Mechanisms of Development 153 (2018) 54–63

Contents lists available at ScienceDirect

Mechanisms of Development

journal homepage: www.elsevier.com/locate/mod

Roles of basal keratinocytes in actinotrichia formation

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ABSTRACT

The embryonic fins and the tip of adult fins of teleost fish are supported by rows of straight, unmineralized fibrils called actinotrichia. The proximal ends of the actinotrichia are bundled and the mineralized bones called lepidotrichia are made along them. Since malformation in actinotrichia causes wavy fin bones, the correct configuration of actinotrichia is essential for the correct construction of the fin shape. Past studies suggested that two types of cells, basal keratinocytes, and mesenchymal cells involve in the formation of actinotrichia. However, the mechanism how these cells contribute is unknown. In this study, we elucidated the role of basal keratinocytes in actinotrichia formation. First, we developed the imaging tool that specifically visualizes the basal keratinocytes and actinotrichia. Then, we established the *in vitro* culture method of the basal keratinocytes and found that the keratinocytes developed fine needle-like structures in it. The TEM image of them showed the specific shadow pattern of actinotrichia, indicating that the fine needle-like structures are the newly made actinotrichia. Finally, we cultured the basal keratinocytes with mature actinotrichia and observed that the basal keratinocytes actively holded actinotrichia with their membrane, and often generated a linear array of cells holding a single actinotrichium. This behavior suggested a mechanism with which long actinotrichia are made by relatively small cells. Our results clarified the role of basal keratinocyte and provided a novel insight into understanding the mechanism of actinotrichia formation.

1. Introduction

The shape and arrangement of vertebrate bones determine the movement of the body. Therefore, the mechanism regulating the bone shape is a major question in the study of morphogenesis. In the fins of adult teleost fish, bones (fin-rays) radially extend toward the tip of the fins. Each fin-ray is segmented and periodically bifurcate to guarantee its flexible movement and uniform strength (Bird and Mabee, 2003).

The process of fin bone generation has been studied extensively in zebrafish. Zebrafish embryos also have fins. However, they are not supported by bones, but by the straight fibers called actinotrichia that radially distribute in the embryonic fin (Fig. 1A, B) (Geraudie, 1977; Wood and Thorogood, 1987; van den Boogaart et al., 2012). The growing tip of adult fins is also supported by actinotrichia, which are bundled in more proximal regions of the fin rays and the lepidotrichia radially develop besides them. After the lepidotrichia are formed, the actinotrichia gradually become restricted to the distal tip of the fin rays (Fig. 1C and Fig. 2E and F) (Becerra et al., 1983; Grandel and Schulte-Merker, 1998; Mari-Beffa and Murciano, 2010). During fin regeneration, actinotrichia are also generated antecedent to the formation of lepidotrichia (Kemp and Park, 1970; Mari-Beffa et al., 1989; Santamaria and Becerra, 1991; Becerra et al., 1996; Quint et al., 2002; Akimenko et al., 2003; Smith et al., 2008; Bockelmann and Bechara, 2009; Pfefferli and Jazwinska, 2015; Thorimbert et al., 2015; Bhadra and Iovine, 2015; Konig et al., 2018). In all cases, actinotrichia play the role of physical support of the fin tip, and look guiding the position where shape the calcified bones are made (Huang et al., 2009). According to the previous studies with zebrafish, the orderly arrangement of actinotrichia is necessary to correctly produce lepidotrichia because the fin-rays often become wavy in the mutants with defects in actinotrichia formation (Huang et al., 2009). Therefore, in order to understand the fin bone formation, clarifying how the actinotrichia are formed and arrayed correctly are pivotal.

Isolated actinotrichia have straight shape and tapered at both ends (Fig. 1D). Although the size of actinotrichia varies, the maximum length is around 100 μm at larval stage (Fig. 1D), which is much larger than the cells in the fin. Actinotrichia are detected by anti-col1 and anti-col2 antibodies, suggesting collagen1 and 2 are the major components of actinotrichia (Duran et al., 2011). Transmission electron microscopic (TEM) images of actinotrichia showed the structural similarity with the collagen fibrils (Kimura and Kubota, 1966; Kemp, 1977; Geraudie and Meunier, 1980; Dane and Tucker, 1985; Wood and Thorogood, 1987; Mari-Beffa et al., 1989).

