Evolution of Developmental Programs for the Midline Structures in Chordates: Insights From Gene Regulation in the Floor Plate and Hypochord Homologues of Ciona Embryos

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In vertebrate embryos, dorsal midline tissues, including the notochord, the prechordal plate, and the floor plate, play important roles in patterning of the central nervous system, somites, and endodermal tissues by producing extracellular signaling molecules, such as Sonic hedgehog (Shh). In Ciona, hedgehog.b, one of the two hedgehog genes, is expressed in the floor plate of the embryonic neural tube, while none of the hedgehog genes are expressed in the notochord. We have identified a cis-regulatory region of hedgehog.b that was sufficient to drive a reporter gene expression in the floor plate. The hedgehog.b cis-regulatory region also drove ectopic expression of the reporter gene in the endodermal strand, suggesting that the floor plate and the endodermal strand share a part of their gene regulatory programs. The endodermal strand occupies the same topographic position of the embryo as does the vertebrate hypochord, which consists of a row of single cells lined up immediately ventral to the notochord. The hypochord shares expression of several genes with the floor plate, including Shh and FoxA, and play a role in dorsal aorta development. Whole-embryo single-cell transcriptome analysis identified a number of genes specifically expressed in both the floor plate and the endodermal strand in Ciona tailbud embryos. A Ciona FoxA ortholog FoxA.a is shown to be a candidate transcriptional activator for the midline gene battery. The present findings suggest an ancient evolutionary origin of a common developmental program for the midline structures in Olfactores.

Keywords: ascidian, Sonic hedgehog, floor plate, endodermal strand, Ciona intestinalis sp. A, notochord, FoxA transcription factors, hypochord
INTRODUCTION

The embryonic midline tissues, notochord, and floor plate are signaling centers that pattern vertebrate embryos (Placzek and Briscoe, 2005; Stemple, 2005). The notochord acts as an axial supportive structure and induces the floor plate in the neural tube and patterns somitic mesoderm via Sonic hedgehog (Shh) secretion. The floor plate then patterns the neural tube along the dorso-ventral axis also using the Shh signal. Thus these midline structures are central elements for construction of the vertebrate body plan.

In anamniote embryos, an endodermal rod-shaped structure, hypochord, transiently appears ventral to the notochord (Franz, 1898; Reinhardt, 1904; Gibson, 1910). Development of the hypochord also depends on signals from the notochord (Cleaver and Krieg, 1998). The hypochord was once thought to be simply a supportive structure (Stöhr, 1895; Corbo et al., 1997a) but several lines of evidence suggest that it plays a role in the positioning of the dorsal aorta (Cleaver et al., 1997; Löfberg and Collazo, 1997; Cleaver and Krieg, 1998; Eriksson and Löfberg, 2000; Hogan and Bautch, 2004), and for determination of left–right axis asymmetry (Danos and Yost, 1996; Lohr et al., 1997). Thus, transient midline tissues originating from different germ layers, the floor plate (ectoderm), the notochord (mesoderm), and the hypochord (endoderm), pattern the embryonic structure in vertebrates.

The hypochord shares expression of several genes with the floor plate and the notochord, including Shh and FoxA (Yan et al., 1995; Appel et al., 1999; Dal-Pra et al., 2011; Peyrot et al., 2011). Although their originating germ layers are different, progenitor cells of these midline tissues locate close to one another in the dorsal marginal zone, such as the Spemann organizer in amphibians and the embryonic shield in zebradish (Shih and Fraser, 1995; Melby et al., 1996; Latimer et al., 2002; Latimer and Appel, 2006; Dal-Pra et al., 2011; Peyrot et al., 2011). These commonalities suggest a tight developmental and evolutionary connection among these midline structures. The notochord is the organ that define the phylum (or superphylum) evolutionary connection among these midline structures. The notochord acts as an axial supportive structure and induces the floor plate in the neural tube (nerve cord) in tunicate Ciona embryos. Comparative genomics between Ciona intestinalis type A and Ciona savignyi revealed that the first intron of hedgehog.A contains highly conserved non-coding regions, which could be candidates for such additional cis-regulatory sequences (Figure 1C). To test this possibility, we placed the first intransequence upstream of the 2.6-kb genomic region in the hedgehog.A > kaeode construct and examined Kaede reporter expression in embryos transfected with this DNA construct (Figure 1D). As expected, higher frequency of Kaede expression in the floor plate was observed (Figures 1D,E). However, the reporter expression in the endodermal strand also remained (Figures 1D,F). The endodermal strand is a caudal midline structure that lies immediately ventral to the notochord and its homology with the vertebrate hypochord has been proposed (Corbo et al., 1997a). Thus, the cis-regulatory regions of Ciona hedgehog.b can activate transcription in the floor plate and hypochord homologues. This observation further prompted us to test an idea that the floor plate and the endodermal strand share a developmental program including the transcriptional machinery.

