

Role of notochord cells and sclerotome-derived cells in vertebral column development in fugu, *Takifugu rubripes*: histological and gene expression analyses

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Abstract Despite the common structure of vertebrates, the development of the vertebral column differs widely between teleosts and tetrapods in several respects, including the ossification of the centrum and the function of the notochord. In contrast to tetrapods, vertebral development in teleosts is not fully understood, particularly for large fish with highly ossified bones. We therefore examined the histology and gene expression profile of vertebral development in fugu, *Takifugu rubripes*, a model organism for genomic research. Ossification of the fugu centrum is carried out by outer osteoblasts expressing *col1a1*, *col2a1*, and *sparc*, and the growing centra completely divide the notochord into double cone-shaped segments that function as intercentral joints. In this process, the notochord basal cells produce a thick notochord sheath exhibiting Alcian-blue-reactive cartilaginous properties and composing the intercentral ligament in cooperation with the external ligament connective tissue. Synthesis of the matrix by the basal cells was ascertained by an in vitro test. Expression of *twist2* indicates that this connective tissue is descended from the embryonic sclerotome. Notochord basal cells express *sox9*, *ihhb*, *shh*, and *col2a1a*, suggesting that the signaling system involved in chondrocyte proliferation and

matrix production also functions in notochord cells for notochord sheath formation. We further found that the notochord expression of both *ntla* and *shh* is maintained in the fugu vertebral column, whereas it is turned off after embryogenesis in zebrafish. Thus, our results demonstrate that, in contrast to zebrafish, a dynamic morphogenesis and molecular network continues to function in fugu until the establishment of the adult vertebral column.

Keywords Vertebral column · Development · Notochord · Sclerotome · *Takifugu rubripes*

Introduction

The vertebral column, which is composed of a repetitive series of bony centra (vertebral body) and joint tissues, is an organ evolved specifically in the vertebrates, discriminating them from invertebrates (Fleming et al. 2015; Mansfield et al. 2015). In teleosts, the molecular developmental biology of the vertebral column has been intensively studied by using zebrafish and medaka as small model organisms (Bensimon-Brito et al. 2012; Fleming et al. 2004; Inohaya et al. 2007; Renn et al. 2013; Willems et al. 2012). Morphological characterization of the vertebral column of several non-model large fish, including perch and salmon, has also been reported (Nordvik et al. 2005; Schmitz 1995). These studies have demonstrated that the anatomy and development of the vertebral column differs significantly between teleosts and tetrapods in several respects, despite the common structure of vertebrates.

For example, although the tetrapod centrum forms by endochondral ossification, in which cartilaginous primordia are formed and then replaced by the bones, the centrum of teleosts forms by membranous ossification, which involves direct ossification of the collagenous matrix (Christ et al. 2000;

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Fleming et al. 2004; Inohaya et al. 2007). In zebrafish, initial membranous ossification of the centrum is carried out by notochord cells, which secrete bone matrix into the notochord sheath (Fleming et al. 2004). In medaka, on the other hand, sclerotome-derived osteoblasts and external ligament connective tissue covering the notochord sheath produce the bone matrix of the centrum (Inohaya et al. 2010). Thus, variation in the membranous ossification process exists among teleosts.

In addition, the intercentral joint of teleosts consists primarily of the notochord; in stark contrast, the joint of tetrapods, the intercentral disk, is formed by chondrogenic sclerotome-derived cells (Inohaya et al. 2010; Schmitz 1995). These data also indicate that the fate of the sclerotome differs significantly between tetrapods and teleosts; the sclerotome of tetrapods forms the cartilaginous primordia of the centrum and intercentral disk, whereas the sclerotome of the teleosts differentiates into osteoblasts and external ligament connective tissue surrounding the notochord sheath (Inohaya et al. 2010).

At present, however, the data are not sufficient to illuminate the cellular and molecular bases of teleost vertebral column development. In a recent study with RNA-seq analysis, we found that various developmental regulatory genes involved in embryonic notochord and sclerotome development, such as *ntl*, *twist2*, and *shh*, are expressed in the post-larval vertebral column of the marine teleost flounder (*Paralichthys olivaceus*), suggesting that these genes continue to function in the vertebral column until the adult stage (Ibaraki et al. 2015). Most teleosts, including flounder, are much larger than zebrafish and medaka, and their centrum is more heavily ossified than that of the smaller fish (Grotmol et al. 2003; Schmitz 1995). Considering that teleosts evolved in the ocean, we hypothesized that features that would enhance our understanding of vertebral column development in teleosts could be more readily identified in larger marine fish, such as fugu (*Takifugu rubripes*), as examined here.

Fugu is a model organism for genomic research (Aparicio et al. 2002) and is an ideal species for research on vertebral column development at the molecular level because of the availability of its genome information and its large body size (approximately 70 cm maximum). To investigate the process of centrum and intercentral joint formation and its interaction with the notochord, we have examined, in this study, the histology and mRNA expression profile of fugu vertebral development. As expected, we have found a dynamic morphogenesis and molecular network that underlies adult-type vertebral column development in fugu and that has not been reported for zebrafish, including the segmentation of the notochord by the centrum into a double cone-shaped chamber that functions as an intervertebral joint and expression of *ntla*, *shh*, *col2a1a*, and *sox9* by the notochord cells in the vertebral column.

Materials and methods

Animals

Fugu embryos were obtained as described previously (Uji et al. 2011). After hatching, larvae were cultured in 500-l plastic tanks and supplied with circulating filtered seawater maintained at 20 °C. Larvae were fed rotifers at 10–25 days post-fertilization (dpf), *Artemia nauplii* at 20–40 dpf, and then a formulated diet. After being anesthetized with tricaine, fish were fixed in 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for skeletal staining and Bouin's fixative for hematoxylin-eosin (H-E) staining and section in situ hybridization (SISH). Fish maintenance and experiments were approved by the University Committee of Animal Experiments.

Skeletal, H-E, and von Kossa staining

Samples fixed in 4 % PFA were bleached by using 1 % H₂O₂/1 % KOH. For cartilage staining, samples were soaked overnight in 0.1 % Alcian blue 8GX (Sigma, St. Louis, MO, USA) dissolved in 1 % HCl/70 % ethanol. Next, the samples were washed with 1 % HCl/70 % ethanol solution and soaked in 80 % glycerol in PBS. For bone staining, samples were treated with Alizarin red S solution (Sigma) prepared according to the manufacturer's directions and soaked in 80 % glycerol/1 % KOH.

Samples fixed in Bouin's fixative were embedded in paraffin and cut into 8- μ m sections. After removal of the paraffin, the sections were subjected to H-E staining.

For Alcian blue and von Kossa double staining, samples fixed with PFA were processed to 8- μ m paraffin sections. After removal of the paraffin, sections were first incubated in 0.1 % Alcian blue 8GX in 1 % HCl/70 % ethanol for 15 min and then treated with von Kossa staining by using the Calcium Stain Kit (ScyTek Laboratories, Logan, UT, USA). von Kossa staining was performed according to the manufacturer's instructions.

Reverse transcription plus polymerase chain reaction

Gill, liver, muscle, skin, vertebral column, and brain were dissected from anesthetized 150-dpf fugu of approximately 13 cm in body length, and total RNA was extracted by using Sepasol-RNA Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's directions. The RNA was treated with DNase I (Takara, Tokyo, Japan) to digest contaminating DNA, after which the RNA was ethanol-precipitated and dissolved to give a concentration of 1 μ g/ μ l in RNase-free water (Takara). Total RNA (1 μ g) was reverse-transcribed to first-strand cDNA by using Moloney murine leukemia virus (M-MLV) reverse transcriptase, ReverTra Ace (Toyobo, Tokyo, Japan), in a total volume of 20 μ l.

