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Short Communication

Foxi1 promotes late-stage pharyngeal pouch morphogenesis through ectodermal Wnt4a activation

Sil Jin[a], Jiyun O[b], Frank Stellabotte[c], Chong Pyo Choe[a,b,*]

[a] Division of Applied Life Science (BK21 Plus Program), Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 52828, Republic of Korea
[b] Division of Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea
[c] School of Allied Health, Business, and STEM, Middlesex Community College, Middletown, CT 06457, USA

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ABSTRACT

The pharyngeal pouches are a series of epithelial outgrowths of the foregut endoderm. Pharyngeal pouches segment precursors of the vertebrate face into pharyngeal arches and pattern the facial skeleton. These pouches fail to develop normally in zebrafish *foxi1* mutants, yet the role Foxi1 plays in pouch development remains to be determined. Here we show that ectodermal Foxi1 acts downstream of Fgf8a during the late stage of pouch development to promote rearrangement of pouch-forming cells into bilayers. During this phase, *foxi1* and *wnt4a* are coexpressed in the facial ectoderm and their expression is expanded in *fgf8a* mutants. *foxi1* expression is unaffected in *wnt4a* mutants; conversely, ectodermal *wnt4a* expression is abolished in *foxi1* mutants. Consistent with this, *foxi1* mutant pouch and facial skeletal defects resemble those of *wnt4a* mutants. These findings suggest that ectodermal Foxi1 mediates late-stage pouch morphogenesis through *wnt4a* expression. We therefore propose that Fox1 activation of Wnt4a in the ectoderm signals the epithelial stabilization of pouch-forming cells during late-stage of pouch morphogenesis.

1. Introduction

Vertebrate craniofacial development relies on precise spatiotemporal interactions and signals between cranial-neural-crest-derived pharyngeal arches, their mesodermal cores, and surrounding ectodermal and endodermal epithelia (Couly et al., 2002; Crump et al., 2004; Piotrowski et al., 2003). The endodermal epithelia exist as segmented pharyngeal pouches, required for patterning and morphogenesis of the craniofacial skeleton. Later, these pouches go on to generate important organs including the Eustachian tube, thymus, parathyroid, and gills in the face and neck (Gordon et al., 2001; Graham et al., 2005; Grevellec and Tucker, 2010; Proctor, 1967; Schwend and Ahlgren, 2009).

Pouch morphogenesis occurs in stages in which pouch-forming cells undergo dynamic epithelial transitions, remodeling, and changes in cell shape and neighbor relationships (Choe et al., 2013). Morphogenesis takes place in two stages which are controlled by multiple ectodermal, mesodermal, and endodermal signaling pathways. During the early stage, endodermal pouch-forming cells lose their columnar morphology, become multilayered, and migrate collectively toward facial ectoderm (Choe et al., 2013). Tbx1-dependent Wnt11r expression from the adjacent mesoderm is responsible for cellular shape changes while mesodermal Tbx1 activation of Fgf8a guides the collective migration of these cells (Choe et al., 2013; Choe and Crump, 2014).

As pouches grow toward the facial ectoderm, ectodermal Wnt4a is required for the junctional localization of Alcama in endodermal pouch-forming cells (Choe et al., 2013). During late-stage pouch morphogenesis, Alcama drives the rearrangement of migrating endodermal cells into the formation of bilayered pouches (Choe et al., 2013). At the end of the late stage, Wnt4a and EphrinB activation of Pak2a maintains the integrity of bilayered mature pouches by further increasing intercellular adhesion (Choe and Crump, 2015). While the mesodermal Tbx1-Wnt11r-Fgf8a pathway that controls early-stage pouch morphogenesis is well understood, genetic control of late-stage, ectodermal Wnt4a expression remains poorly understood (Choe and Crump, 2014). Here, we report that ectodermal *wnt4a* expression is positively regulated by Foxi1, while both *wnt4a* and *foxi1* expression are restricted by Fgf8a in the ectoderm.

Zebrafish *foxi1* mutants and mouse *foxi3* (a *foxi1* functional homologue) mutants display craniofacial defects in otic placode, facial skeleton, and pharyngeal pouch development (Edlund et al., 2014; Nissen et al., 2003; Solomon et al., 2003). Perturbations in survival and proliferation of cranial-neural-crest-derived ectomesenchymes in the pharyngeal arches result in facial skeletal defects (Edlund et al., 2014;

* Corresponding author at: Division of Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea.
E-mail address: cpchoe@gnu.ac.kr (C.P. Choe).

