Eph-Pak2a signaling regulates branching of the pharyngeal endoderm by inhibiting late-stage epithelial dynamics

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ABSTRACT
Branching morphogenesis depends on the precise temporal and spatial control of epithelial dynamics. In the vertebrate head, endodermal branches, called pharyngeal pouches, form through the transient stratification, collective migration and reorganization of epithelial cells into bilayers. Here, we report novel requirements for the EphrinB ligands B2a and B3b, the Ephb4a receptor and the Pak2a kinase in the development of pouches and the posterior facial skeleton that depend on pouches for its segmentation. Time-lapse imaging in zebrafish shows that EphB-Pak2a signaling is required to stabilize pouch epithelial cells at the end of branching morphogenesis. Transgenic rescue experiments further demonstrate that endodermal Eph-ephrin signaling promotes pouch integrity by targeting Pak2a to the plasma membrane, where subsequent activation by Wnt4a-Cdc42 signaling increases junctional E-cadherin in maturing pouches. Integration of Eph-ephrin and Wnt4a signaling through Pak2a thus signals the end of branching morphogenesis by increasing intercellular adhesion that blocks further epithelial rearrangements.

KEY WORDS: Eph-ephrin, PAK, Branching morphogenesis, Craniofacial, Pharyngeal pouches, Zebrafish

INTRODUCTION
During development, epithelial tissues undergo morphogenesis to form elaborately branched organs. Whereas epithelial cells are characterized by apicobasal polarity and strong E-cadherin-mediated intercellular adhesion, dynamic changes in polarity and adhesion facilitate the cell rearrangements that drive epithelial morphogenesis (Wang et al., 2012, 2013). We still know little, however, about what restores polarity and strong adhesion, and hence stops epithelial rearrangements, once branches have formed.

Pharyngeal pouches arise as outpocketings of the foregut endoderm and help to organize later development of the head and neck (Graham, 2008). During their formation in zebrafish, pre-pouch endodermal cells first lose their columnar morphology to become multilayered, which coincides with their collective movement towards the ectoderm (Choe et al., 2013). At the end of outpocketing, cells re-establish columnar morphology and form mature bilayers. These epithelial transitions are guided first by mesodermal Wnt11r, which stimulates the multilayering process, and then by ectodermal Wnt4a, which reorganizes maturing epithelial cells into bilayers. Here, we show that Wnt4a acts together with EphrinB signaling to stabilize mature bilayers.

Eph-ephrin signaling has been implicated in a variety of morphogenetic processes (Dravis et al., 2004; Holmberg et al., 2000; Klein, 2012), with EphB2 and EphB3 being required to stabilize E-cadherin during lumenization of the mouse pancreas (Villasenor et al., 2010). A candidate for mediating these effects of Eph signaling in epithelial morphogenesis is the family of p21-activated kinases (PAKs), as PAKs have been shown to stabilize E-cadherin during zebrafish epiboly (Tay et al., 2010). Whereas these serine/threonine kinases are defined by their activation by small GTPases, such as Cdc42 and Rac (Knaus et al., 1995; Manser et al., 1994; Martin et al., 1995), PAKs are also targets of EphB signaling during nervous system development (Cowan and Henkemeyer, 2001; Kayser et al., 2008). Here, we show that EphB receptors similarly signal through Pak2a in pouch epithelia, with Pak2a terminating pouch outgrowth by stabilizing E-cadherin and inhibiting further cell rearrangements.