Polymerase chain reaction (PCR) primers were prepared for the following genes: fugu no tail a (*ntla*), twist family bHLH transcription factor 2 (*tsist2*), transcription factor Cbfa1 (*runx2*), SRY (sex-determining region Y)-box 9 (*sox9*), sonic hedgehog (*shh*), Indian hedgehog b (*ihhb*), collagen type I alpha 1 (*colla1*), collagen type II alpha 1 (*col2a1*), secreted protein acidic cysteine-rich (*sparc*, also called *osteonectin*), and actin beta-like 2 (β -actin). The gene symbols, GenBank accession numbers, PCR product sizes, and primer sequences are listed in Table 1. Aliquots of 1 μ l cDNA were used for each 20- μ l PCR with *Taq* DNA polymerase (Takara). PCR conditions were as follows: 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 75 °C for 60 s. PCR products were electrophoresed on a 2 % agarose gel (Nacalai), stained with ethidium bromide, and photographed.

In situ hybridization

To prepare templates for synthesizing probes for SISH, cDNA fragments of the genes listed in Table 1 were PCR-amplified by using fugu embryonic cDNA prepared as described previously (Suzuki et al. 2002). The PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), and digoxigenin (DIG)-labeled antisense riboprobes were synthesized by using a DIG RNA Labeling kit (Roche Diagnostics, Mannheim, Germany) with an appropriate RNA polymerase. SISH was performed as described previously (Kurokawa and Suzuki 2002).

Tissue culture

The vertebral column was dissected from 13-cm fugu. After the vertebral column had been cut at the intercentral joint by using a scalpel, notochord tissue was collected, minced into approximately 0.5-mm pieces, and then plated on a Col-1-coated Chamber Slide II (Iwaki, Funahashi, Japan) with DMEM containing 10 % fetal bovine serum and penicillin/streptomycin. After 6 days of culture, the tissues were fixed with 4 % PFA and stained with Alcian blue 8GX in 1 % HCl/70 % ethanol.

Photography

Photomicrographs were taken by using a Leica DFC500 or 450C Digital Camera (Leica, Wetzlar, Germany) attached to a Leica DM2500 microscope. Images were subsequently processed by using Photoshop CS5 (Adobe Systems, San Jose, CA, USA).

Results

Structure of adult fugu vertebral column

We observed fugu skeletal formation at the larval, juvenile, and adult stages histologically (Fig. 1a–g). The terminology of vertebral components used here principally follows that described previously for perch, *Perca flavescens* (Schmitz

Table 1 Sequences of primers used for reverse transcription plus polymerase chain reaction

Gene (protein)	Accession number	Product size (bp)	Primer
<i>ntla</i> (fugu no tail a)	XM_003969076	589	5'-AGAAGGGAGACGCGTCCGAGAGGAA-3' (forward) 5'-ACGTCCTTGTGGTCGCTCCTTTCTT-3' (reverse)
<i>twist2</i> (twist family bHLH transcription factor 2)	NM_001104600	449	5'-TGGTGACCAGTGAGGAGGAGCTGGA-3' (forward) 5'-GTGAGATGCTGACATTGACCAAGCG-3' (reverse)
<i>runx2</i> (transcription factor Cbfa1)	NM_001032643	574	5'-CCTGGTTCGTATGATCAGACGTACCA-3' (forward) 5'-GGGTTGAGAATAGTCGGGGAGTTAC-3' (reverse)
<i>sox9</i> (SRY [sex-determining region Y]-box 9)	XM_003979677	764	5'-CAAGAAAGACCACCCCGACTACAAG-3' (forward) 5'-GGTGGTCTGAATAGTCATACTGCGC-3' (reverse)
<i>shh</i> (sonic hedgehog)	XM_003968034	882	5'-CTCTCTGGCCATCTCCGTGATGAAT-3' (forward) 5'-TGGCCGTCCAAGAGCCACGTTCCCTA-3' (reverse)
<i>ihhb</i> (Indian hedgehog b)	XM_003966649	635	5'-TGCTTCTGTGTTTCAGACCACTCAG-3' (forward) 5'-AGCCAGTGTAATAATCCGCGAGTACC-3' (reverse)
<i>colla1</i> (collagen type I alpha 1)	XM_003964272	632	5'-ACCTTCTGCGTCTCATGTCCACTG-3' (forward) 5'-GCATCCTTGGTGTGGTGATGGTAG-3' (reverse)
<i>col2a1</i> (collagen type II alpha 1)	XM_011602634	703	5'-ACAACCAGATCGAGACCTTGCGTAG-3' (forward) 5'-GGGTTGGGAATCGAGATATCCAC-3' (reverse)
<i>sparc</i> (secreted protein acidic cysteine-rich)	NM_001032550	642	5'-CCTGGACTATCACTGCAAGAAGGGA-3' (forward) 5'-CACTCCTCCAGAGCGATGTAGTTGT-5' (reverse)
β -actin	XM_003964421	324	5'-TCCTCCCTGGAGAAGAGCTA-3' (forward) 5'-GATCCAGACAGAGTATTTACGC-3' (reverse)