Here we provide new evidence that the endodermal strand shares the gene regulatory mechanism with the floor plate in Ciona embryos. Functional analysis of the cis-regulatory region of the floor plate-specific hedgehog.b gene revealed its latent ability to drive transcription in the endodermal strand. Whole-embryo single-cell transcriptome analysis identified a number of genes specifically expressed in both the floor plate and the endodermal strand in Ciona tailbud embryos. These genes and their transcriptional regulation suggest an ancient evolutionary origin of a common developmental program for the midline structures in Olfactores. Our findings also support homology between the vertebrate hypochord and the tunicate endodermal strand.

RESULTS AND DISCUSSION

Transcriptional Activation by Cis-Regulatory Regions of Ciona hedgehog.b in the Floor Plate and Hypochord Homologues

Ciona hedgehog.b is expressed in the floor plate, but not in the notochord during embryogenesis (Takatori et al., 2002; Islam et al., 2010; Figures 1A,B). When the 2.6-kb upstream region of hedgehog.b connected with a Kaede reporter (hedgehog.b > kaeode) was introduced into Ciona embryos, the expression of Kaede reporter was observed in the floor plate at the mid tailbud stage (Figure 1D). In addition to the expression in the floor plate, “ectopic” Kaede expression was observed in the endodermal strand of some embryos (Figure 1D). In contrast, no Kaede expression was observed in the notochord.

Because the reporter expression was only observed in a relatively small proportion of transfected embryos and the ectopic expression in the endodermal strand was observed (Figure 1D), we thought that additional cis-regulatory sequences might be present outside of the 2.6-kb upstream region. Comparative genomics between Ciona intestinalis type A and Ciona savignyi revealed that the first intron of hedgehog.b contains highly conserved non-coding regions, which could be candidates for such additional cis-regulatory sequences (Figure 1C). To test this possibility, we placed the first intron sequence upstream of the 2.6-kb genomic region in the hedgehog.b > kaeode construct and examined Kaede reporter expression in embryos transfected with this DNA construct (Figure 1D). As expected, higher frequency of Kaede expression in the floor plate was observed (Figures 1D,E). However, the reporter expression in the endodermal strand also remained (Figures 1D,F). The endodermal strand is a caudal midline structure that lies immediately ventral to the notochord and its homology with the vertebrate hypochord has been proposed (Corbo et al., 1997a). Thus, the cis-regulatory regions of Ciona hedgehog.b can activate transcription in the floor plate and hypochord homologues. This observation further prompted us to test an idea that the floor plate and the endodermal strand share a developmental program including the transcriptional machinery.

Single-Cell Transcriptomic Analysis Revealed a Gene Battery Shared Among the Midline Tissues

To further investigate the shared developmental program between the floor plate and the endodermal strand, we compared gene expression profiles between these tissues by whole-embryo single-cell transcriptomics at the mid tailbud stage (Table 1 and Figure 2; Horie T. et al., 2018; Horie R. et al., 2018;
Cao et al., 2019). Whole-embryo single-cell transcriptome data clearly revealed that *hedgehog.b* is expressed in the floor plate but not expressed in any other tissues, including the notochord and the endodermal strand (Figure 2B). Among the top 20 genes highly expressed in the endodermal strand, 8 genes were shown to be significantly enriched (p < 0.05) in the floor plate (Table 1). Of these, five genes were highly enriched (p < 0.001) in the floor plate (Table 1 and Figure 2). These genes include *fz4* (gene model ID KH.C6.162) encoding a Frizzled4 receptor, *foxA.a* (KH.C11.313) encoding a FoxA transcription factor, KH.C2.442 encoding a solute carrier family 1 protein, KH.C5.232 encoding a tissue inhibitor of metalloproteinases 4, and KH.C4.230 encoding a calponin/transgelin family protein (*transgelin-related.b*). Interestingly, four of these genes (*fz4,*...
TABLE 1 | Top 20 upregulated genes in the endodermal strand at the mid tailbud stage.

