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ABSTRACT

The vertebrate body plan is thought to be derived during the early Cambrian from a worm-like chordate ancestor. While all three germ layers were clearly involved in this innovation, the role of the endoderm remains elusive. According to the hourglass model, the optimal window for investigating the evolution of vertebrate endoderm-derived structures during cephalochordate development is from the Spemann's organizer stage to the opening of the mouth (Stages 1–7, described herein). Regulatory gene expression, examined during these stages, illustrate that the cephalochordate endoderm is patterned into 12 organ primordia. Early vertebrates inherited at least a portion of 6 of these primordia, while the remainder were lost. Of those that were preserved, we demonstrate that the vertebrate symmetric mouth was built on a vestige of the anterior pre-oral pit, that the pre-existing pharyngeal pouch in this chordate ancestor laid the foundation for the new neural crest cell (NCC)-derived vertebrate-type pharyngeal arches, that the thyroid evolved from the posterior endostyle primordim, that the pancreas was derived from the *Pdx1*-expressing diverticulum primordium, and the small and large intestines originated with the *Cdx1*-expressing hindgut rudiments. This investigation uncovers the evolutionary foundations of vertebrate endoderm-derived structures, and demonstrates that the number of organ primordia were reduced during evolution.

1. Introduction

Paleontological and embryological evidence suggests that the vertebrate body plan arose with the constraints established by its worm-like ancestors and that all three germ layers were inevitably involved in these transitions (Janvier, 2015; Green et al., 2015; Swalla and Moody, 2007a; Shubin et al., 2009). The innovations involving the ectoderm and mesoderm have been comprehensively documented during embryonic patterning, organogenesis and cell-fate determination, however, the role played by the endoderm lineage during this evolutionary process remained ambiguous (Green et al., 2015; Swalla and Moody, 2007a; Shubin et al., 2009; Holland, 2015; Lowe et al., 2015; Bajoghli, 2011). We hypothesize that at least some of these innovations, including those that led to a more energetic lifestyle, were promoted by remodeling and increasing the complexity of endoderm-derived structures.

The evolutionary roots of vertebrate endoderm-derived structures can be traced to early bilaterians (Arendt et al., 2001; Ferrier and Holland, 2001; Martindale, 2005). In living invertebrate bilaterians, endoderm patterning is generally simpler and more conserved than that of the ectoderm and mesoderm (Ferrier and Holland, 2001; Martindale, 2005). Morphological and molecular evidence has shown that the larva of Urbilateria, the common bilaterian ancestor, possessed a tube-shaped gut (Arendt et al., 2001), a unique endoderm-derived structure divided into fore-, mid- and hindgut along the anterior-posterior axis during

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embryonic development (Arendt et al., 2001; Ferrier and Holland, 2001, 2002; Martindale, 2005; Brooke et al., 1998; Fröbius and Seaver, 2006; Arnone et al., 2006; Ikuta et al., 2013; Ferrier, 2016; Fritsch et al., 2016). Patterning of the foregut endoderm shows some divergence between protostomes and invertebrate deuterostomes, while that of mid- and hindgut endoderm is conserved in all invertebrate bilaterians (Ferrier and Holland, 2001, 2002; Martindale, 2005; Brooke et al., 1998; Fröbius and Seaver, 2006; Arnone et al., 2006; Ikuta et al., 2013; Ferrier, 2016; Fritsch et al., 2016). Generally, invertebrate bilaterian mid- and hindgut patterning is regulated by two members of the *ParaHox* cluster, *Pdx* and *Cdx*, and almost all of the *Pdx*-expressing midgut endoderm can form a digestive gland-like ampulla structure or system, while the *Cdx*-expressing hindgut endoderm will develop into an intestine-like structure (Martindale, 2005; Brooke et al., 1998; Ferrier and Holland, 2002; Fröbius and Seaver, 2006; Arnone et al., 2006; Ikuta et al., 2013; Ferrier, 2016; Fritsch et al., 2016).

Studies of the cephalochordate body plan and genome demonstrate that they are "living fossils" of early chordates (Gee, 1996; Yu et al., 2007; Putnam et al., 2008). The embryonic patterning of cephalochordates and vertebrates fits the evolutionary hourglass model, in which the bottleneck represents the phylotypic stage (Holland, 2015; Kalinka et al., 2010). The phylotypic stage is the developmental window during which an organism's basic body plan is assembled. This period, which in vertebrates encompasses organogenesis/mid-gestation, also marks the morphological period during which organisms most resemble one another. This evolutionary model is named to account for the degree of morphological divergence over time: because organisms resemble each other the least during early development, i.e. from fertilization through gastrulation, and later development, i.e. adulthood, the phylotypic period, the period of least divergence, is a bottleneck. Although still contentious, recent molecular data supporting the hourglass model demonstrates that the genes expressed specifically during the phylotypic stage are those that are the most conserved across species (Holland, 2015; Kalinka et al., 2010; Levin and el, 2016; Bogdanović et al., 2016).

We propose that, by examining cephalochordate endoderm patterning during the phylotypic period, the evolutionary prototype of the vertebrate body plan will be revealed (Kalinka et al., 2010; Levin and el, 2016; Bogdanović et al., 2016). However, because the evolutionary comparison of cephalochordate and vertebrate endoderm patterning has not yet been systematically performed, a number of issues remain unsolved. For example, the cephalochordate foregut endoderm expresses *Hhex* in the endostyle primordium, a structure generally regarded as the homolog of the vertebrate thyroid (Yu et al., 2007). However, the nature of this relationship has not yet been elaborated. Furthermore, although the *Pax1/9-Six1-Eya* network is expressed in the pharyngeal region (Kozmik et al., 2007), the relationships among their expression domains during cephalochordate development is as yet unknown. The cephalochordate gills are often used to trace the evolutionary emergence of vertebrate pharyngeal structures and, because the pharyngeal region is crucial to authenticate fossils of the chordate ancestor and early vertebrates (Janvier, 2015; Green et al., 2015; Holland and Chen, 2001; Mallatt and Chen, 2003; Shu et al., 1999; Conway Morris and Caron, 2014), (Fig. 1a), pinpointing the precise expression of the cephalochordate *Pax1/9-Six1-Eya* network is critical to illuminate the origin of vertebrate pharyngeal arch and pouch. Moreover, like other bilaterians, prior to metamorphosis, cephalochordates possess a tube-shaped gut and an asymmetric mouth (Gee, 1996), while all living vertebrates have a symmetric mouth. Thus an investigation of foregut patterning in cephalochordates may reveal the origins of these early vertebrate transitions.