RESULTS AND DISCUSSION

\textbf{efnb2a, efnb3b, ephb4a and pak2a are expressed in developing pouches}

In an \textit{in situ} hybridization screen of zebrafish Eph-ephrin genes, we uncovered expression of one Eph receptor (\textit{ephb4a}) and two EphrinB ligands (\textit{efnb2a} and \textit{efnb3b}) in \textit{her5:GFP} pouch-forming endoderm (Fig. 1A-J). At the onset of pouch formation [18 h post-fertilization (hpf)], \textit{efnb2a} is expressed in migrating neural crest cells of the second arch, whereas endodermal expression of \textit{efnb2a} and \textit{efnb3b} is low. At this time, \textit{ephb4a} is expressed in the posterior pouch-forming endoderm. As arch development proceeds (24 and 30 hpf), endodermal expression of \textit{efnb2a} increases in anterior pouches, whereas \textit{efnb3b} and \textit{ephb4a} become enriched in more posterior pouches. By 30 hpf, co-labeling with neural crest-specific \textit{sox10:GFP} and mesoderm-specific \textit{nkx2.5:GFP} transgenes show that, in addition to endoderm, \textit{efnb2a} and \textit{ephb4a} are also expressed in neural-crest-derived cells and arch mesoderm, respectively (supplementary material Fig. S1). Similar to \textit{efnb2a} transcripts, Efnb2a protein is observed in both maturing pouches and neural-crest-derived mesenchyme, where it localizes to cell membranes (Fig. 1N,O). In addition, we detect dynamic expression of \textit{pak2a} in developing pouches (Fig. 1K-M). Thus, \textit{efnb2a}, \textit{efnb3b}, \textit{ephb4a} and \textit{pak2a} are all expressed in the pouch-forming endoderm, although the restriction of \textit{ephb4a} to posterior pouches suggests the involvement of additional Eph receptors in the anterior pouches.

\textbf{Requirements for Eph-ephrin and Pak2a signaling in pouch development}

Next, we used loss-of-function approaches to determine requirements for Eph-ephrin and Pak2a signaling in pouch development. In \textit{efnb2a} mutants, the normal complement of five pouches formed by 34 hpf, yet pouches were short and disorganized (Fig. 2B). This \textit{hu3393} allele of \textit{efnb2a} is probably a complete null allele, as we detected no Efnb2a protein in mutants (Fig. 2R).
To confirm requirements of Eph-ephrin and Pak2a signaling in pouch development, we also analyzed the ceratobranchial cartilages (CBs) that depend on pouches for their development. As predicted, loss of pak2a or pairwise (but not individual) reductions of efnb2a, efnb3b and ephb4a resulted in losses and/or fusions of CBs (Fig. 2J–M; supplementary material Fig. S2C). In addition, efnb2a and pak2a single mutants displayed fusions of CB1 to the ceratohyal cartilage (CH) of the second arch. As efnb2a is expressed in neural-crest-derived cells of the second arch, we examined whether these CB1-CH fusions might be due to roles of Eph signaling in neural crest migration (Smith et al., 1997). However, analysis of dlx2a expression at 16.5 hpf revealed no intermixing of second and third arch neural crest streams (which generate CH and CB1) in efnb2a mutants, either alone or in combination with reduced efnb3b and ephb4a (supplementary material Fig. S2E). Furthermore, restoration of Efnb2a to the neural crest in sox10;Efnb2a transgenics failed to rescue the CB1-CH fusions and pouch/CB defects of Ephrin-deficient embryos (supplementary material Fig. S2B–D,G). Instead, live imaging of efnb2a−/− arches, followed by cartilage staining in the same individuals, revealed a precise correlation of CB1-CH fusions with earlier shortening of the her5:mCherryCAAX′ second pouch that separates second and third arch sox10;GFP neuronal-crest-derived cells (supplementary material Fig. S2F). Hence, both CB defects and CB1-CH fusions can be explained by earlier requirements of Eph-ephrin and Pak2a signaling in pouch development.