| Gene Model ID | Endodermal strand | Floor plate | Similarity or predicted gene product |
|---------------|-------------------|-------------|--------------------------------------|
| KH2012:KH.L4.15 | 6.44 | 3.63E-35 | log₂ fold change | p-value | log₂ fold change | p-value | Zinc transporter ZIP1 |
| KH2012:KH.C4.693 | 5.54 | 1.39E-30 | −2.18 | 1 | SLIT and NTRK-like protein |
| KH2012:KH.C9.162 | 3.76 | 2.90E-15 | 0.27 | 1 | Regulator of G-protein signaling |
| KH2012:KH.C9.672 | 3.59 | 1.13E-14 | 0.22 | 1 | Regulator of G-protein signaling |
| KH2012:KH.C1.520 | 3.28 | 1.79E-14 | 0.02 | 1 | Secreted frizzled-related protein (sFRP3/4-b) |
| KH2012:KH.C5.232 | 3.26 | 6.66E-13 | 3.41 | 4.04E-05 | Tissue inhibitor of metalloproteinases 4 |
| KH2012:KH.L4.15 | 2.79 | 2.65E-10 | 0.61 | 1 | Uncharacterized protein |
| KH2012:KH.C6.162 | 2.88 | 2.69E-08 | 3.99 | 4.12E-07 | Frizzled receptor (Fz4) |
| KH2012:KH.C4.230 | 2.76 | 4.52E-08 | 4.22 | 3.96E-09 | Transgelin/Calponin/Neuronal protein 25/SM22a (tagln-r.b) |
| KH2012:KH.C2.378 | 2.81 | 4.75E-08 | 3.00 | 3.42E-03 | Brain-enriched hyaluronan-binding protein |
| KH2012:KH.C6.37 | 2.77 | 5.59E-08 | −4.91 | 1 | P-loop containing nucleotide triphosphate hydrolases |
| KH2012:KH.C9.174 | 3.22 | 7.21E-08 | −3.65 | 1 | Hypothetical protein |
| KH2012:KH.C3.203 | 2.58 | 9.24E-08 | 3.55 | 2.97E-05 | Fork head/HNF-3 homologue (FoxA-a) |
| KH2012:KH.C4.230 | 2.51 | 3.56E-07 | −4.57 | 1 | P-loop containing nucleotide triphosphate hydrolases |
| KH2012:KH.L4.17 | 2.09 | 6.46E-05 | −1.17 | 1 | Zinc finger protein (SAL-like protein 1) |
| KH2012:KH.C3.585 | 2.13 | 7.16E-05 | 2.49 | 2.49E-02 | SCRaMblase (phospholipid scramblase) family member (scrm-1) |

foxA.a, KH.C5.23, and KH.C4.230) are also expressed in the notochord (Figures 2D–G). The expression pattern of foxA.a is consistent with the previously reported whole-mount in situ hybridization (Corbo et al., 1997a). These genes may constitute a gene battery co-regulated in the midline tissues at the mid tailbud stage.

For further analysis, we adopted KH.C4.230 as a model to investigate transcriptional regulation in the midline tissues because its expression level is relatively high and the enriched expression in the floor plate, the notochord, and the endodermal strand is strongly supported by the single-cell transcriptomic analysis (p-values, 3.96E-09, 1.49E-14, and 4.52E-08, respectively). KH.C4.230 encodes a protein belonging to the calponin/transgelin family. Calponins and transgelins are actin-associated proteins highly conserved from yeast to mammals (Prinjha et al., 1994; Goodman et al., 2003). We named KH.C4.230 as transgelin-related.b (tagln-r.b) based on the sequence similarity and genomic arrangement (Figure 3). Whole-mount in situ hybridization confirmed that tagln-r.b is expressed in the floor plate, the notochord, and the endodermal strand (Figure 2F).

In the genome of C. intestinalis type A, tagln-r.b is clustered in tandem with five other calponin/transgelin family genes within a 20-kb genomic region (Figure 3A). Whole-embryo single-cell transcriptome and high-throughput in situ hybridization data in the Ghost database (Satou et al., 2005) indicate that at least three of these tagln-r genes (tagln-r.c, tagln-r.d, and tagln-r.e) are also specifically expressed in the floor plate, the notochord, and the endodermal strand (Supplementary Figure 1; spatial expression patterns of tagln-r.e can be found at http://ghost.zool.kyoto-u.ac.jp/cgi-bin/phototgh.cgi?inkey=CLSTR02020). Thus the clustered tagln-r genes are likely to be co-regulated as a member of the gene battery above mentioned.