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Nissen et al., 2003; Solomon et al., 2003). In zebrafish the survival and proliferation deficits of these ectomesenchymes were partially rescued by activating Fgf3/8 signaling in the facial ectoderm at 20 h post-fertilization (hpf), yet they could not rescued by activating Fgf3/8 signaling after 22 hpf when most pouches have developed in zebrafish (Edlund et al., 2014). Since it has been demonstrated that pouches play a role in ectomesenchymal survival (Brito et al., 2006), the facial skeletal defects seen in *foxi1* mutants could also be attributed to defective pouch formation. *foxi1* is expressed in the pharyngeal endoderm as well as in the facial ectoderm, but not in the ectomesenchymes of the arches during pharyngeal arch development (Nissen et al., 2003; Solomon et al., 2003). Previously, it was proposed that a Fgf3- and Foxi1-dependent regulation of Pax8 may pattern the pharyngeal endoderm autonomously to form pouches, as Foxi1 regulates Pax8 expression in the pouches through endodermal Fgf3 expression (Nissen et al., 2003). However, the role that ectodermal Foxi1 plays in remodeling endoderm into pouches has yet to be analyzed. Here, we show that ectodermal Foxi1 promotes rearrangements of pouch-forming cells into bilayered pouches during late-stage pouch morphogenesis through ectodermal Wnt4a activation.

2. Results

2.1. Foxi1 is required for remodeling of pouch-forming cells during late-stage pouch morphogenesis

In order to understand how Foxi1 controls pouch morphogenesis at the cellular level, we first reanalyzed *foxi1* mutants using Alcama immunohistochemistry to label pouches. While five bilayered mature pouches were present at 34 hpf in wild-type siblings (Fig. 1A), fewer abnormally shaped pouches formed in *foxi1* mutants whose anterior pouches were typically missing (Fig. 1B, M). Upon close inspection these pouch-forming cells were inappropriately multilayered compared to wild-type pouches (Fig. 1B, F), which suggests that pouch morphogenesis fails to progress to the late stage when pouches become bilayered. Previously, we reported that pouch patterning mutants can be divided into two groups: one which includes *tbx1* and *wnt11r* mutants that display early-stage defects including a delay or failure of pouch outgrowth, and a second group including *wnt4a* and *ephrinb2a* mutants, that display late-state defects such as multilayered, immature or missing pouches (Choe et al., 2013; Choe and Crump, 2014, 2015). Even though we cannot completely rule out that the absence of the anterior two pouches in *foxi1* mutants could be a consequence of early defects in pouch outgrowth, the failure of pouch-forming cells to mature into a bilayer in mutants suggests that Foxi1 is required for late-stage pouch morphogenesis.