As seen in other invertebrate bilaterians, *Pdx1* and *Cdx1* are expressed in the cephalochordate midgut and hindgut endoderm, though it is not clear which specific tissues express these genes. For example, the diverticulum, a protruding structure produced by the cephalochordate midgut endoderm, is believed to be a homolog of the vertebrate liver (Muller, 1844; van Weel, 1937; Barrington, 1937) (Fig. 1a). Verifying this relationship would be an important step in understanding the evolution of the vertebrate body plan. Similarly the cephalochordate hindgut endoderm produces only a very short intestine-like structure, and its possible segmentation cannot be assessed by morphology alone (Fig. 1a). To resolve these issues, we examine and compare the expression of conserved endoderm-patterning genes.

The regulation of vertebrate endoderm-derived organogenesis and the resulting diversity of cell types and tissue functions are more complex than in any other deuterostome group (Fig. 1b) (Grapin-Botton and Melton, 2000; Zaret, 2008). Despite this complexity, the early developmental stages of vertebrate endoderm patterning appear to be more simply regulated than in other deuterostomes. During early vertebrate development, three factors, *Sox2*, *Pdx1* and *Cdx1*, pattern the vertebrate endoderm into three domains along the anterior-posterior axis (Grapin-Botton and Melton, 2000) (Fig. 1b). *Sox2* and *Pdx1* are expressed in the foregut endoderm, while the *Cdx1*-expressing region defines the hindgut (Zaret, 2008). Within the broad domain of *Sox2* expression, *Pax 9* is expressed in the pharyngeal pouch primordium, and *Foxn1* in the thymus primordium (Grapin-Botton and Melton, 2000). Proximal to the *Sox2* expression domain, *Hhex* expression demarcates the thyroid primordium, while *Pdx1* is expressed in the dorsal and ventral pancreatic bud as well as the duodenum (Grapin-Botton and Melton, 2000; Zaret, 2008). Proximal to the ventral pancreatic bud, the *Hhex* and *Prox1*--expressing region marks the hepatic primordium (Grapin-Botton and Melton, 2000; Zaret, 2008; Jung et al., 1999). In the posteriormost endoderm, the *Cdx1*-expressing region develops into small and large intestines (Grapin-Botton and Melton, 2000).

Numerous fundamental developmental mechanisms are conserved

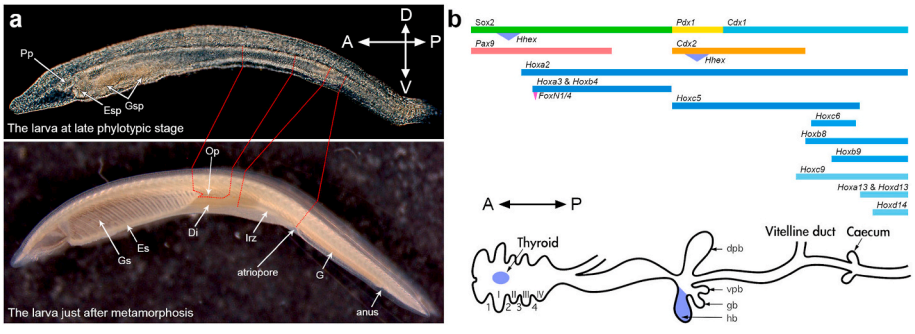

Fig. 1. Comparison of endoderm patterning in cephalochordates and vertebrates. a. Cephalochordate endoderm patterning and its derived structures. Although celphalochordate endoderm patterning and regulatory gene expression have not yet been systematically investigated, endoderm-derived structures can be roughly deduced using morphological methods. **b.** A patterning map of the vertebrate endoderm. Vertebrate endoderm patterning is controlled by highly conserved regulatory genes and *Hox* members from all four clusters. A↔P, the anterior-posterior axis; D↔V, the dorsal-ventral axis; Pp, pre-oral pit; Esp, endostyle primordium; Gsp, gill-slit primordia; Gs, gill slits; Es, endostyle; Op, Oesophagus; Di, diverticulum; Irz, ileo-colon-ring zone; G, gut; 1–4, pharyngeal arches 1–4; I–IV, pharyngeal pouches I–IV; dpb, dorsal pancreas bud; vpb, ventral pancreas bud; gb; gallbladder bud; hb, hepatic bud.

between cephalochordates and vertebrates. For example, the vertebrate organizer (Spemann's organizer in amphibians/the node in birds and mammals) is evolutionarily conserved between cephalochordates and vertebrates (Yu et al., 2007). Given that the organizer stage precedes the chordate organogenic/phylotypic stage, studying the expression of endoderm pattering genes during basal chordate organogenesis in the context of the hourglass model should provide the optimal setting to examine the evolution of vertebrate endoderm-derived structures. To examine cephalochordate endoderm patterning using this model, we focus on and define the following stages: stage 1 (S1), hatching (mid--neurula); stage 2 (S2), 6–8 somites; stage 3 (S3), 10–12 somites (late neurula); stage 4 (S4), 1 gill-slit primordium (early pharyngula); stage 5 (S5), 2 gill-slit primordia; stage 6 (S6), 3 gill-slit primordia; stage 7 (S7), mouth opening (the end of pharyngula) (see Method). We use gene expression during these stages of cephalochordate development in conjunction with re-described fossil samples (Janvier, 2015; Gee, 1996; Holland and Chen, 2001; Mallatt and Chen, 2003; Shu et al., 1999; Conway Morris and Caron, 2014) to address several unresolved issues in vertebrate evolution, including the emergence of the pharyngeal pouch, the relationship of the endostyle and the thyroid, the origins of the new vertebrate symmetric mouth, the origin of the liver and pancreas, and gut segmentation during chordate evolution.

2. Results

2.1. The pharyngeal pouch and the origin of vertebrate branchia

The endoderm-derived gill is one of the most important features of invertebrate deuterostomes (Janvier, 2015; Green et al., 2015; Swalla and Moody, 2007b). This structure is shared among early deuterostomes (Han et al., 2017), the ancestors of echinoderms (Jefferies, 1968; Dominguez et al., 2002), hemichordates (Gillis et al., 2012), cephalochordates (Swalla and Smith, 2008) and urochordates (Swalla and Moody, 2007b). However, the vertebrate branchia are more complex and are composed of both endoderm-derived pouches and neural crest cell (NCC)-derived arches. While the evolution of the vertebrate gill remains a long-standing question, the emergence of the pharyngeal pouch is similarly unclear (Green et al., 2015). We provide evolutionary developmental evidence suggesting that the endoderm-derived pharyngeal pouch is a feature that distinguishes chordates from other deuterostomes (Fig. 2 and Figs. S1, 2).