The Role of FoxA.a as a Common Transcriptional Activator for the Midline Gene Battery

Because the expression profile of foxA.a (Figure 2F) was very similar to that of tagln-r.b (Figure 2G), FoxA.a seemed to be a good candidate for a common transcriptional activator in the midline tissues. To test this possibility, we examined distribution of the FoxA.a binding sites in the upstream of the putative transcription start sites of each of the clustered tagln-r genes using a set of ChIP-on-chip data of FoxA.a (Kubo et al., 2010). As expected, FoxA.a binding sites are enriched in the 5′ flanking region of each tagln-r gene (Figure 3B). To analyze the transcriptional regulatory mechanism of tagln-r.b, its 2.8-kb upstream region was connected with the coding sequence of Kaede (Figure 4A) and introduced into Ciona embryos. The tagln-r.b > kaede DNA construct recapitulated the endogenous expression pattern of tagln-r.b; it was expressed in the floor plate, the notochord, and the endodermal strand (Figure 3C), suggesting that the 2.8-kb upstream region contains cis-regulatory sequences sufficient for transcription in the midline tissues.

The 2.8-kb upstream region of tagln-r.b contains eight putative Fox binding sites (Figure 4A). Among these sites, three distal sites [binding sites (BS) 1–3] are conserved between C. intestinalis type A and Ciona savignyi, whereas five proximal sites (BS4–8) are not conserved. To test functional importance of putative Fox BSs, three conserved sites (BS1–3) or all eight BSs (BS1–8)
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FIGURE 2 | Whole-embryo single-cell RNA-seq analysis of midline tissue-specific genes. (A) A t-distributed stochastic neighbor embedding (t-SNE) projection map of mid-tailbud stage embryos obtained in a previous study (Horie T. et al., 2018). Each dot corresponds to the transcriptome of a single cell, and cells possessing similar transcriptome profiles map near each other. The major tissue types in tailbud-stage embryos were identified. Identification of tissue types is based on the expression of known marker genes as previously described (Horie T. et al., 2018). Clusters of cells corresponding to the floor plate, the notochord, and the endodermal strand are encircled. (B) The t-SNE projection map highlighting hedgehog.b-expressing cells (red dots) in the floor plate. (C–G) The t-SNE projection map showing the expression profiles of representative genes that are specifically expressed in both the floor plate and the endodermal strand (Table 1). Red and orange dots indicate cells expressing KH.C2.442 (C), KH.C6.162 (frizzled4, D), KH.C5.232 (E), KH.C11.313 (foxA.a, F), and KH.C4.230 (tagln-r.b, G). (H) Expression pattern of tagln-r.b at the tailbud stage visualized by whole-mount in situ hybridization. The arrow and the arrowhead indicate the floor plate and the endodermal strand, respectively. Scale bar, 50 µm.

were mutated in the tagln-r.b > kaede construct (Figure 4A) and the reporter expression was examined in the mid tailbud embryos transfected with these DNA constructs (Figure 4B). When only the conserved sites were mutated (∆BS1-3), the reporter expression was significantly reduced in the floor plate and the endodermal strand, whereas it was moderately reduced in the notochord. Additional mutations in the non-conserved BSs (BS4-8) did not further decrease the reporter expression
in each tissue. These results suggest that a Fox transcription factor, presumably FoxA.a, serves as a transcriptional activator of tagln-r.b in the midline tissues via direct interaction with the upstream region. Our observation also suggests that a greater contribution of FoxA.a to transcriptional activation of tagln-r.b in the floor plate and the endodermal strand than in the notochord. Because disruption of all Fox BSs in the cis-regulatory region of tagln-r.b had only slightly reduced the reporter expression in the notochord (Figure 4), it is plausible that Brachyury is the main activator for tagln-r.b in the notochord.

FIGURE 3 | The calponin/transgelin family gene cluster in the Ciona genome. (A) Schematic diagram of the gene cluster. Six genes, tagln-r.a, tagln-r.b, tagln-r.c, tagln-r.d, tagln-r.e, and tagln-r.f, each encoding a calponin/transgelin family protein are clustered in a 20-kb genomic region. (B) The transcriptional landscape of the tagln-r loci. Transcription start sites (TSSs) and spliced leader (SL) trans-splicing sites (Yokomori et al., 2016) and FoxA.a binding sites determined by ChIP-on-chip analysis (Kubo et al., 2010) were mapped on the Ghost Genome Browser (Satou et al., 2005, 2008). (C) Localization of Kaede reporter expressed under the control of the upstream cis-regulatory region of tagln-r.b. Arrows, arrowheads, and asterisks indicate the floor plate, the endodermal strand, and the notochord, respectively. Scale bar, 50 µm.

