation with bone development during endochondral ossification (Zelzer and Olsen 2005; Eshkar-Oren et al., 2009) and bone repair (Street et al., 2002). Despite the importance of Vegf in these processes, comparative expression data for endochondral versus intramembranous bones is not available. We then inhibited Vegf by implanting inhibitor-soaked beads adjacent to individual papillae at HH 35, the onset of ossicle induction. A combination of ink-injection and alkaline phosphatase staining was used to determine the effect of this Vegf inhibition on scleral vasculature and ossicles, respectively.

Our results demonstrate that Vegf plays a key role in regulating ossicle growth, but *vegfa* is not expressed by osteoblasts themselves. Instead, the papillae and surrounding superficial mesenchyme act as a source of Vegf beginning just prior to ossicle induction and ending as the papillae degenerate. Not surprisingly, Vegf inhibition causes an increase in the size of the avascular zone surrounding the bead. Collectively, the data shows that papilla-derived Vegf is an important regulator of vascular growth and patterning. Furthermore, Vegf inhibition results in abnormal sclerotic ring development, indicating that Vegf may also be important for condensation formation and/or osteoblast differentiation.

2. Materials and methods

2.1. Chicken embryos

Fertilized White Leghorn (*Gallus gallus*) eggs were obtained from the Nova Scotia Agricultural College (Truro, NS) or Cox Brothers Farm (Truro, NS) depending on availability. Eggs were incubated at 37 ± 1 °C, 40% humidity and were turned 1–2 times daily. On the third day of incubation, eggs were windowed as previously described (Silver, 1960; Korn and Cramer, 2007). Chicken embryos were staged according to the Hamburger and Hamilton (1951) staging series.

2.2. In situ hybridization

Plasmids containing *vegfa* were a kind gift from Todd Camenisch (University of Arizona, USA). Whole-mount *in situ* hybridization was performed as previously described (Nieto et al., 1996; Franz-Odendaal, 2008) with modification. Half-heads were reacted with antisense probe at each stage examined. Embryos receiving either no probe or sense probe were used as negative controls. Whole-mount stained tissues were cryosectioned at 10–12 μm thickness.