Zhang et al. (2010) identified gene family named *actinodin* (*and*) that is assumed to contribute to the formation of actinotrichia (Zhang et al., 2010). The *and* gene family consists of four homologues: *and1*, *and2*, *and3* and *and4* (Zhang et al., 2010). Double morphants of *and1* and *and2* showed hypoplasia during actinotrichia formation in zebrafish larvae, suggesting these genes are crucial for the formation of actinotrichia (Zhang et al., 2010).

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https://doi.org/10.1016/j.mod.2018.08.010
Received 9 March 2018; Received in revised form 21 August 2018; Accepted 22 August 2018
Available online 06 September 2018

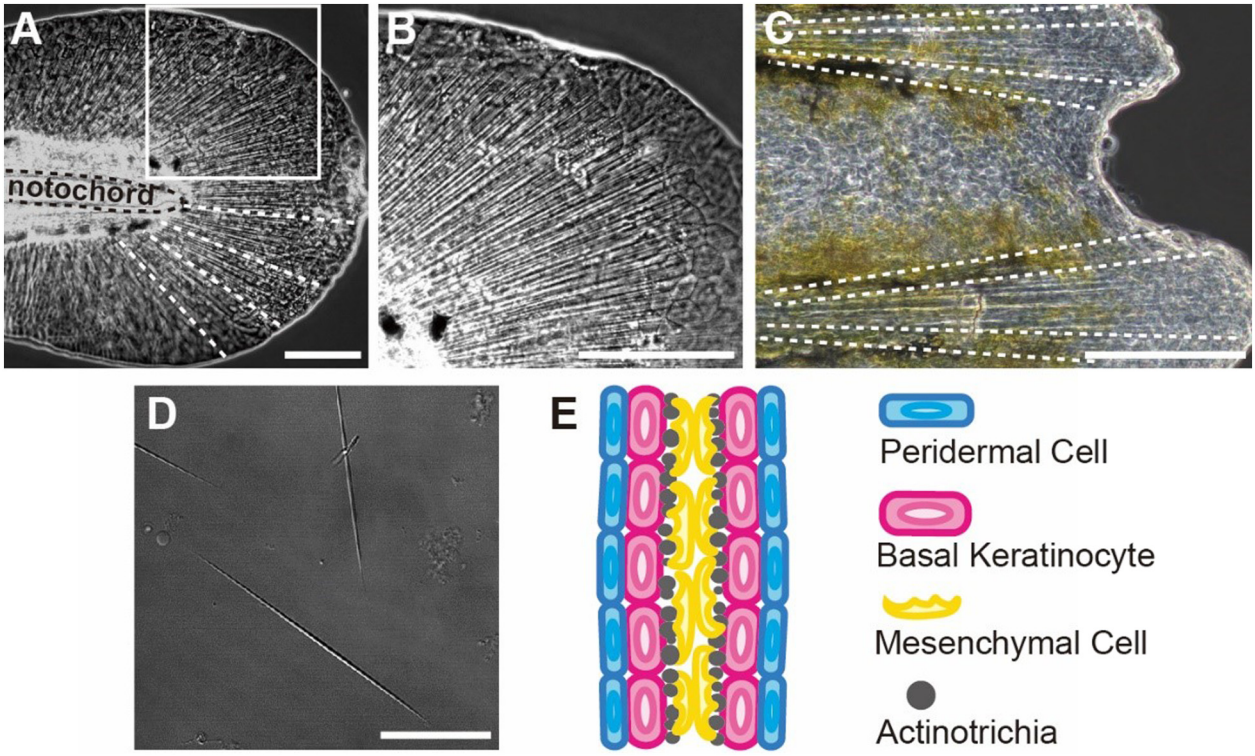

Fig. 1. The distribution pattern of actinotrichia in zebrafish fin. (A) Phase contrast image of the median fin fold in larva at 3 days post-fertilization (dpf). Actinotrichia are radially distributed throughout the whole fin. (B) Magnified view of the box region in (A). (C) Distal tip of the adult caudal fin. Actinotrichia radially extend from the edge of each fin-ray to distal fin tip. White dot lines in (A and C) indicate actinotrichia orientation. (D) Confocal image of the actinotrichia isolated from larval fin. (E) Schematic diagram of the transverse section at the very tip of fin modified from Dane and Tucker (1985) and Wood and Thorogood (1987). Actinotrichia are located inside the epidermis constituted by two epidermal cell layers. Scale bar is 100 μm in (A–C) and 50 μm in (D).