Among 29 Fox transcription factors identified in C. intestinalis type A (Imai et al., 2004; Satou et al., 2005), FoxA.a is the most plausible candidate as the transcription factor that interacts with Fox BSs in the upstream region of tagln-r.b for three reasons. First, as mentioned above, the ChIP-on-chip data demonstrated FoxA.a binding to the upstream region of tagln-r.b (Kubo et al., 2010). Second, expression patterns of foxA.a and tagln-r.b are similar to each other. Third, none of the other Fox family genes show similar expression patterns (Imai et al., 2004). In a strict sense, however, the present analysis does not exclude the possibility that a Fox transcription factor other than FoxA.a is involved in the transcriptional activation of tagln-r.b. To further assess the role of FoxA.a in tagln-r.b expression in the midline tissues, functional manipulations of FoxA.a, such as...
FIGURE 4 | Functional analysis of putative Fox binding sites (BSs) in the cis-regulatory region of tagln-r.b. (A) Schematic diagram of the Kaede reporter constructs. Cyan and blue boxes indicate the 5′ untranslated region and a partial coding region, respectively, of tagln-r.b. Green boxes indicate the coding sequence of Kaede. Substitution mutations were introduced into putative Fox BSs. Colored ovals indicate the putative Fox BSs and black crosses indicate mutated BSs. (B) Expression of the Kaede reporter in the midline tissues of tailbud embryos. Localization of Kaede was detected by immunofluorescence staining in mid tailbud (12 hpf) embryos that developed from fertilized eggs electroporated with tagln-r.b>kaede fusion constructs. Vertical bars indicate the percentage of Kaede-positive embryos. Error bars represent SEM from three independent experiments. The total number of embryos scored for each construct was 172 for tagln-r.b>kaede (wild-type), 152 for tagln-r.bΔBS1-3>kaede, and 147 for tagln-r.bΔBS1-8>kaede. Statistical analysis was carried out using the standard Student t-test (**P < 0.01, *P < 0.05).

overexpression of wild-type and a repressor form and tissue-specific knockdown, will be required in future studies.

Disruption of all Fox BSs in the cis-regulatory region of tagln-r.b did not completely abolished the reporter expression in the floor plate and the endodermal strand (Figure 4). This suggests that other transcription factors are involved in transactivation of tagln-r.b. Future identification of transcription factors that interacts with the cis-regulatory region of tagln-r.b will contribute to the elucidation of the gene regulatory networks for the development of the floor plate and the endodermal strand.

Developmental Roles of the Endodermal Strand in Ciona Embryos

The hypochord, transient rod-like structure situated under the notochord, is first described in embryos of elasmobranchs (Leydig, 1852). Many morphological studies on this structure were reported in embryos of lampreys, fishes, and amphibians in the late 19th and early 20th centuries (Hatta, 1893; Franz, 1898; Klaatsch, 1898; Reinhardt, 1904; Gibson, 1910). Since then, however, the hypochord has been neglected by researchers for many years, and its function remains elusive. An inductive role in the formation of the dorsal aorta has been proposed (Cleaver et al., 1997; Löfberg and Collazo, 1997; Cleaver and Krieg, 1998; Eriksson and Löfberg, 2000). Although it is uncertain whether the hypochord has a structural counterpart in embryos of higher vertebrates, a similar inductive role of the dorsal endoderm in blood vessel patterning has been proposed in avian embryos (Hogan and Bautch, 2004).