During patterning of the hemichordate foregut endoderm, *Pax1/9*, *Six1* and *Eya* form a basic character identity network (ChIN) that regulates gill pore development (Gillis et al., 2012). In cephalochordates, the *Pax1/9-Six1-Eya* expression network can be separated into two parts (Fig. 2b–d & Fig. S1). *Pax1/9* is not expressed in the gill pore primordia, but rather in the developing pharyngeal endoderm (Fig. 2c & Fig S1a-c). As in hemichordates, *Six1* and *Eya* are expressed in the gill pore

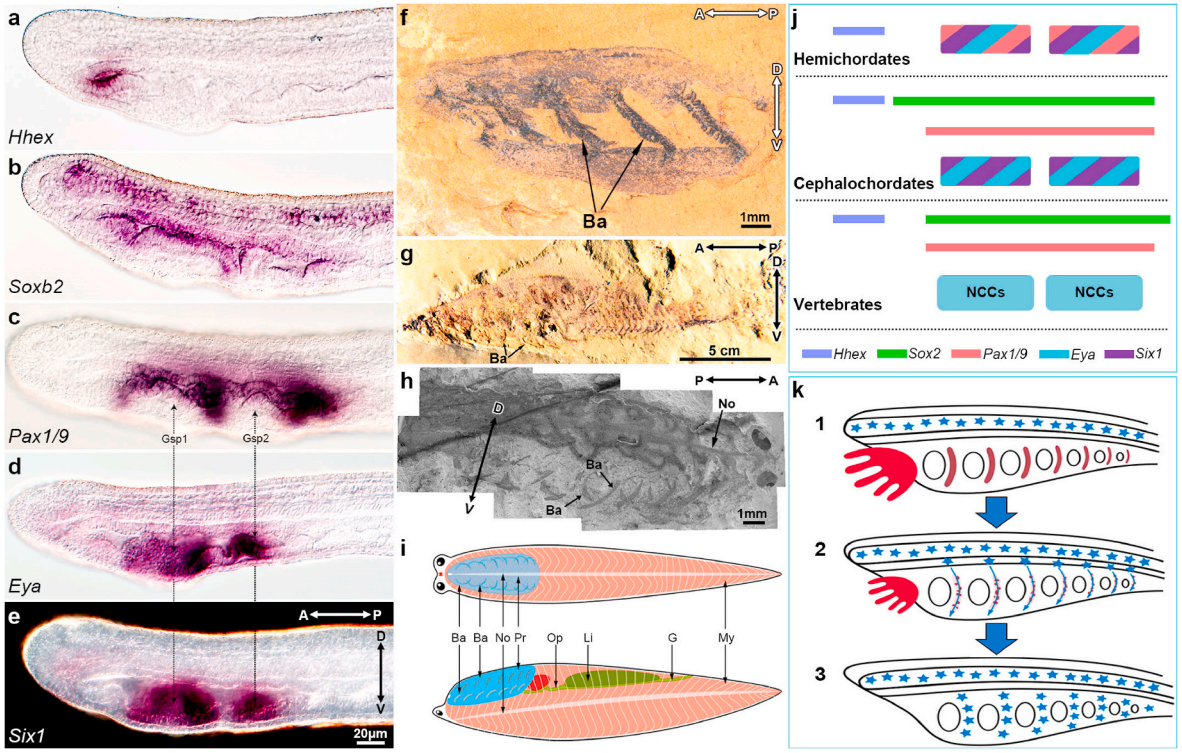

Fig. 2. The evolutionary origin of vertebrate branchiae. a-e. The patterning map of the foregut endoderm reveals that the cephalochordate gill is in a transitional state, laying the groundwork for the emergence of vertebrate branchiae. The primitive *Pax1/9-Eya-Six1* network is separated into two parts (c–e). The developing pharyngeal endoderm is homologous to the vertebrate pharyngeal pouch and both express *Hhex*, *Sox2* (*Soxb2*) and *Pax1/9* (a–c), while the gill slit rudiments are homologous to those of hemichordates and express *Eya* and *Six1* (d, e). **f**. The branchial structure of *Haikouella* (*Haikouella lanceolata*). The gills of *Haikouella* had vertebrate-type branchial arches. **g-h**. The branchial structure of early vertebrates. The myllokunmingiids (*Haikouichthys ercaicunensis*, IGYGB HZ-f-12-127) and *Metaspriggina* (*Metaspriggina walcotti*, ROM62933. Images courtesy of Jean-Bernard Caron, Royal Ontario Museum, Toronto, Canada) typify early vertebrates and both had NCC-derived pharyngeal arches. **i.** A reconstruction of an early vertebrate body plan (*Metaspriggina*) reveals NCC-derived pharyngeal arches within endoderm-derived pharyngeal pouches. **j**. A comparison of foregut endoderm patterning among deuterostomes suggests that the pharyngeal pouch is a distinct chordate feature, and that the pre-existing pharyngeal pouch of early chordates provided a niche for proto-NCCs. **k**. The proposed evolutionary origin of vertebrate branchial structures. At stage 1, the early chordates, like amphioxus, had endoderm-derived gills with acellular cartilage (purple). At stage 2, proto-NCCs (blue stars in pharyngeal region) began to migrate into the pre-existing pharyngeal pouches (blue arrows) to form vertebrate-like pharyngeal arches. At stage 3, early vertebrates had NCC-derived pharyngeal arches. Ba, branchial arch; No, notochord; Pr, pharyngeal region; Li, liver; G, gut; My, myomere. NCCs, neural crest cells. For other abbreviations, see Fig. 1.

rudiments (Fig. 2d, e & Fig. S1d-j). In the expression of *Hhex*, *Sox2* and *Pax1/9*, the developing cephalochordate pharyngeal endoderm is homologous to the vertebrate pharyngeal pouch (Fig. 2a–c, j). The cephalochordate homolog of vertebrate *Sox2* includes *Soxb1* and *2* (Meulemans and Bronner-Fraser, 2007). Like vertebrate *Sox2*, *Soxb2* is stably expressed in the developing pharyngeal endoderm (Fig. 2b), and its expression never overlaps with *Hhex* during the phylotypic stage (Fig. 2a and b) (Grapin-Botton and Melton, 2000; Zaret, 2008). Thus, the separate expression of *Pax1/9-Eya-Six1* network and the spatiotemporal expression of *Hhex*, *Soxb2* and *Pax1/9* suggest that the cephalochordate gill represents a transitional state between the patterning seen in hemichordates and in vertebrates (Fig. 2j). Furthermore, these data suggest that cephalochordate gill pores are homologous to those of hemichordates, while the cephalochordate pharyngeal endoderm is homologous to the vertebrate pharyngeal pouch. The emergence of the pharyngeal pouch in early chordates is crucial for a better understanding of the origin of the vertebrate body plan, since this pre-existing structure serves as a foundation upon which the proto-NCCs could construct the new NCC-derived pharyngeal arch, replacing the primitive endoderm-derived gill bars (Green et al., 2015) (Fig. 2j).