2.2. Foxi1 regulates late-stage pouch morphogenesis through ectodermal Wnt4a

We examined the effect of the *foxi1* mutation on late-stage pouch development. It has been proposed that Foxi1 modulates downstream cellular responses to Fgf3 signaling during pouch development based on a loss of *fgf3* expression in *foxi1* mutant pouches (Nissen et al., 2003). Consistent with this model, we predicted that similar pouch defects would be found in both *foxi1* and *fgf3* mutants. We tested this possibility by first confirming the reduction in *fgf3* expression seen in *foxi1* mutant pouches. In wild-type embryos, *fgf3* is expressed in pouches, the strongest expression in nascent pouches (David et al., 2002). As pouches formed we noticed an anterior-to-posterior wave of increasing *fgf3* expression with the most robust expression found in the last three pouches (Fig. 2A). In *foxi1* mutants, we observed a similar *fgf3* expression pattern in the posteriormost two to three pouches, but we found that *fgf3* expression in the nascent pouches was weaker relative to wild-type expression, confirming the positive regulation of Fgf3 by Foxi1 in the endoderm (Fig. 2B). We next analyzed whether Foxi1 regulated late-stage pouch morphogenesis through Fgf3 signaling. As Fgf3 has been implicated in pouch development by controlling pouch cell migration (Crump et al., 2004; Herzog et al., 2004), we compared *fgf3* mutant pouches with *foxi1* mutant pouches expecting to see similar, multilayered immature pouches in both. In *fgf3* mutants, the anterior two pouches formed, while the posterior pouch-forming cells failed to migrate out (Fig. 1C, G, M). These *fgf3* mutant phenotypes differ markedly from *foxi1* mutant phenotypes and suggest that posterior pouch morphogenesis fails to initiate in *fgf3* mutant pharyngeal endoderm. We also analyzed ceratobranchial (CB) cartilages whose development is contingent upon appropriate pouch formation in 5 dpf-old *fgf3* mutants. In wild-type embryos, five CB cartilages form from the posterior pharyngeal arches that are segmented by posterior pouches (Fig. 1I, M). In *fgf3* mutants, vestiges of the anterior CB cartilages form, yet the posterior CB cartilages form as one fused cartilage, consistent with the absence of posterior pouches (Fig. 1K, M). The defects in outgrowth of pouch-forming cells and the fused giant CB cartilages seen in *fgf3* mutants strongly suggest that pouch formation fails to initiate during early-stage pouch morphogenesis, which is in contrast to the defects expected in *foxi1* mutants. We also noted that the CB cartilage defects found in *foxi1* mutants differed from those of *fgf3* mutants. Instead of a single, fused CB, we found a reduction in the number of normally shaped CBs in *foxi1* mutants (Fig. 1J, M). Though we cannot completely rule out the possibility that Fgf3 signaling acts with Foxi1 to control late-stage pouch morphogenesis, we are impeded from testing this as the *fgf3* mutant pouches fail to progress beyond the early stage. Therefore, we are unable to find any evidence suggesting that Foxi1 regulates late-stage pouch morphogenesis through Fgf3.

We found that the multilayered pouch phenotypes and the missing CB cartilage phenotypes seen in *foxi1* mutant pouches were more severe yet reminiscent of the *wnt4a* mutant phenotypes (Fig. 1D, H, L, M; Choe et al., 2013). These common phenotypes between *foxi1* and *wnt4a* mutants suggest that Foxi1 and Wnt4a are genetically linked to control late-stage pouch morphogenesis. We found these common phenotypes intriguing and examined the expression of *foxi1* and *wnt4a* during late-stage pouch morphogenesis. While *wnt4a* is expressed segmentally in the facial ectoderm (Fig. 2D; Choe et al., 2013), *foxi1* is expressed in the ectoderm as well as pouch endoderm during wild-type pouch morphogenesis (Fig. 2F; Nissen et al., 2003; Solomon et al., 2003). We found that *wnt4a* and *foxi1* were co-expressed in the ectoderm of wild-type embryos (Fig. 2C), while *wnt4a* expression was significantly reduced in *foxi1* mutants (Fig. 2D, E). However, the endodermal and ectodermal *foxi1* expression was unaffected in *wnt4a* mutants (Fig. 2F, G). This epistatic analysis indicates that Foxi1 acts upstream of Wnt4a to positively regulate its ectodermal expression during pouch development. We therefore propose that Foxi1 controls late-stage pouch morphogenesis through ectodermal Wnt4a, organizing pouch-forming cells into bilayered pouches.

2.3. Fgf8a acts as a negative regulator of ectodermal foxi1 expression during the pouch morphogenesis

In order to further understand the genetic hierarchies at play during pouch morphogenesis, we analyzed *foxi1* expression in *fgf3*, *tbx1*, and *fgf8a* mutants that showed pouch defects (Crump et al., 2004; Herzog et al., 2004; Piotrowski et al., 2003; Piotrowski and Nusslein-Volhard, 2000). Both endodermal and ectodermal *foxi1* expression were unaffected in *fgf3* mutants compared to wild-type embryos (Fig. 3A, B, E, F, I, J). In *tbx1* mutants, the endodermal *foxi1* expression was significantly reduced, whereas the ectodermal expression was expanded, suggesting that Tbx1 regulates *foxi1* expression positively and negatively in the endoderm and ectoderm, respectively (Fig. 3C, G, K). Interestingly, in *fgf8a* mutants, the ectodermal expression of *foxi1* was dramatically expanded, yet normal *foxi1* expression was seen in the disorganized endoderm (Fig. 3D, H, L).

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