Endodermal roles of Eph-ephrin and Pak2a signaling in pouch development

To test whether Eph-ephrin and Pak2a function in the endoderm for pouch and cartilage development, we next blocked the activity of these pathways just in the endoderm, using dominant-negative transgenes. Specifically, we used an endodermal nkx2.3:Gal4VP16 driver (Choe et al., 2013) to express kinase-dead (KD) forms of EphB4a (Zisch et al., 1998) and Pak2a (Sells et al., 1997; Tang et al., 1997). Similar to loss-of-function embryos, endoderm-specific disruption of EphB or Pak2a activity resulted in reduced numbers of dysmorphic pouches, as well as losses and/or fusions of CB cartilages (Fig. 2F,N; supplementary material Fig. S2B,C). In addition, an nkx2.3:Efnb2a transgene, which selectively restored Efnb2a protein to endoderm, partially rescued the pouch and CB cartilage defects of efnb2a−/−; efnb3b-MO embryos (Fig. 2G,O,S,T). A hallmark of transmembrane B-type Ephrins is their ability to both activate Eph receptors in neighboring cells (forward signaling) and signal cell-autonomously (reverse signaling) (Kullander and Klein, 2002). However, a truncated version of Efnb2a only competent for forward signaling (Xu and Henkemeyer, 2009) also rescued pouch and CB defects (Fig. 2H,P,T). These results suggest that forward signaling within the endoderm regulates pouch development, although we cannot rule out additional roles of EphB signaling in other arch tissues. One possibility then is that distinct subsets of endodermal cells express either the ligand or receptor, thus allowing signaling in trans rather than cis-attenuation, with expression and signaling shifting as pouches mature from anterior to posterior.

Disintegration of pouch epithelia in the absence of Eph-Pak2a signaling

To investigate the requirements of Eph-Pak2a signaling in pouch cell behaviors, we performed time-lapse imaging of her5:mCherryCAAX−labeled pouches in signaling-deficient embryos. In wild-type siblings, pouches developed in an anterior-to-posterior progression, with subsets of cells becoming multilayered, collectively migrating and rearranging into bilayers as migration ceases (Fig. 3A–D; supplementary material Movie S1A). In contrast, we observed common pouch cell behavior defects in embryos lacking EphrinBs, EphB activity in the endoderm or pak2a (Fig. 3E–P; supplementary material Movie S1B–D). Although pouch initiation was unaffected, these embryos all displayed progressive detachment of cells from the epithelium during the branching process, with cells breaking off individually or as small clusters. Anti-Alcama staining of fixed
embryos similarly revealed disorganized pouch bilayers and clusters of dissociated epithelial cells (Fig. 3Q-T; supplementary material Fig. S3). In particular, pouches deficient for Eph-ephrin or Pak2a displayed misaligned apical membranes, abnormal multi-layering and/or monolayers where bilayers should be present. Whereas we interpret this as a function of Eph-Pak2a signaling in increasing intercellular adhesion to stabilize pouches, Eph-Pak2a signaling might also help to maintain adhesion within the transitional epithelium.

EphrinBs and Wnt4a cooperatively activate Pak2a

As EphrinB-deficient and pak2a mutant embryos display similar pouch defects, we investigated whether Eph-ephrin signaling might activate Pak2a. PAK activation involves autophosphorylation of a conserved serine residue in an amino-terminal inhibitory domain (S143 in Pak2a) (Tay et al., 2010). During normal development, we observed pPAK-S143 staining within pouch epithelia, which increased as pouches matured (Fig. 4A; supplementary material Fig. S4A). Consistent with forward Eph-ephrin signaling activating Pak2a, pPAK-S143 staining was decreased in KD-EphB4a-expressing endoderm and efnb2a mutants, and further decreased in efnb2a−/−; efnb3-MO embryos (Fig. 4B,E,S; supplementary material Fig. S4D). PAK phosphorylation was also reduced upon endodermal expression of dominant-negative Cdc42 (supplementary material Fig. S4D), consistent with the known roles of small GTPases in activating PAK (Manser et al., 1994). As Wnt4a activates Cdc42 during pouch formation (Choe et al., 2013), we explored whether EphrinB and Wnt4a signaling might function together to maximally activate Pak2a. Indeed, we observed a reduction of pPAK-S143 staining in wnt4a mutants and a near-complete loss in efnb2a−/−; efnb3−/−; efnb3b−/−; wnt4a mutants (Fig. 4C,D,S). Wnt4a mutants also displayed much more severe pouch and CB cartilage defects than either single mutant alone (Fig. 4D,J,S). This synergism is unlikely due to Wnt4a regulation of Efnb2a protein localization or ephb4a expression, as these were unaffected in wnt4a mutants (supplementary material Fig. S4B,C). Instead, the strong genetic interactions between Efnb2a and Wnt4a are consistent with Eph-ephrin and Wnt4a-Cdc42 signaling coordinately activating Pak2a.