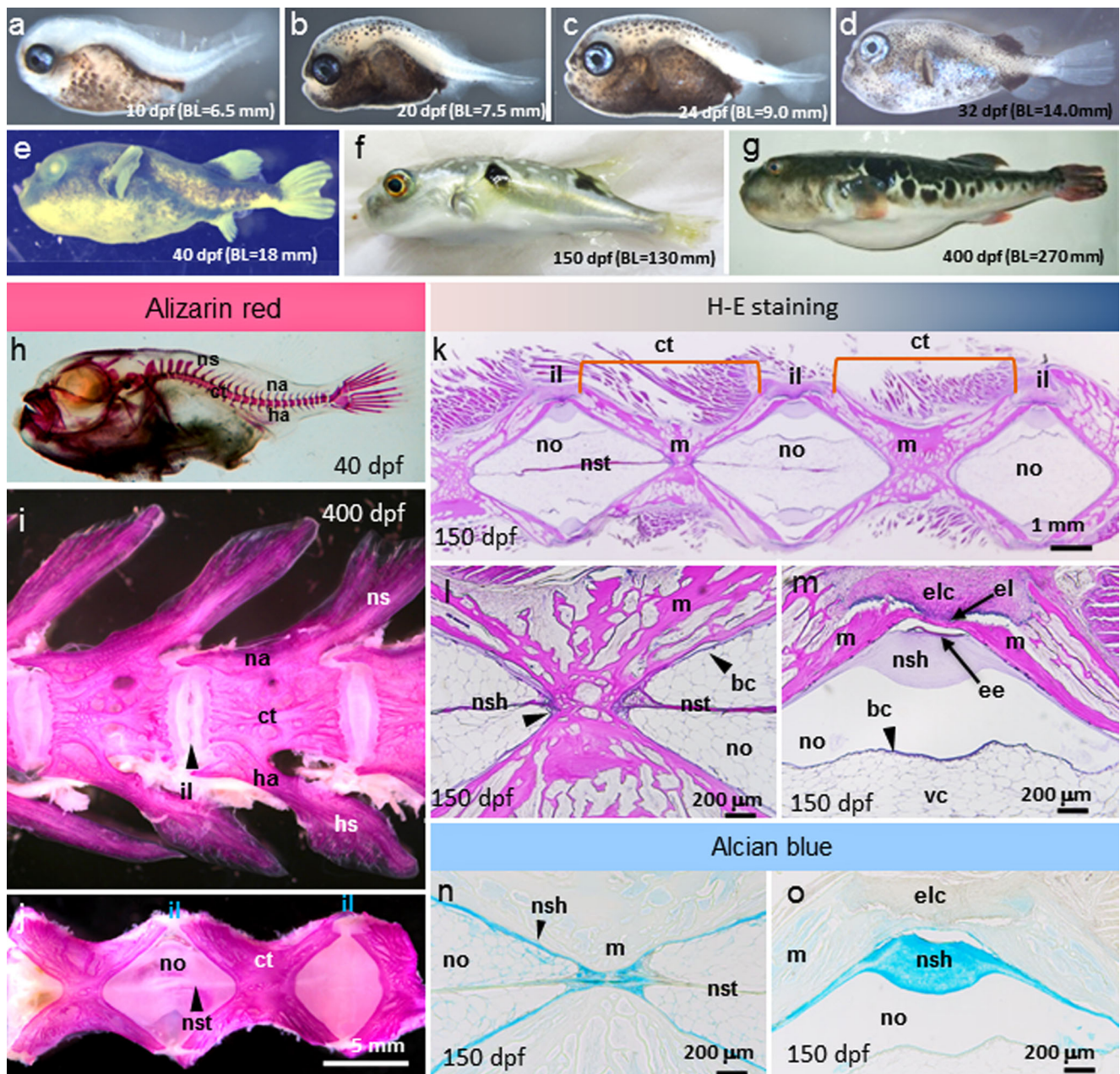


Fig. 1 Structure of fugu vertebral column. **a–g** Lateral view of fugu at stages of skeletal observation (*dpf* days post-fertilization, *BL* body length). **h** Alizarin red staining in whole mount. **i** Vertebral column stained with Alizarin red. **j** Horizontal fracture of vertebral column. **k** Hematoxylin-eosin (*H-E*)-stained section. **l** Higher magnification of segmented part of notochord. **m** Higher magnification of intercentral ligament. Space between notochord sheath and basal cells is formed by

an artifact, and they originally attach closely as shown in Fig. 3g. **n, o** Alcian blue staining of vertebral column sections adjacent to **l** and **m**, respectively (*bc* notochord basal cell, *ct* centrum, *ee* elastic externa, *el* external ligament, *elc* external ligament connective tissue, *ha* hemal arch, *hs* hemal spine, *il* intercentral ligament, *m* bone matrix, *na* neural arch, *no* notochord, *ns* neural spine, *nsh* notochord sheath, *nst* notochord string, *vc* vacuolated cell)

1995). The vertebral column of fugu examined here is composed of 22 centra, with non-ossified intervertebral ligaments between the centra (Fig. 1h, i). The neural and hemal arches extend from the centra and are attached to the spine (Fig. 1i). Dissection of the vertebral column showed that the centrum is highly ossified up to the central region, and a double cone-shaped chamber, consisting of the notochord segmented by

adjacent centra, exists between the centra (Fig. 1j, k). Thus, the vertebral column of adult fugu consists of a repetitive pattern of segmented centra and intercentral joints composed of a segmented notochord.

The notochord forms two types of extracellular matrix: an outer notochord sheath and an inner notochord string (Fig. 1k–m). The notochord sheath consists of non-ossified

tissue with reactivity to Alcian blue covering the inner surface of the vertebral column (Fig. 1n, o). The notochord string comprises fibrous tissue with strong affinity for eosin (Fig. 1l). The notochord sheath forms intercentral ligaments with the elastic externa, external ligament, and outer elastic connective tissue (Fig. 1m; see also below). The notochord string runs through the median line of the notochord chamber, connecting the inner side by the adjacent centra (Fig. 1i, j). Thus, both the notochord sheath and notochord string function to connect the adjacent centra. A layer of notochord basal cells is attached to the inner surface of the notochord sheath, and vacuolated cells fill the notochord chamber (Fig. 1l, m).

Early skeletogenesis

The early phase of larval vertebral skeletogenesis was observed by whole-mount staining of cartilage and bone (Fig. 2). Fugu larvae hatch at 3 dpf at 20 °C (Uji et al. 2011). No skeletal elements have developed by the time of hatching (data not shown). At 10 dpf, auditory capsules attached to the notochord, pharyngeal arches, and pectoral fins began cartilaginous skeletogenesis, but vertebral column formation had not yet begun (Fig. 2a, b). The vertebral elements that first appeared at 15–20 dpf were the anterior-most neural arches extending dorsally from the notochord to surround the spinal cord (Fig. 2e, i). These arches formed by membranous ossification without the formation of cartilage (Fig. 2c–g). Next, the centrum began to form from the anterior-most elements by membranous ossification in order to ring the notochord from the point of contact with the neural arch (Fig. 2e, j, k). All vertebrae, 22 in total, had appeared by 40 dpf (Fig. 1h), and intercentral ligaments (which do not react with Alizarin red) formed between the adjacent centra (Fig. 2f, g, h). Concomitantly, the hemal arches and neural and hemal spines also formed by membranous ossification (Fig. 1j, k). Thus, the entire fugu vertebral structure formed by membranous ossification without the production of any cartilage.

Ossification of centrum and segmentation of notochord

We then histologically followed the formation of the vertebral column through the establishment of the adult form (Fig. 3). The bone matrix of the centrum, which is strongly eosinophilic and stained by von Kossa, began to accumulate between 10 and 24 dpf (Fig. 3e, g, l, m), and synthesis of bone matrix was active at the anterior and posterior part of the centra (Fig. 3b, n, o). It then formed highly branched trabeculae (Fig. 3h, k). By 400 dpf, highly ossified adult-type centra were established (Fig. 1i, j). Double-staining with von Kossa and Alcian blue clearly showed that bone matrix accumulation of the centrum began along the outer surface of the notochord sheath, which was reactive with Alcian blue (Fig. 3l, m). During further

growth of centrum, calcium deposition took place only at the outer surface of the notochord sheath (Fig. 3l, m).

The notochord, which was a simple tube-like structure at 10 dpf (Figs. 2a, 3e), began to segment by 24 dpf, forming the pre-pattern of the periodic segments of the centra and intercentral ligaments; the notochord expanded at the intercentral ligaments and contracted by the accumulating bone matrix of the centra (Fig. 3g). The notochord contracted further by the inward growth of the centra (Fig. 3a, h) and was finally completely segmented by the centra at 62 dpf (Fig. 3d, k). The segmented notochord formed double cone-shaped chambers between the adjacent centra, producing the intercentral joints (Fig. 3k; see also below).

Formation of notochord sheath, notochord string, and intercentral ligaments

At 10 dpf, the notochord consisted of an outer thin notochord sheath (stainable by Alcian blue) and inner vacuolated cells (Fig. 3e, f). The notochord sheath grew into a thick acellular layer covering the inner surface of the bone matrix of the centrum by 40 dpf (Fig. 3j, n, o). The notochord sheath grew extremely thick between the fringes of adjacent centra and composed the intercentral ligaments in combination with the elastic externa, elastic ligaments, and external elastic connective tissue (Fig. 3i, o). The external ligament connective tissue consisted of densely packed fibroblastic cells and was attached to the elastic ligaments and the peripheral region of the bone matrix of the centrum (Fig. 3i, o). The elastic ligament was a thin matrix continuing to the centrum bone matrix, and the elastic externa consisted of highly eosinophilic dense matrix (Fig. 3i). The notochord basal cells covering the inner surface of the notochord sheath became visible at 24 dpf (Fig. 3g) and were thick at the intercentral ligaments (Fig. 3i, o).