The identification of a pharyngeal pouch in early chordates may settle the longstanding controversy about the origin of vertebrate pharyngeal structures. Furthermore, by incorporating our data with newly re-described fossil evidence, the evolutionary origin of the vertebrate gill can be more clearly understood. For example, a sister group of vertebrates, *Haikouella* from the Chengjiang biota, is closer to early vertebrates than to cephalochordates, and has six paired branchia with arches and pouches (Janvier, 2015; Gee, 1996; Holland and Chen, 2001; Mallatt and Chen, 2003) (Fig. 2f). The stem vertebrates, myllokunmingiids *Myllokunmingia* and *Haikouichthys,* also from the Chengjiang biota, are more "fishlike" and retain pharyngeal arches and pouches (Janvier, 2015; Shu et al., 1999; Swalla and Smith, 2008) (Fig. 2g). Similarly, another stem vertebrate, *Metaspriggina* from the Burgess Shale, displays unequivocal bipartite pharyngeal arches within pouches (Conway Morris and Caron, 2014) (Fig. 2h and i). Character analysis has authenticated that the pharyngeal arches in *Haikouella*, myllokunmingiids, and *Metaspriggina* are derived from the NCC (Janvier, 2015; Swalla and Smith, 2008). Consequently, the evolutionary origin of vertebrate branchial structures can be divided into three stages, as follows: stage 1: early chordate stage; chordates at this stage possessed pharyngeal pouch (es) and endoderm-derived gill structures. Stage 2: transitional stage. At this stage, proto-NCCs migrated into the pharyngeal pouches to form pharyngeal arches, replacing endoderm-derived acellular cartilage. Stage 3: early vertebrate stage. As in myllokunmingiids and *Metaspriggina*, the branchial structures of this stage were vertebrate-like, with pharyngeal pouches and well-developed NCC-derived pharyngeal arches (Fig. 2k).

2.2. The thyroid evolved from a portion of the endostyle

The *Hhex*-expressing foregut endoderm appears conserved in deuterostomes and is also found in hemichordates (Satoh et al., 2014) (Fig. 2j). In cephalochordates, *Hhex* is robustly and specifically expressed in the developing endostyle (Fig. 3a and Fig. S3). *Foxq1* marks the endostyle primordium of invertebrate chordates (Ogasawara and Satou, 2003; Mazet et al., 2005), however, *Foxq1* is relegated to the posterior portion of the cephalochordate endostyle (Fig. 3b). The vertebrate thyroid is the homologous structure of the endostyle, and is regulated by a basic transcriptional network that includes *Hhex*, *Nkx2.1*, *Pax 8* and *Foxe1* (De Felice and Di Lauro, 2011; Ogasawara, 2000). Like *Hhex*, *Nkx2.1* is also expressed in endostyle primordium (Venkatesh et al., 1999) while *Foxe1* is detected in another endoderm-derived cephalochordate-specific structure, the club-shaped gland, which is lost in adults (Yu et al., 2002). Like *Foxq1*, *Pax2/5/8* is expressed in the posterior endostyle (Fig. 3c). Moreover, *Mnxa/b*, a member of homeobox superfamily, is similarly expressed in the posterior endostyle (Fig. 3d). Together, this cephalochordate expression analysis suggests that the anterior endodstyle was lost during vertebrate evolution, while the posterior endostyle gave rise to the vertebrate thyroid. Based on this basic molecular network, after recruiting *Foxe1* and losing *Foxq1*, the genetic and morphological features of the posterior endostyle facilitated the formation of the novel vertebrate-type thyroid (Mazet, 2002).

2.3. The new symmetric vertebrate mouth

During the early phylotypic stage, *Pitx2* expression is detected on the

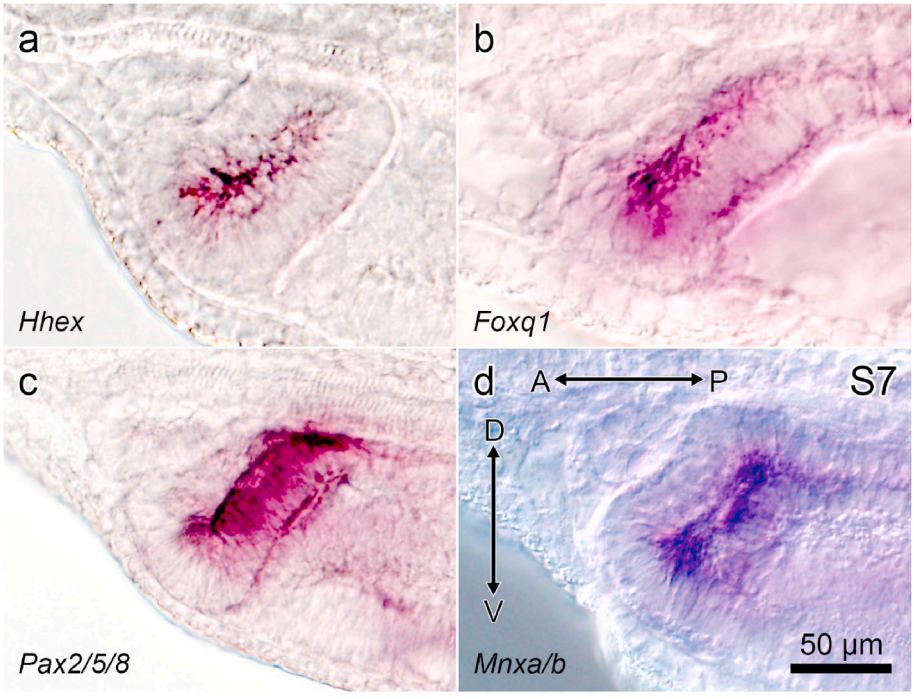

Fig. 3. The posterior endostyle is homologous to the thyroid. a-d. Expression of the thyroid development regulatory network in the endostyle primordium during the phylotypic stage. Like the vertebrate thyroid, *Hhex* is expressed in cephalochordate endostyle primordium throughout the phylotypic stage (a); however, *Pax2/5/8*, *Foxq1* and *Mnxa/b* expression is confined to the posterior fold of the primordium (b–d). The expression pattern of the endostyle development regulatory network suggest that the posterior portion is homologous to the thyroid, while the anterior portion has been lost in vertebrates. S7, Stage 7. For other abbreviations see Fig. 2.

left side of the embryo, throughout the foregut endoderm that produces the mouth primordium, anterior endostyle, and posterior pre-oral pit (Fig. 4a). Slightly later in development, these three primordia are separated by other intervening endoderm-derived structures, but maintain *Pitx2* expression (Fig. 4b and c). The *Nodal-Lefty-Pitx* signaling cascade controls chordate left-right asymmetry (Soukup et al., 2015; Li et al., 2017). During the late phylotypic stage, *Pitx* marks the left-sided mouth (Kaji et al., 2016) (Fig. 4b). *Pitx* and *Soxb2* expression divide the pre-oral pit primordium into two parts (Fig. 4c and d). The anteriormost site of cephalochordate *Soxb2* expression is in the anterior pre-oral pit, while the anteriormost vertebrate *Soxb2* expression domain is in the developing mouth (Fig. 1b). The vertebrate *Sox2*-expressing foregut endoderm is continuous, not interrupted by any other developing structure, and the vertebrate mouth is symmetric throughout embryogenesis (Fig. 1b) (Grapin-Botton and Melton, 2000; Zaret, 2008). Furthermore, the cephalochordate pre-oral pit does not have a vertebrate structural homolog. This evidence shows that the evolutionary route to vertebrates from cephalochordates involved the loss of the *Pitx*-expressing foregut endoderm. If the vertebrate mouth were indeed remodeled from the anterior pre-oral pit, the patterning of the cephalochordate foregut endoderm is otherwise similar to that of vertebrates (Fig. 4e).