The configuration of the cells and actinotrichia in the fins is studied in the past reports (Dane and Tucker, 1985; Wood and Thorogood, 1987) (Fig. 1E). The tip of the zebrafish fin is composed of epidermal cell layer and mesenchymal cells. Epidermal cell layer consists of two distinct cell layers, outer peridermal cells and inner basal keratinocytes (Dane and Tucker, 1985; Wood and Thorogood, 1987; Hatzold et al., 2016) (Fig.1E). Previously, Lalonde et al. (2016) identified cell expressing *and1* using GFP (Lalonde et al., 2016). When GFP was expressed under the control of *and1* promoter (2 kb promoter), a basal layer of cells (basal keratinocytes) and mesenchymal cells locating inside of the actinotrichia array were found to express GFP (Lalonde et al., 2016). *In situ* hybridization of *and* genes also confirmed the expression in these two types of cells (Zhang et al., 2010). Zhang and Lalonde concluded that these cells cooperatively involve in the formation of actinotrichia (Zhang et al., 2010; Lalonde et al., 2016; Lalonde and Akimenko, 2018). However, the detailed roles of these cells remain unknown.

The formation of actinotrichia is a dynamic phenomenon that is carried out by multiple types of cells at the growing tip of the fins. Therefore, understanding its mechanisms *in vivo* is quite difficult. To overcome such difficulty, a standard method is to isolate each cell type and to examine its function in a condition where other cell types do not exist. In this paper, therefore, we focused on the role of basal keratinocytes and observed the behavior of the isolated basal keratinocytes *in vitro*. Results of the *in vitro* study suggested that basal keratinocytes were able to initiate the production of actinotrichia by themselves and elongate it by generating an array of cells holding a single actinotrichium.

2. Results

2.1. Visualization of the basal keratinocytes and the actinotrichia

First, we tried to identify the promoter region that is active only in the basal keratinocytes. *and1* gene is expressed in both basal keratinocytes and mesenchymal cells (Zhang et al., 2010). We dissected the promoter of the *and1* gene and identified a 1.4 kb fragment (see Supplemental Fig. 1) that is specifically active only in the cell layer beneath the layer of epidermal cells. Fig. 2A–C shows the expression of GFP-CaaX (CaaX is a peptide adaptor that makes the protein stays in the membrane) driven by the *keratin4* promoter and mCherry-CaaX driven by 1.4 kb of the *and1* promoter, respectively. *keratin4* is specifically expressed in the peridermal cells (Gong et al., 2002; Lee et al., 2014; Hatzold et al., 2016). As shown in Fig. 2A–C, the 1.4 kb *and1* promoter was inactive in the peridermal cells. Fig. 2C shows an optical section of the fin. At the middle part of the fin where the mesenchymal cells locate, no mCherry expression was observed. These data confirmed that the expression due to 1.4 kb *and1* promoter was limited in basal keratinocytes.

Second, we tried to label the actinotrichia with GFP. Because the And1 protein is one of the component of the actinotrichia (Zhang et al., 2010; Thorimbert et al., 2015; Konig et al., 2018), we fused GFP to the full-length And1 protein. However, this construct showed very faint fluorescence in actinotrichia (data not shown), suggesting that the GFP protein was inhibiting the aggregation of And1 protein to the other components of actinotrichia. Then, we dissected the coding region of the *and1* gene and found the And1^{480bp}-GFP construct (see Supplemental Fig. 1) brightly visualize the actinotrichia in both embryo and grown fish (Fig. 2D–F).

The image obtained by the GFP is significantly different from that of anti-type II collagen antibodies used in the previous studies