The eosinophilic matrix began to accumulate in the notochord chamber at the contracting portion (Fig. 3h, j) and finally formed the fibrous notochord string connecting the adjacent centra at 32–62 dpf (Fig. 3k). The notochord string was more eosinophilic than the bone matrix (Fig. 3k). These data thus demonstrate that, during segmentation by the centra, the notochord undergoes dynamic morphogenesis, including the formation of the thick notochord sheath and elastic externa that compose the intervertebral ligaments, the notochord string connecting adjacent centra, and a large chamber filled with vacuolated cells.

The diameter of the notochord was approximately 200 μm at 10 dpf, and by 400 dpf, the maximum diameter of the segmented notochord was approximately 5 mm, indicating that the notochord continued to grow during vertebral column formation. The size of the vacuolated notochord cells did not change and was approximately 80 μm (Fig. 3e, k).

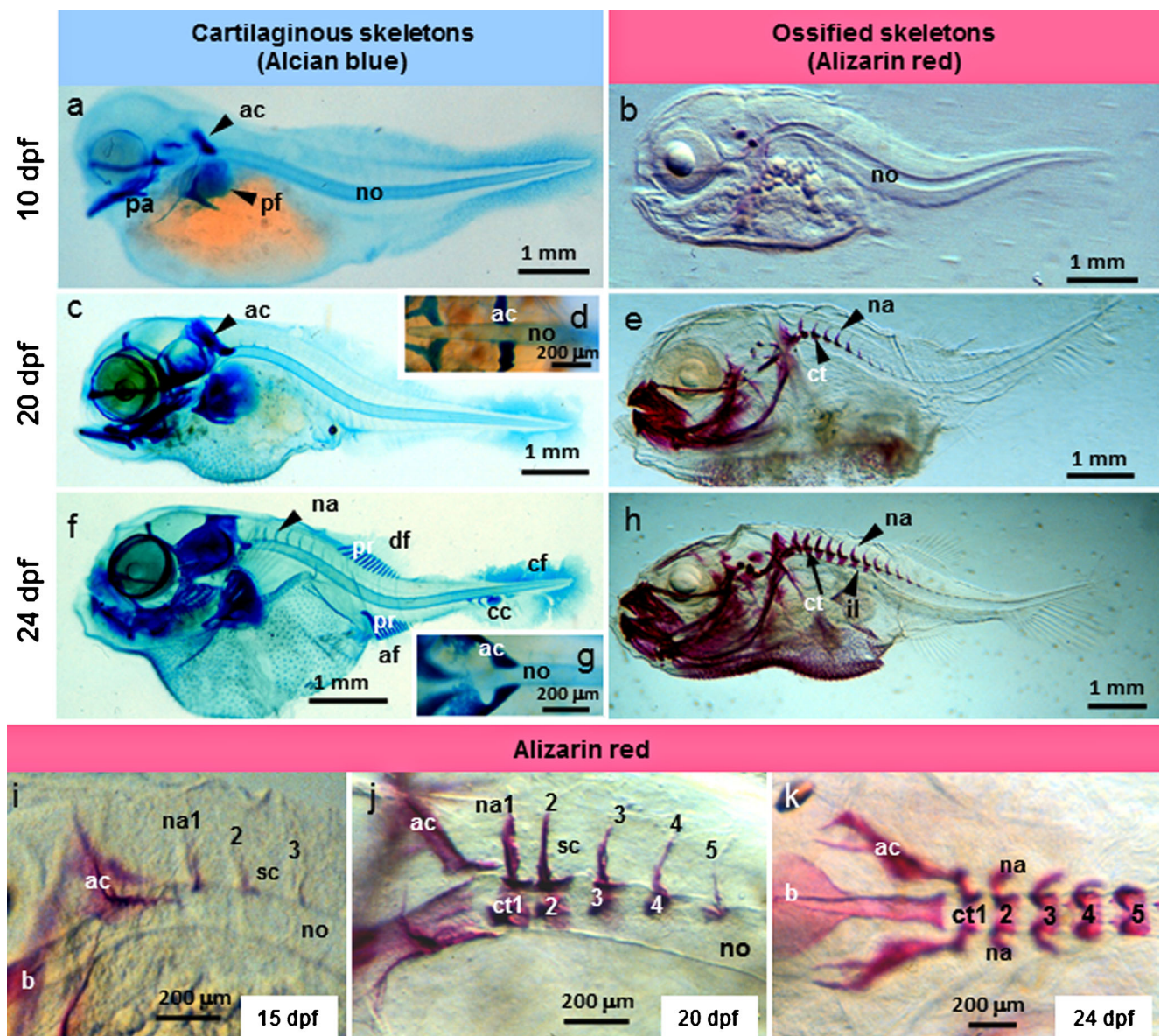


Fig. 2 Early development of cartilaginous and bony skeleton in fugu. **a–h** Cartilaginous and ossified skeletons visualized by Alcian blue and Alizarin red staining in whole mount. **d, g** Higher magnification views of rostral end of notochord and attached cartilage (dorsal view). **i–k** Higher magnification views of anterior vertebral column stained with

Alizarin red at initial phase of skeletogenesis (*ac* auditory capsule, *af* anal fin, *b* basioccipital bone, *cc* caudal fin cartilage, *cf* caudal fin, *ct* centrum, *df* dorsal fin, *ha* hemal arch, *il* intercentral ligament, *no* notochord, *na* neural arch, *pa* pharyngeal arch, *pf* pectoral fin, *pr* proximal radial, *sc* spinal cord)

Gene expression in vertebral column

Using RT-PCR, we examined the tissue expression of nine genes related to notochord and skeletal formation in 150-dpf fugu: *ntla*, *shh*, *ihhb*, *twist2*, *runx2*, *sox9*, *col2a1a*, *colla1*, and *sparc* (Table 1). All of these genes were expressed in the vertebral column, and the expression of *ntla* and *ihhb* was specific to the vertebral column (Fig. 4a). The genes *shh*, *twist2*, *runx2*, *col2a1a*, *colla1*, and *sparc* were also expressed in the gill and skin, both of which have skeletal elements (Fig. 4a).

Using SISH, we determined those cells in the vertebral column expressing each of the nine genes examined by PCR. Both basal and vacuolated cells in the notochord expressed *ntla* (Fig. 4b, c). Of note, the vacuolated cells expressed only *ntla* among the nine genes examined, whereas the basal cells expressed *shh*, *ihhb*, *runx2*, *sox9*, *col2a1a*, and *sparc*, in addition to *ntla* (Fig. 4b–e, g–j, m). Thus, of the nine genes examined, the notochord basal cells expressed all except *twist2* and *colla1*.