Membrane targeting of Pak2a rescues pouches in EphrinB-deficient embryos

As we detected phosphorylated PAK at the apicolateral membranes of maturing pouch cells, we tested whether Eph-ephrin signaling might activate Pak2a by targeting it to the membrane. Consistently, pouch-specific expression of Pak2a with a membrane-targeting farnesylation sequence (nkx2.3:Pak2aCAAX) partially rescued pouch and CB defects and pPAK-S143 staining in efnb2a−/−; efnb3-MO embryos (Fig. 4F,L,S). This rescue is unlikely due to increased general levels of Pak2a, as a non-membrane-targeted nkx2.3:Pak2a transgene failed to rescue (supplementary material Fig. S4E,G,H,J). Instead, rescue by membrane-targeted Pak2a
suggests that Eph-ephrin signaling activates Pak2a by targeting it to the plasma membrane, where it could then be activated by Cdc42.

Eph-Pak2a signaling increases junctional E-cadherin localization in pouches

As the maturation of developing pouch epitelial into bilayers coincides with increased E-cadherin levels (Choe et al., 2013), we examined whether the disorganized bilayers of signaling-deficient embryos might be due to reduced junctional E-cadherin. Consistently, we observed a moderate decrease in junctional E-cadherin in efnb2a mutants, which was further exacerbated by reduction of efnb3b (Fig. 4N,Q,S). These reduced E-cadherin levels might be causative for pouch defects, as expression of a dominant-negative version of E-cadherin specifically in the nkx2.3+ endoderm resulted in similar pouch and CB defects to loss of EphrinBs (supplementary material Fig. S5). Moreover, the loss of junctional E-cadherin is probably due to reduced activation of Pak2a, because (1) similar reductions in E-cadherin were observed in pak2a mutants and dominant-negative Pak2a embryos (supplementary material Fig. S4F); (2) membrane-targeted Pak2a rescued E-cadherin levels in Ephrin-deficient embryos (Fig. 4R); and (3) the synergistic loss of phosphorylated PAK in efnb2a; wnt4a compound mutants was reflected by further reductions of E-cadherin levels compared with single mutants alone (Fig. 4P).

Previously, we had shown that Wnt4a promotes adherens junction stability in part through junctional localization of the Alcama immunoglobulin protein (Choe et al., 2013). However, the role of Wnt4a in activating Pak2a appears to be distinct from its role in localizing Alcama, as we detected no apparent changes in Alcama localization in pak2a mutants or embryos deficient for Eph-ephrin signaling (Fig. 3R-T; supplementary material Fig. S3). In addition, a sub-phenotypic dose of alcama-MO further decreased E-cadherin localization and enhanced the pouch and CB defects of pak2a mutants (supplementary material Fig. S4F-J). These results suggest that Eph-ephrin and Wnt4a signaling increase E-cadherin levels, and intercellular adhesion through parallel Pak2a and Alcama pathways, which together would stop pouch outgrowth by inhibiting further epithelial rearrangements.

MATERIALS AND METHODS

Zebrafish strains

Published lines include efnb2a<sup>-<i>ahu3393</i></sup> (Cavodeassi et al., 2013), pak2a<sup>-<i>vh411</i></sup> (Buchner et al., 2007), Tg(<i>sox10:LOXP-GFP-LOXP-Hsa.DLX3</i>)<sup>MYO</sup> (Das and Crump, 2012), and wnt4a<sup>-<i>hu3393</i></sup>, Tg(her5:mCherryCAAX)<sup>MYO</sup>, Tg(nkx2.3:GAL4-VP16,myl7:EGFP)<sup>MYO</sup> and Tg(UAS:cdc42:Gfp)<sup>MYO</sup>. These MOs were obtained from Genetools and 1 nl of a 300-μM solution was injected at the one-cell-stage. alcama-MO is published (Choe et al., 2013). Tg(nkx2.3:efnb2a), Tg(nkx2.3:pak2a:CAAX), Tg(UAS:KD-Ephb4a) and Tg(UAS:KD-Pak2a) transgenic constructs were generated using the Gateway (Invitrogen) Tol2kit (Kwan et al., 2007). See supplementary materials and methods for primers.