2.4. The evolutionary rudiments of the liver and pancreas

The cephalochordate diverticulum and ileo-colon ring are both derived from the midgut endoderm. Although the diverticulum has been regarded as the prototype of the vertebrate liver for over 150 years (Muller, 1844; van Weel, 1937; Barrington, 1937), this homology has not yet been supported with evolutionary developmental biology evidence (Lecroisey et al., 2015). In the cephalochordate midgut endoderm, *Pdx1* marks the diverticulum and ileo-colon ring rudiments, but unlike in vertebrates, its expression is not contiguous with *Cdx1*-expressing endoderm (Fig. 5). During the early phylotypic stage, the cephalochordate diverticulum and ileo-colon ring are both derived from a *Pdx1* and *Nkx6* co-expressing endoderm progenitor (Fig. S4a-c). At the late phylotypic stage, *Pdx1* expression is high in the diverticulum primordium and gradually weakens in the ileo-colon ring primordium. During the same developmental window, *Nkx6* has the opposite expression pattern, and is low in the diverticulum but high in the ileo-colon ring primordium (Fig. S4d-f). By the end of phylotypic stage, *Pdx1* expression is confined to the diverticulum primordium and *Nkx6* is expressed mainly in the ileo-colon-ring primordium (Fig. 5). The diverticulum and ileo-colon ring primorida are flanked by *Nkx2.2*-expressing endoderm throughout the phylotypic stage (Fig. 5 and Fig. S4b, e).

During the late phylotypic stage, the diverticulum expresses *Pdx1*, *Nkx6,* and *Gata4/5/6*, as well as *Mnxa/b* in the dorsal region (Fig. 5 and Fig. S5). Similarly, the expression of *Sox2* (*Soxb1* and *2*) (Fig. S5), *Pdx1,* and *Nkx6* in the ileo-colon ring are all developmentally regulated, and *Sox7/17* expression is confined to the dorsal region (Fig. 5 and Fig. S6). During vertebrate endoderm patterning, *Sox2* is invariantly expressed anterior to *Pdx1* (Grapin-Botton and Melton, 2000; Zaret, 2008) (Fig. 1b), suggesting a reason for the disappearance of the ileo-colon ring from the vertebrate body plan. Thus, the vertebrate posterior foregut derivatives must arise from the diverticulum primordium, specifically its posterior region. Because *Pdx1* is a developmental marker of the vertebrate pancreas but not the liver (Grapin-Botton and Melton, 2000), and *Nkx6*, *Gata4/5/6* and *Mnxa/b* participate in pancreas but not liver development (Zaret, 2008), we propose that the diverticulum is the evolutionary predecessor of the pancreas. Because the vertebrate liver is derived from *Hhex* and *Prox*-expressing endoderm, but *Hhex* expression is limited to the cephalochordate endostyle (Fig. S3) and *Prox* cannot be successfully annotated in the amphioxus genome (Putnam et al., 2008), we suggest that cephalochordates do not form a liver-like organ.

2.5. The evolutionary rudiments of the gut

Regulatory gene expression divides the late phylotypic stage hindgut endoderm into three segments along the anterior/posterior axis (Fig. 5 and Fig. S6). The first segment, which we have termed gut primordium 1 (gut 1), expresses *Soxb2*, *Nkx2.2*, and *Gata4/5/6,* while expression of *Sox7/17* and *Mnxa/b* are dorsally restricted. The second segment, gut primordium 2 (gut 2), is marked by *Cdx1* and *Nkx2.2*, while *Sox7/17* and *Mnxa/b* remain dorsally restricted. The posteriormost primordium, gut 3, is mainly marked by *Cdx1* alone, although the dorsal aspect of this primordium weakly expresses *Mnxa/b*.

Because the gut 1 primoridium gives rise to an accessory structure of the ileo-colon ring (Young, 1981), a structure that lacks a homolog in vertebrates, and because the gut 1 primordium expresses the vertebrate foregut marker *Sox2* (*Soxb2*) while the vertebrate hindgut expresses only *Cdx2*, we suggest that gut 1 was lost in the transition to vertebrates. Furthermore, if the entire *Sox2*-expressing region of the cephalochordate mid- and hindgut endoderm is lost, then as observed in

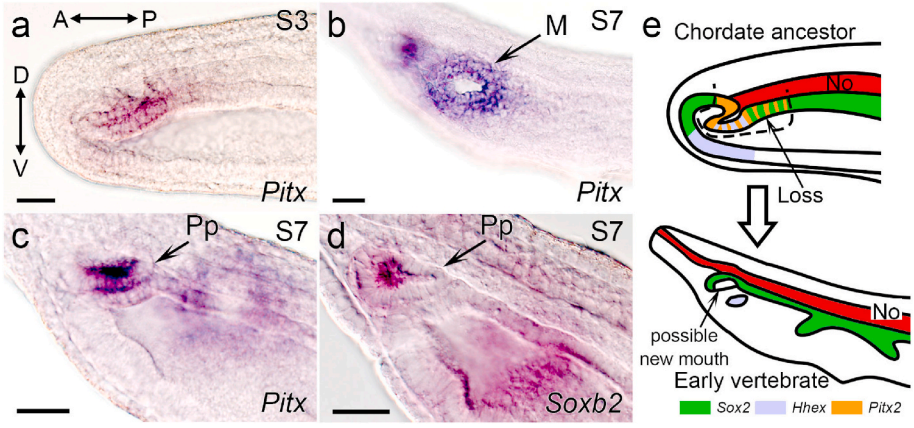

Fig. 4. The vertebrate mouth is remodeled from the anterior region of the cephalochordate pre-oral pit. a-d. The expression of *Pitx* and *Soxb2* in the cephalochordate foregut endoderm (a, Stage 3; b-g, Stage 7). During the early phylotypic stage, the primordia of the mouth, anterior endostyle and posterior pre-oral pit comprise the *Pitx*-expressing region of the left foregut endoderm (a). These primordia are separated during development by intervening endoderm-derived structures (b, c). During the late phylotypic stage *Pitx* and *Sox2* are expressed in different portions of the pre-oral pit primordium (c, d). The anterior pre-oral pit represents the anteriormost site of *Sox2* expression (d), while in vertebrates the anteriormost region of endodermal *Sox2* expression is the developing mouth. **e**. This evidence suggests that if the *Pitx* expressing foregut endoderm were lost, the cephalochordate foregut would be patterned similar to the vertebrate foregut. Furthermore this data suggests that the new vertebrate mouth is a remodeled relic of the anterior domain of the cephalochordate pre-oral pit. S3, stage 3; M, mouth. Scale bars: 20 μm. For other abbreviations, see Figs. 1–3.