The cells covering the outer surface of the bone matrix of the centrum expressed *colla1* and *sparc*, indicating that these

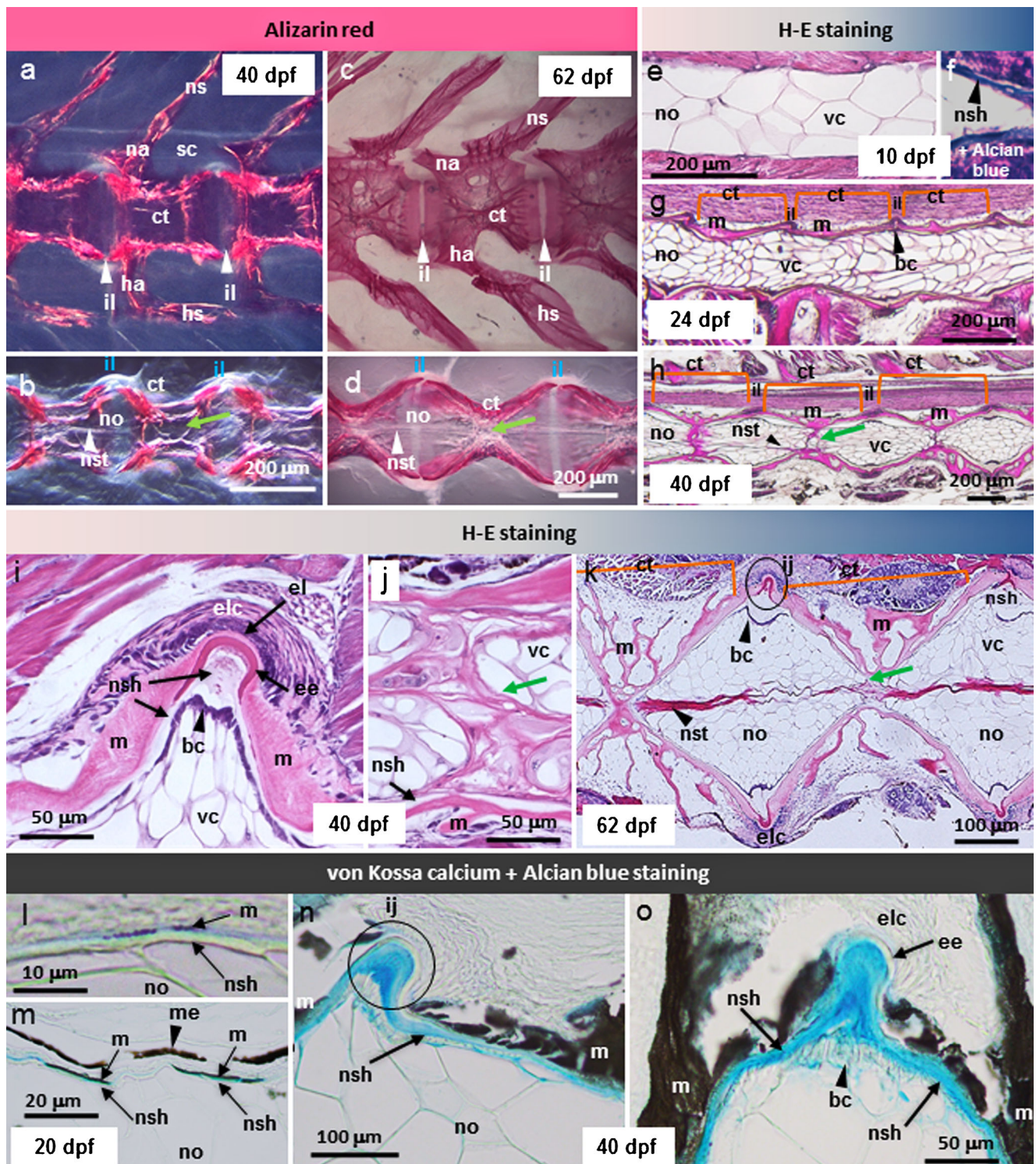


Fig. 3 Histology of notochord, centra, and intercentral ligaments in developing vertebral column. **e, f, k** Horizontal sections; all others are sagittal sections. **a, c** Vertebral column stained with Alizarin red in lateral view. **b, d** Horizontal fractures. **e–k** H-E-stained sections; Alcian blue staining was also carried out (**f**). **i–o** Double-staining with von Kossa and Alcian blue to discriminate notochord sheath and calcium deposition of bone matrix (*green arrows* positions at which notochord segmented by

centra, *bc* notochord basal cells, *ct* centrum, *ee* elastic externa, *el* external ligament, *elc* external ligament connective tissue, *ha* hemal arch, *hs* hemal spine, *il* intercentral ligament, *ij* intercentral joint [encircled], *m* bone matrix, *me* melanophore, *no* notochord, *na* neural arch, *nsh* notochord sheath, *nst* notochord string, *ns* neural spine, *sc* spinal cord, *vc* notochord vacuolated cells)