Staining

Immunohistochemistry was performed as described (Crump et al., 2004) using antibodies against Alcama/ZNF8 (Zebrafish International Resource Center; 1:400), E-cadherin (610181, BD Biosciences; 1:250), pS143-PAK (pPAK144, a gift from Edward Manser, National University of Singapore; 1:250), GFP (TP401, Torrey Pines Biolabs; 1:1000) and Efnb2a (AF1088, R&D Systems; 1:500). Alcian Blue staining and fluorescent in situ hybridizations were as described (Zuniga et al., 2011) using probes against nkx2.3a,b, efnb2ahu3393 and efnb3b<sup>-<i>sh</i></sup>. Imprinting-blocking (5′-ACTCC-CATCAAAGCGGTGGCGGGA-3′), splice-blocking (5′-TGTTGTGTTT-ACTACTCTGTCGCCACT-3′) and control (antisense to splice-blocking, 5′-AGTGG-GACAGAGTGAGTAACACA-3′) efnb3b<sup>-<i>MO</i></sup> were obtained from Genetools and 1 nl of a 300-μM solution was injected at the one-cell-stage. alcama-MO was published (Choe et al., 2013), using primers for Tg(nkx2.3:EFnb2a), Tg(nkx2.3:pak2a:CAAX), Tg(UAS:KD-Ephb4a) and Tg(UAS:KD-Pak2a) transgenic constructs were generated using the Gateway (Invitrogen) Tol2kit (Kwan et al., 2007). See supplementary materials and methods for primers.

Imaging and scoring

Fluorescent images were acquired on a Zeiss LSM5 confocal microscope. Dissected craniofacial cartilages were photographed on a Leica DM2500 upright microscope. To quantitate pouch defects, misshapen or normal
Fig. 4. Requirements for Eph-ephrin and Wnt signaling in PAK activation and E-cadherin levels. (A-F) Immunohistochemistry shows Alcama (green) and the activated form of PAK (pPASK143, red) at 34 hpf. In wild types, pPAK-S143 is higher in anterior pouches (p1-p3) compared with less mature posterior pouches (p4 and in particular p5). Compound efnb2a; wnt4a mutants produce fewer pouches and have further reduced pPAK-S143 staining than single mutants alone. In Ephrin-B-deficient embryos, forced expression of membrane-targeted Pak2a (nkx2.3:Pak2aCAAX) rescues both pouches and PAK autophosphorylation. Scale bar: 40 μm. (G-L) Ventral views of facial cartilage at 5 dpf show losses of CBs and CH-CB1 fusions in mutants and rescue of these defects in Ephrin-B-deficient embryos by nkx2.3:Pak2aCAAX. (M-R) Immunohistochemistry shows E-cadherin protein (green) within pouches p4 and p5. In wild types, E-cadherin localizes to cell-cell contacts along apicolateral membranes. Compound efnb2a; wnt4a mutants have further reduced E-cadherin staining than single mutants alone, and nkx2.3:Pak2aCAAX rescues E-cadherin levels in Ephrin-B-deficient embryos. Scale bar: 20 μm. (S) Quantification of pouch and CB defects, and scoring of reduced pPAK and E-cadherin staining. Data represent mean±s.e.m. ***P<0.001.

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References

Statistics
We employed the multiple comparison test of Tukey–Kramer to quantify pouchnCB defects and pPAK/E-cadherin staining. See supplementary material Table S1 for numbers of animals employed.

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Competing interests
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Author contributions
C.P.C. designed and performed the experiments. C.P.C. and J.G.C. interpreted results and wrote the manuscript.

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