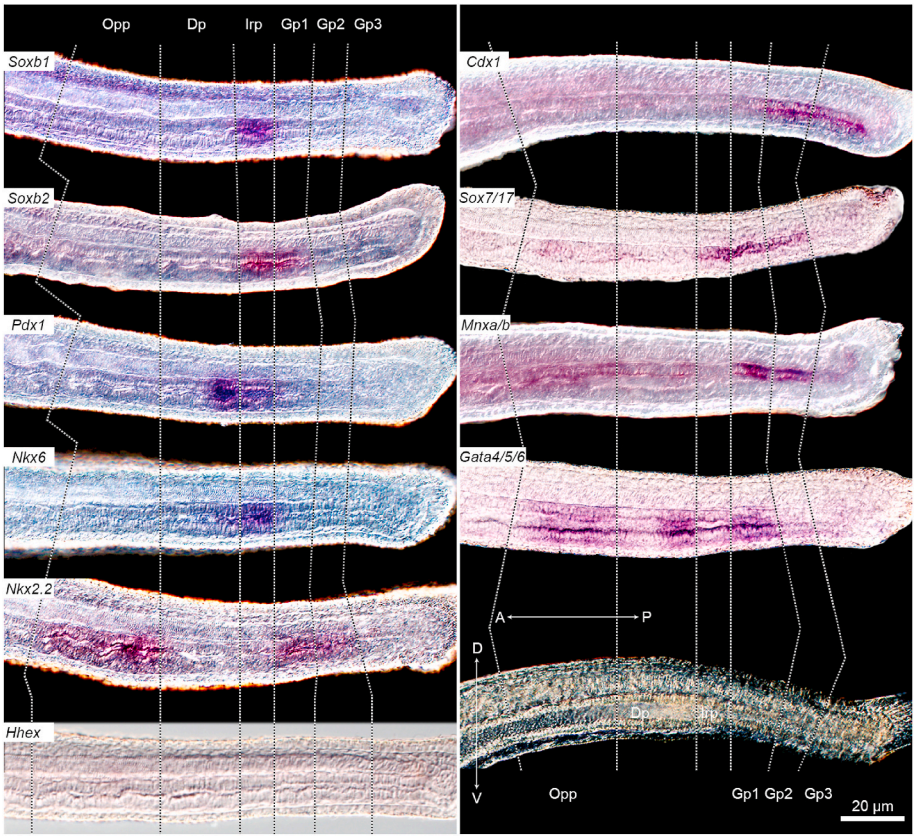

Fig. 5. Patterning of the cephalochordate midgut and hindgut endoderm reveals the evolutionary rudiments of the vertebrate pancreas and gut. During the late phylotypic stage, patterning genes divide the anterior-posterior axis of the cephalochordate midgut and hindgut into five regions, i.e. the primordia of the diverticulum, ileo-colon ring and guts 1–3. The primordium of the diverticulum expresses *Pdx1*, *Nkx6* and *Gata4/5/6*, and is negative expression of *Hhex*, which is vertebrate hepatic developing marker gene. The ileo-colon-ring primordium is marked by *Sox2* (*Soxb1* and *2*), *Nkx6* and *Pdx1* expression. The gut 1 primordium expresses *Sox2* (*Soxb2*), *Nkx2.2* and *Gata4/5/6*. The primordium of gut 2 expresses *Cdx1* and *Nkx2.2*, and that of gut 3 is marked only by *Cdx1*. *Mnxa/b* is expressed throughout the dorsal part of the mid- and hindgut, except for the ileo-colon ring primordium. *Sox 17* is expressed in the dorsal region of the primordia of the ileo-colon ring, gut 1 and gut 2. This analysis demonstrates that, in the absence of the cephalochordate *Sox2*-expressing domains in the mid- and hindgut endoderm, the *Pdx1* and *Cdx1*-expressing region becomes homologous to that of vertebrates. Opp, Dp, Irp and Gp1–3, the primordia of the oesophagus, diverticulum, ileo-colon ring and guts 1–3 respectively. For other abbreviations, see Fig. 2.

vertebrates, the *Pdx1* and *Cdx1*-expressing region would be contiguous (Fig. 1b). Similar to the vertebrate small and large intestine, gut primordia 2 and 3 express *Cdx1* (Fig. 6). Although cephalochordate gut patterning occurs in the absence of the spatiotemporal regulation provided by the *Hox* clusters, other transcription factors may function to segment gut primordia 2 and 3 (Fig. S6). Consequently, we propose that the cephalochordate gut 2 and 3 segments are homologous to the vertebrate small and large intestines, respectively, revealing that the basic patterning of these hindgut domains occurred prior to the emergence of vertebrates.

2.6. Other endoderm-derived structures

Above, we have shown that posterior to the *Sox2/Pax1/9*-expressing pre-existing pharyngeal pouch, the late phylotypic stage cephalochordate endoderm is divided into seven segments along the anterior-posterior axis (Fig. S6). The primordia we have yet to discuss include the rudiments of gill slits 4–n and the oesophagus. Both primordia are located between the cephalochordate *Sox2* and *Pdx1* expression domains, which, in vertebrates, are directly apposed. Based partly on the logic that loss of these primordia would result in a vertebrate-like mid/hindgut patterning map, we propose that gill slits 4-n and the oesophagus primordia were lost during the vertebrate transition. Furthermore, because the vertebrate oesophagus is derived from the *Sox2*-expressing foregut endoderm, located posterior to the pharyngeal pouch, and its patterning is simultaneously regulated by *Pax1/9* and *Hox* clusters (Grapin-Botton and Melton, 2000) (Fig. 1b), an evolutionary developmental biology based analysis suggests that a narrow oesophagus that supported the food-filtering system of the chordate ancestor was also bound to be lost in the new vertebrate body plan.