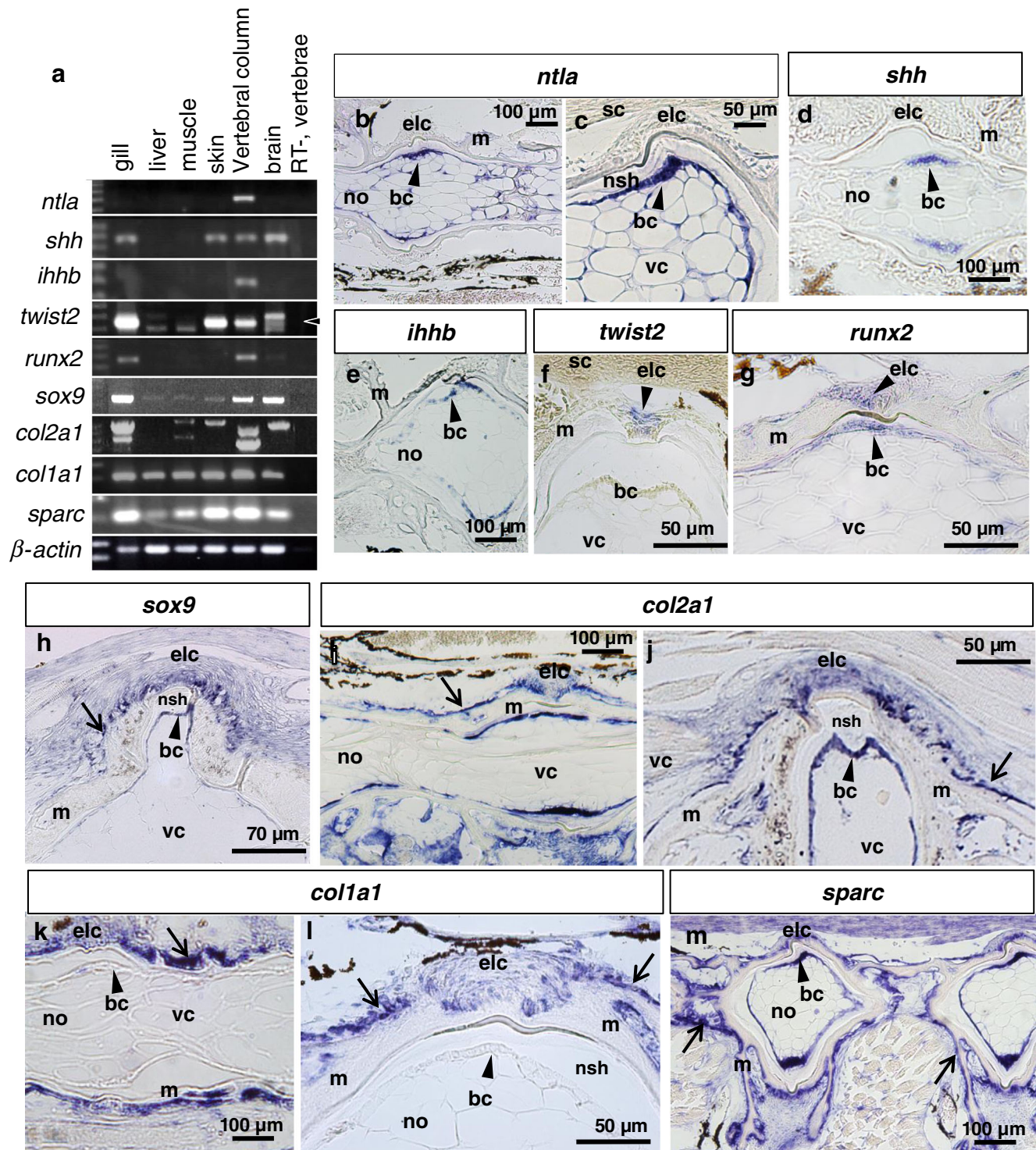


Fig. 4 Expression of *ntla*, *shh*, *ihhb*, *twist2*, *runx2*, *sox9*, *col2a1*, *col1a1*, and *sparc* in the vertebral column of 40-dpf fugu (for an explanation of gene names, see Table 1). **a** Reverse transcription plus polymerase chain reaction (PCR) analysis of tissue expression (*arrowhead* in *twist2* position of expected PCR product). Production of multiple PCR bands for *col2a1a* is presumably

attributable to alternative splicing in the region selected for amplification. **b–m** mRNA localization by section in situ hybridization (SISH). All sections are sagittal in orientation (*arrows* SISH signals in osteoblasts covering the bone matrix of the centrum, *bc* notochord basal cell, *elc* external ligament connective tissue, *no* notochord, *vc* notochord vacuolated cell)

cells are osteoblasts (Fig. 4k–m). The osteoblasts also expressed *col2a1a* (Fig. 4i). The external ligament connective

tissue expressed *twist2*, *runx2*, *sox9*, *col2a1a*, *col1a1*, and *sparc* (Fig. 4f, g, j, l, m).

Matrix synthesis by notochord basal cells

Histologically, we observed that the cytoplasm of notochord basal cells in the proximity of intervertebral ligament included Alcian-blue-positive substances (Fig. 5a). To verify this observation, we collected notochord explants from the vertebral column of 150-dpf fugu and cultured them *in vitro* (Fig. 5b, c). The notochord basal cells formed aggregations, and an Alcian-blue-positive substance was produced in the cell aggregates (Fig. 5d). We could detect *ihh* expression in the cultured cells, indicating that major components of the cell aggregate were the notochord basal cells (Fig. 5e). Thus, the matrix production observed *in vitro* was reproduced by the *in vitro* culture experiment. These data suggest that the notochord basal cells synthesize and secrete extracellular matrix components, including cartilaginous proteoglycan, to form the notochord sheath.

The data regarding the developmental process and tissue mRNA expression in vertebral column are summarized in Fig. 6.

Discussion

Centrum and intervertebral joint formation

In medaka and zebrafish, the notochord is not completely segmented by centra, even in adult fish, and there is no notochord string (Bird and Mabee 2003; Haga et al. 2009; Inohaya et al. 2007). This adult vertebral column structure is similar to that of 24- to 40-dpf fugu larvae. In fugu, we observed that the centra continued to grow by active bone matrix formation and completely divided the notochord into double cone-shaped segments, forming intervertebral joints. During this process, the notochord cells formed a thick notochord sheath covering the inner surface of the centra and fibrous notochord string. The notochord sheath formed intervertebral ligaments connecting the edges of adjacent centra, and the notochord string also connected the adjacent centra from the inside. Thus, in fugu, the centra and notochord continue coordinated dynamic morphogenesis to establish a repetitive series of

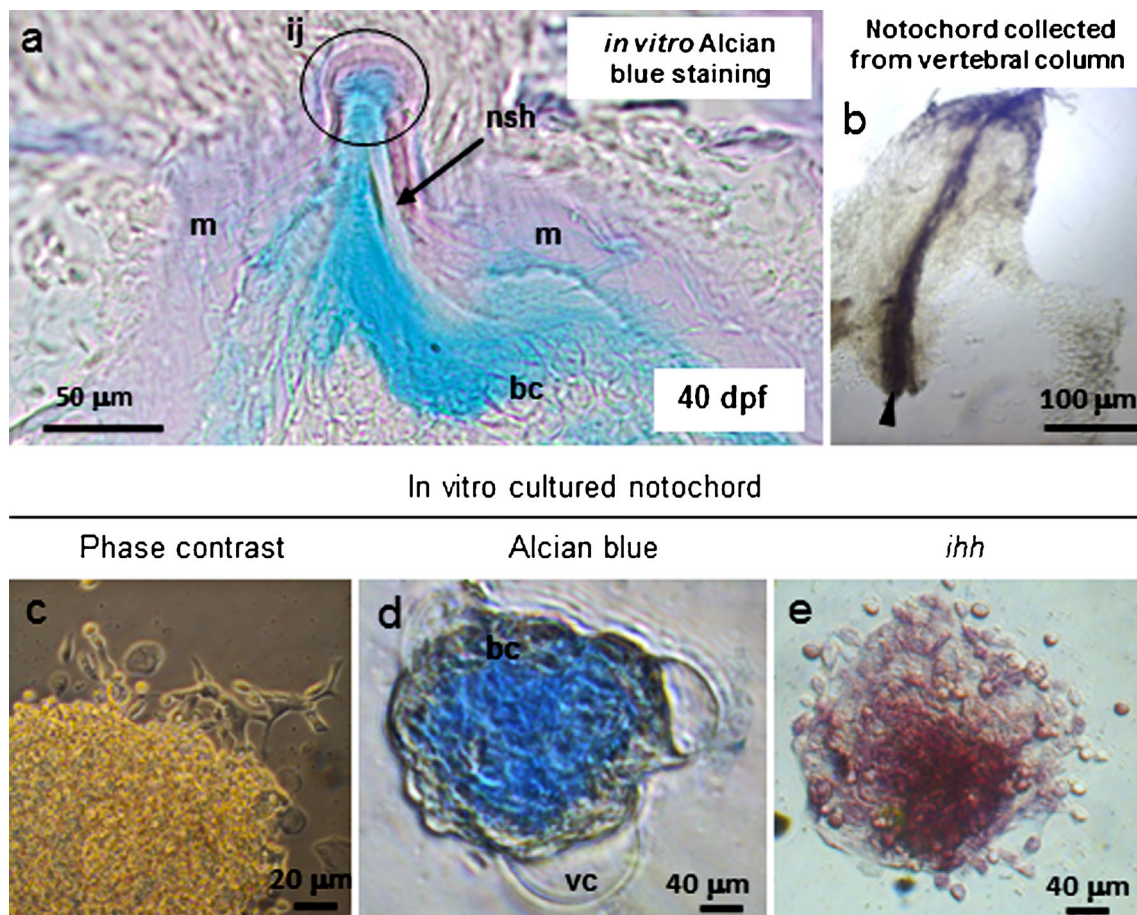


Fig. 5 Matrix synthesis by notochord basal cells and *in vitro* culture of notochord cells. **a** Production of Alcian-blue-positive matrix by notochord basal cells in proximity of intervertebral ligament (*bc* notochord basal cell, *ij* intercentral joint [encircled], *m* bone matrix, *nsh* notochord sheath). **b** Notochord tissue collected from 150-dpf fugu vertebral column (*arrowhead* thickened notochord basal cells at the

intervertebral ligament). **c** Culture notochord cells forming a cellular aggregate. **d** Alcian blue staining of cultured notochord cells. Alcian-blue-positive matrix is synthesized in the cell aggregate (*bc* notochord basal cell, *vc* notochord vacuolated cell). **e** Expression of *ihh* in cultured notochord