The only function of the endodermal strand known to date is its role as the precursor of the adult intestine (Hirano and Nishida, 2000; Nakazawa et al., 2013). The similarity between the hypochord and the endodermal strand prompted us to ask whether the Ciona endodermal strand has an inductive role similar to that of the vertebrate hypochord. In vertebrate embryos, the blood vessel precursor angioblasts migrate toward the hypochord or dorsal endoderm to form the dorsal aorta (Cleaver and Krieg, 1998; Eriksson and Löfberg, 2000; Hogan and Bautch, 2004). To test whether similar cell migration
occurs in *Ciona* embryos, we labeled trunk mesenchyme cells with the photoconvertible fluorescent protein Kaede (Ando et al., 2002) and fluorescence emitted by Kaede was converted from green to red by irradiation with 405-nm violet light at 10 hpf. The *kaede* transgene was expressed using an upstream regulatory region of *Ciona pax2/5/8.a*, which could drive the reporter gene expression in trunk mesenchyme cells. These embryos were analyzed by time-lapse imaging from late tailbud (12 hpf) to larval (24.5 hpf) stages (Figure 5). Some of the Kaede-labeled mesenchyme cells were shown to migrate into the tail along the endodermal strand (Figure 5 and Supplementary Video 1). The *Ciona* endodermal strand may exert an inductive cue for the migratory mesenchyme cells, suggesting a functional similarity between the vertebrate hypochord and the *Ciona* endodermal strand.

The top 10 predominantly expressed genes in the endodermal strand include genes encoding extracellular ligands and receptors, including SLIT and NTRK-like protein (KH.C4.693), secreted frizzled-related protein (KH.C1.520), and frizzled receptor (KH.C6.162) (Table 1). Expression of these genes suggests an active interaction between the endodermal strand and other tissues. In zebrafish, the hypochord expresses the *frzb/sfrp3* gene that encodes a secreted frizzled-related protein (Thisse et al., 2001; Tendeng and Houart, 2006), showing a further similarity between the endodermal strand and the hypochord. Functional analysis of these genes may give insights into the role of the endodermal strand in *Ciona* embryos.

**Conserved Developmental Programs for Midline Tissues in Olfactores**

The present findings, along with a number of previous studies, illustrate common features and the difference of midline development between vertebrates and tunicates (Figure 6). The gene regulatory network for notochord development in ascidian embryos has been extensively studied (Imai et al., 2006; Hotta et al., 2008; Passamaneck et al., 2009; Kubo et al., 2010; José-Edwards et al., 2015; Reeves et al., 2021). *Brachyury* is a key specifier gene for the notochord formation. FoxA.a is an upstream activator of *Brachyury* (Imai et al., 2006; Hotta et al., 2008; Kubo et al., 2010), but it also directly activates notochord-specific genes (Passamaneck et al., 2009; José-Edwards et al., 2015; Reeves et al., 2021).

Co-expression of Brachyury and FoxA family transcription factors is required for notochord development both in vertebrates and tunicates (Herrmann and Kispert, 1994; Teillet et al., 1998; Friedman and Kaestner, 2006; Imai et al., 2006; Hotta et al., 2008; Passamaneck et al., 2009; José-Edwards et al., 2015). The notochord patterns the central nervous system, somitic mesoderm, and dorsal endoderm by secreting Shh in vertebrate embryos, whereas *hedgehog* genes are not expressed in the notochord of tunicate embryos (Takatori et al., 2002). FoxA and Shh/Hedgehog.b are co-expressed in the floor plate of both vertebrates and tunicates (Tessier-Lavigne et al., 1988; Placzek et al., 1990a,b; Takatori et al., 2002; Imai et al., 2009; Dal-Pra et al., 2011; Peyrot et al., 2011). FoxA and Shh are

![FIGURE 5](#) Migration of mesenchyme cells along the endodermal strand during larval development. (A–D) Time-lapse fluorescent images of a late-tailbud embryo expressing Kaede under the control of the cis-regulatory region of *Pax2/5/8.a* at the time indicated. Kaede fluorescence was photo-converted from green to red (shown in magenta) by 405-nm laser irradiation at 12 hpf (A). At 12 hpf, photo-converted Kaede fluorescence was observed in the central nervous system (asterisks) and mesenchyme cells (arrowhead) in the trunk region, whereas no cells were labeled in the tail region. As development proceeded (B–D), a few cells labeled with photoconverted-Kaede appeared in the tail region and posteriorly migrated along the endodermal strand (white arrows). Cells synthesized Kaede after photo-conversion were labeled with green fluorescence (green arrows in panels B–D). Scale bar, 100 µm.