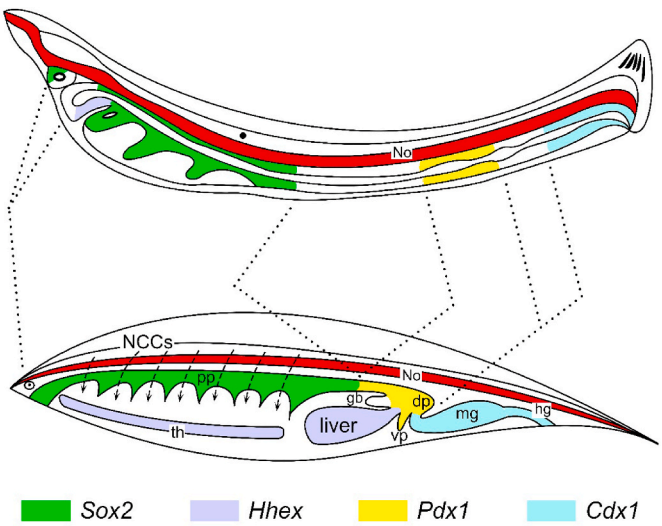

Fig. 6. The evolutionary rudiments of the vertebrate body plan: insights from the endoderm. Our evolutionary developmental biology analysis reveals that the primordia of posterior pre-oral pit, anterior endostyle, gill pores, gill slits 4-n, oesophagus, ileo-colon ring and gut 1 are absent from the patterning map of the vertebrate endoderm. All of these evolutionary losses may result in improvements in food acquisition, allowing for filter feeders to prey on food. These innovations in endoderm patterning played a central role in the origin of vertebrate body plan. For example, in *Metaspriggina*, the new vertebrate NCC-derived pharyngeal arches and muscles strengthened the pharyngeal-derived structures, enhancing its ability to swallow food, and the newly derived digestive organs, including the liver, improved its ability to digest food and metabolize nutrients. Pp, pharyngeal pouches; th, thyroid; dp, dorsal pancreas; vp, ventral pancreas; mg, midgut; hg, hindgut. For other abbreviations, see Figs. 1 and 2.

3. Discussion

3.1. The pharyngeal pouch and the evolutionary route of early vertebrates

Over the past 150 years, many characters have been analysed in research into the origin of the vertebrate body plan from the early deuterostome clade, however, there is no one unchallenged feature which can distinguish chordates from other deuterostomes—not even the notochord (Janvier, 2015; Jefferies, 1968; Dominguez et al., 2002). For instance, the calcichordate group, of early echinoderms also had a structure similar to the notochord (Jefferies, 1968; Dominguez et al., 2002). The presence of a pre-existing pharyngeal pouch in cephalochordates, as described above, provides chordates with the unambiguous and distinguishing feature of an endoderm-derived pharyngeal pouch, suggesting that chordates could also be called pouchates.

Under the pouchate framework, the evolution of early vertebrates from worm-like animals to real craniates can be more explicitly understood (Fig. S7). The early chordate ancestor evolved a pharyngeal pouch from pharyngeal endoderm. Prior to the emergence of stem vertebrates, olfactory function was added. At the *Haikouella* stage, the NCC-derived pharyngeal arches emerged. At the stage of myllokunmingiids and *Metaspriggina*, stem vertebrate genesis was complete, and the vertebrate-type pharyngeal arches and mouth evolved. At the cyclostome stage, the NCC-derived head skeleton emerged. At the ostracoderm stage, cranial skeleton evolution was complete. Finally, after the emergence of the jaw, the basic foundations of the vertebrate body plan were all in place (Fig. S7).

3.2. Evolutionary loss of endoderm-derived structures, and lifestyle changes

Most endoderm-derived structures play a role in the digestive system, and we argue that innovations in these structures played a central role in the origin of the vertebrate body plan. Our ancestors evolved from acquiring food by filter feeding to predation. Using a combination of regulatory gene expression and morphological observations, we demonstrate that, during the phylotypic stage, the cephalochordate endoderm can be divided into 12 primordia, including the pre-oral pit, pharyngeal pouch, pharyngeal gill pores, asymmetric mouth, endostyle, gill slits 4-n, oesophagus, diverticulum, ileo-colon ring and guts 1–3 (Fig. 1a and Fig S6). Our analysis suggests that the primordia of the posterior pre-oral pit, anterior endostyle, gill pores, gill slits 4-n, oesophagus, ileo-colon ring and gut 1 were lost in the evolutionary transition to vertebrate-like endoderm (Fig. 6). On the developmental fates and functions of these 12 primordia in amphioxus, pre-oral pit forms the presumable homolog of vertebrate anterior pituitary, and however its authentic function is unclear (Soukup and Kozmik, 2016); pharyngeal pouch packages and protects endoderm-derived gill arch (Young, 1981); pharyngeal gill pores form gill arches and participate in filtering food particles (Young, 1981); asymmetric mouth will be symmetric and filters food particles (Young, 1981; Soukup and Kozmik, 2016); endostyle is the homolog of vertebrate thyroid from both development and functions (Young, 1981); gill slits 4-n form the 4-n pharyngeal pouch and gill pores (Young, 1981); oesophagus plays an important role in the behavior of filtering food particles by controlling food size (Young, 1981); diverticulum is the homolog of vertebrate pancreas, and however which is a special intracellular digestive organ and can phagocytize food particles directly (He et al., 2018); ileo-colon ring is an auxiliary digestive organ and can pump partial food particles into the lumen of diverticulum (He et al., 2018); guts 1–3 form the adult gut, which can also phagocytize some food particles and forming feces (Young, 1981; He et al., 2018). In vertebrate body plan, the homolog of anterior pre-oral pit develops into salivary glands (Urkasemsin and Ferreira, 2019), which can help digesting foods by exocrine function; pharyngeal pouch packages and protects the mesoderm-derived gill arch (Young, 1981); the homolog of anterior endostyle form thyroid, which is classical endocrine organ and can regulate physiological behaviors by synthesizing and releasing hormones, such as triiodothyronine (T3) and thyroxine (T4) and peptide hormone calcitonin, etc. (Young, 1981); most vertebrate mouths are symmetric throughout embryogenesis (Young, 1981); the homolog of diverticulum forms more complex pancreas with both exocrine and endocrine functions (He et al., 2018); the homolog of gut 2 forms small and large intestines, which digest foods in their lumens and absorb nutrients (Young, 1981); the homolog of gut 3 form rectum, which forms feces and can reabsorb some water and nutrients (Young, 1981). We argue that all of these evolutionary losses were related to changes in the manner of acquiring and digesting food, and that this innovation is reflected by the body plan of *Metaspriggina* (Conway Morris and Caron, 2014). The new symmetric mouth, broad pharynx, NCC-derived pharyngeal arches/muscles, and shortened oesophagus ensured that early vertebrates could transition from passive filter-feeding to actively swallowing larger foods. Since the sorting function of the food-filtering system (Young, 1981) would no longer be necessary under these new circumstances, this transition also explains the loss of the ileo-colon ring from the body plan of early vertebrates.