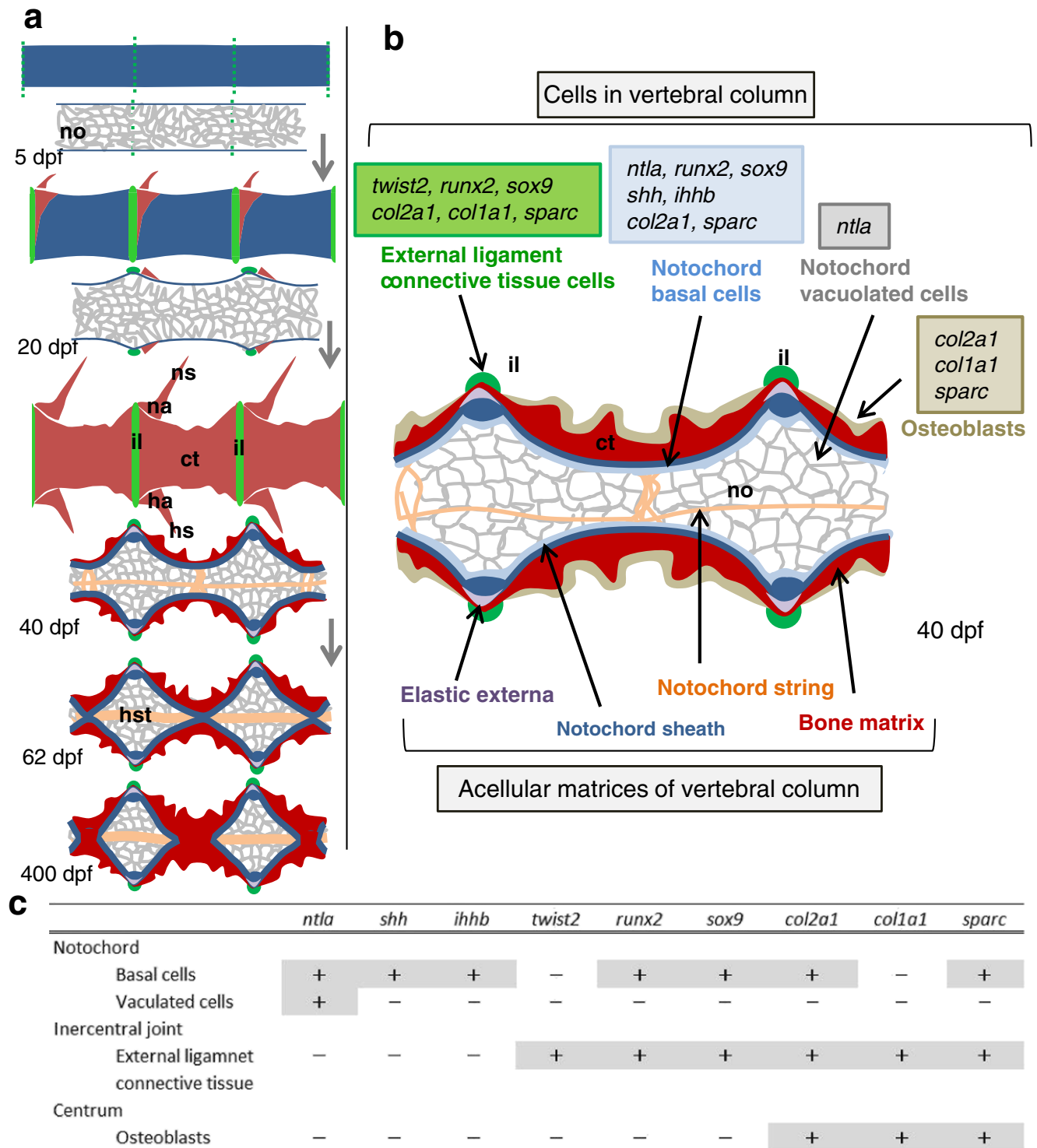


Fig. 6 Summary of developmental process and gene expression profile in fugu vertebral column. **a** Representation of developmental process of vertebral column, highlighting the segmentation of the notochord by growing centra. **b** Histology of the vertebral column indicating tissue

mRNA expression. **c** List of signal-positive (+) and signal-negative (-) cells in the SISH (*ct* centrum, *ha* hemal arch, *hs* hemal spine, *il* intercentral ligament, *no* notochord, *na* neural arch, *ns* neural spine, *nst* notochord string)

segmented centra and intervertebral joints until the adult form of the vertebral column is established (Fig. 6a). With regard to

the origin of the bone matrix that occupies the center of centra, the centra is formed as highly branched trabeculae and is

equipped with a space for osteoblasts between trabeculae (Figs. 11, 3k); we suppose that such a highly branched structure allows the inward growth of the centrum to segment the notochord into intervertebral joints.

A notochord string is also found in the notochord chamber of perch (Schmitz 1995). We therefore hypothesize that, in large fish, the highly ossified centra, thick notochord sheath, and fibrous notochord string function to give physical strength to the vertebral column, and that the filling of the notochord chamber with vacuolated cells helps reduce the weight of the vertebral column.

Centrum ossification

Reports concerning the ossification of the centra in teleosts indicate that the process takes place by membranous ossification, unlike in tetrapods (Fleming et al. 2004; Inohaya et al. 2007; Schmitz 1995). We ascertained that the centra and all other vertebral elements develop by membranous ossification in fugu. In zebrafish, membranous ossification of the centra is reportedly initiated by the notochord cells, which supply bone matrix to the notochord sheath (Fleming et al. 2001). On the other hand, in medaka, osteoblasts and external ligament connective tissue cells located on the outer surface of the notochord sheath produce the centrum bone matrix (Inohaya et al. 2007). Col1 and Sparc, which form a complex, are major components of the bone matrix (Ricard-Blum 2011; Termine et al. 1981). In fugu, we have found that the notochord cells do not express *colla1*, and that osteoblasts expressing both *colla1* and *sparc* are located on the outer surface of the centrum (Fig. 4k, m). Histologically, we have shown that the notochord sheath synthesized by the notochord cells is not ossified tissue but rather has cartilaginous properties, and that ossification of the bone matrix takes place along the outer surface of the notochord sheath (Figs. 3l–o, 6b, c). These data suggest that, in fugu, the notochord cells do not contribute to bone matrix production and instead indicate that outer osteoblasts are responsible for matrix production and centrum ossification. The process of centrum ossification in fugu is thus more similar to that in medaka than that in zebrafish. The control of centrum growth by outer osteoblasts is understandable for large fish that form highly developed centra, such as fugu.

The osteoblasts of zebrafish and gar (*Lepisosteus oculatus*) are reported to express not only *colla1*, but also *col2a1*, which is specific for chondrocytes in tetrapods (Eames et al. 2012; Ricard-Blum 2011). Similarly, in the present study, we have found that fugu centrum osteoblasts express *col2a1*, in addition to *colla1* (Fig. 6b, c). Therefore, the production of Col2 in addition to Col1 as the bone matrix seems to be a general feature of teleosts.