![FIGURE 6](#) Comparison of developmental programs for midline tissues between tunicates and vertebrates. Co-expression of Brachyury and FoxA family transcription factors is required for notochord development both in vertebrates and tunicates. Both the notochord and the floor plate secrete Shh in vertebrate embryos, whereas *hedgehog* genes are not expressed in the notochord of tunicate embryos. FoxA and Shh/Hedgehog.b are co-expressed in the floor plate of both vertebrates and tunicates. FoxA and Shh are also co-expressed in the hypochord precursor. The tunicate endodermal strand expresses FoxA.a and has a latent transactivation potential of *hedgehog*.*b*.
also co-expressed in the hypochord or its primordium (Ruiz i Altaba, 1998; Dal-Pra et al., 2011; Peyrot et al., 2011). The tunicate endodermal strand expresses FoxAa (Corbo et al., 1997a) and has a latent transactivation potential of hedgehog.b as shown in this study.

In conclusion, the present study suggests that the floor plate and the hypochord homologue of Ciona embryos share a gene battery, which is regulated by a common transcription activator FoxAa. The FoxA transcription factor seems to be a key regulator for midline development both in ascidians and vertebrates. The endodermal strand may have an inductive role for a novel population of migratory trunk cells, which further reveals a common feature shared between the endodermal strand and the hypochord. Altogether, the present findings suggest an ancient origin of a common developmental program for and common developmental roles of the midline structures in Olfactores.

MATERIALS AND METHODS

Ciona Adults and Embryos

Mature adults of C. intestinalis type A (also called Ciona robusta) were provided by the Maizuru Fisheries Research Station of Kyoto University and by the Misaki Marine Biological Station of the University of Tokyo through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and were maintained in indoor tanks of artificial seawater (ASW) (Marine Art BR; Tomita Pharmaceutical, Tokushima, Japan) at 18°C. The adults were also collected from the pond on the campus of Kobe University, Kobe, Japan and from the fishing harbor in Murotsu, Hyogo, Japan. Eggs and sperm were obtained surgically from the gonoducts, and the eggs were fertilized in vitro. After insemination, the embryos were raised in ASW containing 50 µg/ml streptomycin sulfate (S6501; Sigma-Aldrich, St. Louis, MO, United States) at 18°C.

Whole-Mount in situ Hybridization

The cDNA clones for hedgehog.b (Gene Collection ID R1CGC41g12) and tagln-r.b (Gene Collection ID R1GCGC29n19) were obtained from the Ciona Gene Collection release 1 (Satou et al., 2002) and used as the templates to synthesize probes. To linearize the plasmid DNA for probe synthesis, cDNA clones were digested with XhoI (for hedgehog.b) or EcoRI (for tagln-r.b). Antisense RNA probes were synthesized with T7 RNA polymerase by using a DIG RNA Labelling Kit (Sigma-Aldrich, St. Louis, MI, United States). Ciona intestinalis type A embryos were fixed at the early tailbud stage in 4% paraformaldehyde in 0.1 M MOPS (pH 7.5) and 0.5 M NaCl at 4°C for 16 h, prior to storage in 80% ethanol at −30°C. Whole-mount in situ hybridization was carried out as described (Oonuma and Kusakabe, 2019).