3.3. Remodeling of endoderm-derived structures and whole genome duplication

In this investigation, we reveal that, although the resultant cell types and physiological functions of the homologous structures are more varied, many of the core regulatory genes involved in vertebrate endoderm patterning and organogenesis are similarly expressed in the cephalochordate endoderm during the phylotypic stage (Fig. S8) (Grapin-Botton and Melton, 2000; Zaret, 2008). While the cephalochordate endoderm is segmented like vertebrates, the regulation of cephalochordate endoderm patterning remains at the level of ChIN, and does not recruit the dynamic inducing signaling networks and hierarchical gene regulatory networks (GRNs) found in vertebrates (Van de Peer, 2004; Van de Peer et al., 2009). We have demonstrated the activity of ChINs during the phylotypic stage in the primordia of all cephalochordate endoderm-derived structures (Van de Peer et al., 2009). We propose that the function of ChINs in these primordia promotes limited cell-fate differentiation, while the GRNs are required to promote the diversity of the endoderm-derived cell types and organs present in vertebrates (Grapin-Botton and Melton, 2000; Zaret, 2008; Van de Peer et al., 2009). Furthermore, while cephalochordates have only a single *Hox* cluster (Putnam et al., 2008), the more complex functions of the vertebrate endoderm require the participation of *Hox* members from all four clusters (Grapin-Botton and Melton, 2000) (Fig. 1b). These facts suggest that the evolutionary path from an amphioxus-like ancestor to craniates requires the recruitment of large numbers of novel regulatory elements to induce, remodel and refine the primitive ChINs (Van de Peer et al., 2009; Nowotschin et al., 2019). We propose that many of these innovations were dependent on the two rounds of whole-genome duplications (2 R) that occurred during the evolution of chordates (Van de Peer, 2004) (Fig. S8).

4. Conclusion

Utilizing the hourglass model of evolution, we examined cephalochordate endoderm-derived structures during the phylotypic period. We demonstrate that the vertebrate symmetric mouth evolved from the anterior pre-oral pit, that, during development, the cephalochordate *Pax1/9*-expressing pharyngeal endoderm is homologous to the vertebrate pharyngeal pouch, that the thyroid is homologous to the posterior endostyle, that the vertebrate pancreas evolved from the *Pdx1*-expressing endoderm of early chordates at the phylotypic stage, and that the small and large intestine are homologous to the cephalochordate gut 2–3 segments (Fig. 6).

5. Methods

Animal and embryo procurement. The adult cephalochordate *Branchiostoma belcheri* samples were collected from Zhanjiang Bay, Guangdong province, China, during the summer breeding season (June 20th to July 10th), and cultured with mixed algal powder at 28–32 °C at Beihai Marine Station of Nanjing University, Guangxi province, China. Gametes were acquired by heat stimulation (35–37 °C) and fertilized in filtered seawater. Embryos and larvae were cultured at 30 ± 2 °C.

Gene identification. cDNA and amino acid sequences were aligned using ClustalW, and the neighbour-joining trees constructed with Mega 6 software using 1000 bootstrap. The cDNA sequences are deposited in Genbank (LIBEST_026,676), and the phylogenetic trees are deposited in https://pan.seu.edu.cn:443/link/EAF803D337EE7B68D39C2D625EF2EA50, where thy can be freely accessed.

***In situ* hybridization.** The samples were fixed in MEMPLA at room temperature for 1 h or at 4 °C for 12 h, prior to storage in 75% ethanol at −20 °C. The *in situ* hybridization experiments on embryos and larvae were performed as described by Holland (Holland et al., 1996) and Holland (Holland et al., 1999) with some modifications. Total RNA was isolated by TRIzol® LS Reagent (Thermo Fisher Scientific, 10,296,028) and treated with DNase I (Thermo Fisher Scientific, 18068015), and reverse transcribed with SuperScript® III (Thermo Fisher Scientific, 18080093). Target genes were amplified according to the open genome database of *B. floridae* in NCBI and JGI. The PCR reactions were performed using *Premix Taq*™ (*Ex Taq*™ Version 2.0 plus dye) (TaKaRa, RR902A), the DNA fragments inserted into pGEM-T Easy Vector Systems II (Promega, A1380), and the templates amplified by M13 primers. The digoxigenin (DIG)-labelled riboprobes were synthesized by T7 or Sp6 RNA polymerase (Roche, 11,175,025,910) and precipitated with 4 M LiCl/100% ethanol (40 μl/1000 μl) for 12 h. Hybridizations were performed in Netwells™ (Corning, 3479). To reduce background noise, the probe concentration was decreased to 0.1–0.2 ng μl^{-1} and the hybridization was performed at 60–65 °C for 12–16 h. Post-hybridization washes were performed in 50% deionized formamide, 5X SSC and 0.1% Tween20 for 3 × 30 min at the hybridization temperature, in 50% deionized formamide, 2 x SSC and 0.1% Tween20 for 2 × 30 min at the hybridization temperature, in 50% deionized formamide, 1X SSC and 0.1% Tween20 for 2 × 30 min at the hybridization temperature, in 0.2 x SSC and 0.1% Tween 20) for 30 min at 37 °C and finally in NaPBSTw (0.02 M PBS and 0.1% Tween 20 for 3 × 10 min at room temperature with gentle rocking. RNase treatments were omitted. The anti-digoxigenin-AP (Roche, 11,093,274,910) was visualized by 0.5 x NBT/BCIP (Roche, 11,681,451,001) at 2–4 °C for 30 min to 14 days. The cephalochordate endoderm-patterning regulatory gene expression patterns are deposited at https://pan.seu.edu.cn:443/link/EAF803D337EE7B68D39C2D625EF2EA50 and are freely accessible.

Photography. Embryos and larvae were photographed by DP72 CCD on IX81 microscopes (Olympus, Tokyo, Japan). For differential interference contrast (DIC), samples were transferred into 0.02 M NaPBS in *Nunc*™ *Lab-Tek*™ *Chamber* Slides™ (Thermo Fisher Scientific, 155,380). Images were acquired and processed using cellSens software (Olympus, Tokyo, Japan). Images were combined together using Adobe Photoshop CS3 or CS5. The fossil samples were photographed on a stereomicroscope (Zeiss, SteREO Discovery. V20).

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “On the origin of vertebrate body plan: insights from the endoderm using the hourglass model”.

CRediT authorship contribution statement

Chunpeng He: Conceptualization, Writing - original draft, Formal analysis, Writing - review & editing, Methodology. **Tingyu Han:** Conceptualization, Writing - original draft, Formal analysis, Writing - review & editing. **Xin Liao:** Methodology. **Rui Guan:** Formal analysis. **J.-Y. Chen:** Conceptualization, Writing - original draft. **Kimberly D. Tremblay:** Conceptualization, Writing - original draft, Writing - review & editing. **Zuhong Lu:** Conceptualization, Writing - original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gep.2020.119125.

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