External ligament connective tissue

The structure of the fugu intervertebral ligament observed in the present study, consisting of the notochord sheath, elastic externa, elastic ligament, and outer elastic connective tissue, is similar to that of perch (Schmitz 1995). Regarding the fate of embryonic sclerotome cells, these cells in medaka have been demonstrated to differentiate into the external ligament connective tissue cells and osteoblasts that form the centrum (Inohaya et al. 2007). We have found that *twist2*, a marker of sclerotome cells (Gitelman 1997; Yasutake et al. 2004), is expressed by the external ligament connective tissue (Fig. 6b, c), suggesting that this tissue descends from the sclerotome in fugu, as suggested for medaka (Inohaya et al. 2007).

We have further found that the fugu external ligament connective tissue expresses matrix protein genes, including *colla1*, *col2a1a*, and *sparc* (Fig. 6b, c), suggesting that the external ligament connective tissue participates in bone matrix production together with osteoblasts, as suggested for medaka (Inohaya et al. 2007). In addition, *runx2*, a master transcription factor for osteoblast differentiation (Komori et al. 1997), is expressed in this tissue, indicating that the external ligament connective tissue cells have osteoblastic properties for bone matrix production, similar to osteoblasts.

Function of notochord cells

The notochord sheath of lamprey reportedly contains Col2 fibers (Eikenberry et al. 1984). In mice, the notochord cells secrete Col2 as a component of the notochord sheath (Smits and Lefebvre 2003). Post-embryonic notochord cells are classified into two types: the basal cells, which line the inner surface of the notochord sheath, and the inner vacuolated cells (Schmitz 1995). In agreement with data from lamprey and mouse studies, we have found that fugu notochord basal cells express *col2a1* (Fig. 6b, c). In addition, we have observed that the notochord sheath contains cartilaginous proteoglycan that is reactive with Alcian blue (Fig. 1n, o), and in tests in vitro, cultured notochord basal cells synthesize this proteoglycan (Fig. 5d), suggesting that fugu notochord basal cells secrete Col2 and proteoglycan to produce the notochord sheath. We therefore conclude that the synthesis of Col2 for notochord sheath formation is a conserved function of notochord cells in vertebrates, from the cyclostomes to the lineages of both teleosts and mammals.

We have also found that fugu notochord basal cells co-express *sparc* and *col2a1* (Fig. 6b, c). In mammals, even though Sparc is a major component of bone matrix in complex with Col1, Sparc is also synthesized by chondrocytes together with Col2 under certain physiologic conditions (Nakamura et al. 1996). Therefore, in fugu, Sparc and Col2 synthesized by notochord basal cells might form the matrix in the notochord sheath.

In mammals, the transcription factor Sox9 regulates *Col2* expression in chondrocytes, and Ihh signaling stimulates the

proliferation of chondrocytes (Lefebvre et al. 1997; St-Jacques et al. 1999). In addition, Shh reportedly stimulates *Sox9* and *Col2a1* expression in mesenchymal stem cells; thus, *Sox9*, *Ihh*, and *Shh* are upstream regulators of cartilaginous skeletal development in tetrapods (Warzecha et al. 2006). For chondrocytes, *Runx2* functions to control their proliferation by inducing *Ihh* expression (Yoshida et al. 2004). In the present study, we have found that fugu notochord basal cells express *ihhb*, *shh*, *sox9*, *runx2*, and *col2a1* (Fig. 6b, c), suggesting that the system that regulates Col2 synthesis in tetrapod chondrocytes also functions in the notochord basal cells of fugu for notochord sheath synthesis. Thus, the notochord basal cells of fugu, unlike those of zebrafish, are not osteoblastic but rather are unique cells that have a cartilaginous property and that function specifically in notochord sheath synthesis.

Expression of *ntla* and *shh* by notochord cells

The transcription factor *ntla* plays an essential role in notochord development and is expressed by embryonic notochord cells in all chordates. In zebrafish, *ntla* expression ceases after embryogenesis (Schulte-Merker et al. 1992). Expression of *shh* in the notochord induces sclerotome formation in the somite and ceases after embryogenesis in zebrafish (Krauss et al. 1993). Thus, the expression of both *ntla* and *shh* in the notochord ceases after embryogenesis in zebrafish. In contrast, in the present study, we have found that the expression of both *ntla* and *shh* is maintained in the fugu notochord during the development of the vertebral column; *ntla* is expressed by all notochord cells, and *shh* is expressed by notochord basal cells (Fig. 6b, c). We have observed the expression of both *ntla* and *shh* at least up to 150 dpf (data not shown). Thus, our data suggest that, unlike zebrafish, *ntla* and *shh* continue to function in the notochord cells until the adult stage in fugu. Using a transgenic line, Haga et al. (2009) have reported that, in zebrafish, the *shhb* (previously named *tiggy-winkle hedgehog*) promoter drives *gfp* expression in the intervertebral joint, although in situ hybridization (ISH) does not detect a signal for *shhb* from the vertebral column. Therefore, *shhb* might be expressed in the intervertebral joint, also in zebrafish, at a level lower than that of ISH sensitivity.

The zebrafish genes for which transcription is directly controlled by *ntla* have been identified (Morley et al. 2009). Several genes that we have found to be expressed by fugu notochord basal cells, including *shh*, *ihhb*, *sox9*, *col2a1a*, and *sparc*, are not included among the genes identified by Morley et al. (2009). As such, the genes downstream of *ntla* that are involved in vertebral column development in fugu remain unidentified. However, our data indicate that the notochord cells retain some role in vertebral column development and growth regulated by *ntla* from the embryonic to adult stages.

Comparison with tetrapod vertebral development

Our research on fugu demonstrates that the fate of the sclerotome in teleosts (external ligament connective tissue cells that synthesize bone matrix) differs from that in tetrapods (chondrocytes of the centrum and intercentral disk primordia), as suggested for medaka (Inohaya et al. 2007). In the present study, we have found that, in fugu, the external ligament connective tissue, which descends from the sclerotome, expresses both *col2a1a* and *sox9* (Fig. 6b, c). Therefore, the regulation of Col2 synthesis by *Sox9*, which is typical of tetrapod chondrocytes, is conserved in the sclerotome-derived cells that form the centra by membranous ossification in teleosts. We also speculate that specialization of sclerotome-derived cells to osteoblastic cells differentiated from chondrocytes took place in the teleost lineage after the branching of the tetrapod lineage, because in ancestral fish, including the Chondrichthyes and Cyclostomata, the vertebral column is cartilaginous (Fleming et al. 2015).

We have found that fugu notochord cells synthesize Col2 as a matrix for the notochord sheath, as shown in tetrapods (Smits and Lefebvre 2003). In tetrapods, the notochord degenerates into a rudimentary tissue, the inner nucleus pulposus, in the intervertebral disk (O'Rahilly and Müller 1994). In contrast, in fugu, the notochord keeps growing and synthesizing extracellular matrix components to produce the thick notochord sheath until the adult stage is reached, and the notochord basal cells maintain some functions initiated during the embryonic stage via the *ntl* and *shh* signaling network.

Thus, in fugu, the notochord plays multiple important roles in vertebral development, including segmentation, imparting physiologic strength and flexion, and reducing the weight of the vertebral column. The profile of vertebral development described here for fugu possibly represents the original vertebral column developmental system in large teleosts that evolved in the ocean and might provide clues for future studies of the evolutionary diversity of vertebral column development in teleosts and tetrapods.

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