Preparation of Reporter Constructs and Electroporation

To make the hedgehog.b > kaede plasmid, the 2.6-kb upstream region of Ciona hedgehog.b (Gene Model ID KH.C5.544) was amplified from the genomic DNA of C. intestinalis type A by PCR using a pair of nucleotide primers (5’-ATCTGCAGGCGCATTTAGTGACACAGA-3’ and 5’-ATGGATCCCGCTGATCCAGATG-3’), digested with PstI and BamHI, and then inserted into the PstI/BamHI sites of the pSP-Kaede vector (Hozumi et al., 2010). To make the hedgehog.b (+int) > kaede construct, the first intron sequence of hedgehog.b was amplified from the genomic DNA using a pair of nucleotide primers (5’-TTCTCAGAGCCAGTATGTGCCAC-3’ and 5’-CCCTGCGACCATCCAAGCTTCCGATAAC-3’), digested with XhoI and PstI, and then inserted into the XhoI/PstI sites of the hedgehog.b > kaede plasmid. The tagln-r.b > kaede plasmid was made by inserting the 2.8-kb upstream region of Ciona tagln-r.b (Gene Model ID KH.C4.230) into the pSP-Kaede plasmid using an In-Fusion HD Cloning Kit (Takara Bio, Japan). The 2.8-kb upstream region of tagln-r.b was amplified from the genomic DNA by PCR using a pair of nucleotide primers (5’-AAACTCGAGTACACACGAAATTAAGCAGAGC-3’ and 5’-TTTCTCTGTTGGCCGATT-3’). To generate mutant constructs, tagln-r.b∆BS1-3 > kaede and tagln-r.b∆BS1-8 > kaede, putative Fox binding sites (RYYAYA; Chen et al., 2016) were mutagenized by the PCR-based method as previously described (Oonuma and Kusakabe, 2019). Oligonucleotide primers used for the mutagenesis of fox binding sites (BS1-8) were: 5’-GTACGCGAAAAGCTGTTTTTAAATATC-3’ and 5’-CTGGT1gggcGTACCTTTACCTTTGAGTTG-3’. The 2.8-kb upstream region of Ciona tagln-r.b was amplified from the genomic DNA by PCR using a pair of nucleotide primers (5’-AAACTCGAGTACACACGAAATTAAGCAGAGC-3’ and 5’-TTTCTCTGTTGGCCGATT-3’). To generate mutant constructs, tagln-r.b∆BS1-3 > kaede and tagln-r.b∆BS1-8 > kaede, putative Fox binding sites (RYYAYA; Chen et al., 2016) were mutagenized by the PCR-based method as previously described (Oonuma and Kusakabe, 2019). Oligonucleotide primers used for the mutagenesis of fox binding sites (BS1-8) were: 5’-GTACGCGAAAAGCTGTTTTTAAATATC-3’ and 5’-CTGGT1gggcGTACCTTTACCTTTGAGTTG-3’. The 2.8-kb upstream region of Ciona tagln-r.b was amplified from the genomic DNA by PCR using a pair of nucleotide primers (5’-AAACTCGAGTACACACGAAATTAAGCAGAGC-3’ and 5’-TTTCTCTGTTGGCCGATT-3’).

Immunofluorescence Staining

Immunofluorescent staining was carried out according to the method described by Nishitsui et al. (2012). To visualize the localization of Kaede, a rabbit anti-Kaede polyclonal antibody
(PM012; Medical & Biological Laboratories, Nagoya, Japan; for Kaede) was diluted 1:1000 in 10% goat serum in T-PBS (0.1% Triton X-100 in PBS) and used as the primary antibody. The secondary antibody was an Alexa Fluor 488-conjugated anti-rabbit IgG (A11008; Thermo Fisher Scientific, Waltham, MA, United States). Fluorescent images were obtained by using a laser scanning confocal microscope (FV1200 IX83; Olympus, Tokyo, Japan). Confocal images were collected at 1-µm intervals in the z-axis.

Whole-Embryo Single-Cell Transcriptomic Analysis
A published single-cell transcriptome dataset of mid-tailbud embryos obtained using the 10x Genomics Chromium system (Horie T. et al., 2018; Horie R. et al., 2018; Cao et al., 2019) was used to analyze expression profiles of genes in the midline tissues. The dataset is available through GEO (GSE120035): https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120035. The t-distributed stochastic neighbor embedding (t-SNE) analysis was performed using the Loupe Cell Browser 3.1.1 software (10x Genomics, Pleasanton, CA, United States). The processed data in a Loupe Cell Browser file (.cloupe) is available through the Mendeley data repository: http://dx.doi.org/10.17632/n4pxpr28cb.1. Differentially expressed genes were identified and ranked by statistical significance as previously described (Horie T. et al., 2018).

Time-Lapse Live Imaging and Photo-Conversion of Kaede
Embryos electroporated with pax2/5/8.a > kaede were reared in ASW and mounted on a glass slide with ASW containing 1.5% methylcellulose at 10 hpf. Photoconversion of Kaede was performed as described (Oonuma et al., 2016). Fluorescent images were taken every 15 min for 12.5 h at 18°C by using a laser scanning confocal microscope (FV1200 IX83; Olympus, Japan). Confocal images were collected at 1-µm intervals in the z-axis.

DATA AVAILABILITY STATEMENT
The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

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AUTHOR CONTRIBUTIONS
TK conceived the project and wrote the manuscript. KO, KS, TH, and TK designed the experiments. KO, MY, NM, NO, MM, ST, HS, EN, YH, and KS performed the experiments. TH, ST, MS, and TK analyzed the single-cell RNA-seq data. TH provided the essential materials. KO, NO, and TK analyzed and interpreted the data. KO, TH, and TK edited the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.704367/full#supplementary-material

Supplementary Video 1 | Time-lapse of an embryo showing migration of mesenchyme cells labeled with photo-converted Kaede shown in Figure 5.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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