Ancestral mesodermal reorganization and evolution of the vertebrate head

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Abstract

Introduction: The vertebrate head is characterized by unsegmented head mesoderm the evolutionary origin of which remains enigmatic. The head mesoderm is derived from the rostral part of the dorsal mesoderm, which is regionalized anteroposteriorly during gastrulation. The basal chordate amphioxus resembles vertebrates due to the presence of somites, but it lacks unsegmented head mesoderm. Gastrulation in amphioxus occurs by simple invagination with little mesodermal involution, whereas in vertebrates gastrulation is organized by massive cell movements, such as involution, convergence and extension, and cell migration.

Results: To identify key developmental events in the evolution of the vertebrate head mesoderm, we compared anterior/posterior (A/P) patterning mechanisms of the dorsal mesoderm in amphioxus and vertebrates. The dorsal mesodermal genes gsc, bra, and delta are expressed in similar patterns in early embryos of both animals, but later in development, these expression domains become anteroposteriorly segregated only in vertebrates. Suppression of mesodermal involution in vertebrate embryos by inhibition of convergence and extension recapitulates amphioxus-like dorsal mesoderm formation.

Conclusions: Reorganization of ancient mesoderm was likely involved in the evolution of the vertebrate head.

Keywords: Amphioxus, Head mesoderm, Vertebrate body plan, Somites

Introduction

How the highly complex vertebrate head—composed of brain, head muscles, and skull—evolved from non-vertebrate ancestors is a fundamental question in current evolutionary and developmental biology [1–3]. Recent comparative studies of the cephalochordate amphioxus and vertebrates suggest that a region homologous to the vertebrate fore/mid/hind brain is also present in the rostral part of the central nervous system (CNS) of amphioxus [4–6]. Amphioxus is a basal chordate that has somites extending to the rostral end of the body, and is considered the best proxy for understanding the origin of the vertebrate body plan [7].

The homology between amphioxus and the vertebrate CNS indicates that the unsegmented vertebrate head mesoderm evolved directly from the rostral somites of amphioxus [8]. Expression of en in the rostral somites of amphioxus (Branchiostoma floridae) and en2 in the ventral part of the mandibular head mesoderm of shark (Scyliorhinus torazame) embryos supports this hypothesis [9, 10]. However, Bpax3/7, a homologue of pax3 that serves as a somite marker in vertebrates, is expressed in the rostral somites, suggesting that the vertebrate head mesoderm did not evolve by simple modification of rostral somites of an amphioxus-like ancestor, but rather by fundamental reorganization that occurred in the dorsal mesoderm [2, 10].

During embryogenesis, the vertebrate head mesoderm derived from the rostral part of the dorsal mesoderm is regionalized along the A/P axis by a gradient of Wnt/β-catenin signalling [11, 12]. In the regionalization of the dorsal mesoderm, downstream regional marker genes of Wnt/β-catenin signalling are expressed in the progenitor domains; gsc is expressed in the head mesoderm, whereas bra is expressed in the presumptive notochord during the late-gastrula stage [13, 14]. Additionally, in the trunk mesoderm, delta expression is detected in the presumptive somite region [15, 16]. Previous functional studies have...
shown that overexpression of *Xenopus laevis* dkk1 (negative regulator of Wnt/β-catenin signalling) expands the gsc expression domain posteriorly in *Xenopus* embryos, whereas bmt expression is activated by Wnt/β-catenin signalling [11, 17, 18]. Additionally, delta has an essential role in somitogenesis, and is under the control of Wnt/β-catenin signalling [19].

In amphioxus, gsc and bra are co-expressed in the presumptive notochordal region at the gastrula stage [20, 21]. The presumptive somite marker delta is expressed in the first and second somites in the late-gastrula stage [22]. Loss of gsc expression in the notochord and gain of gsc expression in the head mesoderm of vertebrates compared with amphioxus indicates that A/P re-arrangement of mesodermal gene expression occurred in the lineage of vertebrates. Excessive Wnt/β-catenin signalling in amphioxus embryos induced by inhibition of GSK-3α/β does not affect the expression of regional marker genes of the dorsal mesoderm, such as bra and fgf8/17/18, during the gastrula stage [23]. This suggests that, unlike in vertebrates, Wnt/β-catenin signalling does not play a role in dorsal mesoderm regionalization in amphioxus. If vertebrate embryos did evolve a rearrangement of gene expression in the dorsal mesoderm to generate the head mesodermal region, what was the key developmental event in this process? We consider that re-arrangement of gene expression in the vertebrate dorsal mesoderm from an ancestral chordate evolved as described previously [29]. Adult sharks (*S. torazame*) were collected from Nakaminato Bay, Ibaraki, Japan in October and maintained in a seawater tank at 16 °C. Eggs were obtained from the adult females and maintained in the seawater tank until they developed to the described stages [30].

In situ hybridization

Whole-mount in situ hybridization of amphioxus, lamprey, and shark embryos was performed as described in previous studies [10, 29, 31]. For section in situ hybridization of *Xenopus* embryos, larval-stage embryos were fixed using MEMFA for 2 h at room temperature then section in situ hybridization was performed as described [32]. For fluorescence in situ hybridization, the protocol used for amphioxus whole-mount in situ hybridization [23] was applied and an antibody (Anti-DIG-POD, Roche, Basel, Switzerland) and TSA system (PerkinElmer, Waltham, MA, USA) were used. Cellmask deep red (Life Technologies, Carlsbad, CA, USA) (1/1000) were used to stain the plasma membrane or nuclei. A Zeiss LSM 780 (Zeiss, Jena, Germany) was used to collect confocal images.

Plasmid construction and gene markers

The sequences of Bfdkk1/2/4 [21], Bfgsc [21], Bbra [20], Bfwnt8 [21], Bdelta [22], Stddkk1 (KF551566), Stgsc (KF564642), Stdelta (KF551567), Stbra (KF551568), and Stwnt8 (KF551569) were amplified by PCR and cloned into a TOPO cloning vector. Xldkk1-pCS2 (NM_001085592), Xlgsc-pCS2 (NM_001087809), Xldelta–2-pCS2 (NM_001086082), Xlbra–pSP64 (M77243.1), Xlwnt8–pCS2 (NM_001088168), and XlmyoD–pCS2 (NM_001085897) were gifts from Dr. Yoshiki Sasai from RIKEN CDB, Japan. Xltxbl1 (NM_001090445) was amplified by PCR and cloned into the pCR-TOPO vector. Lfgsc (KF551572), Ldelta (KF564639), Lbra (AB501127), and Lwnt8 (KF551570) were amplified
by PCR and cloned into a TOPO cloning vector. Xldd1-myc-pCS2, myc-XldshdelDEP-pCS2, and Brrnd1-pCS2 were linearized using NotI and transcribed using SP6 polymerase (mMessage mMachine, Ambion, Austin, TX, USA).

**Xenopus experiments**

Embryos were staged according to the Normal Table published by Nieuwkoop and Faber [33]. The mRNA, in 1× modified Barth’s saline, was injected into the embryos using a fine glass capillary tube and a pressure injector (Narishige, Tokyo, Japan). The embryos were then transferred to 0.1× Barth’s saline until further manipulation or harvest. For histological analysis, the embryos were fixed using Bouin’s fixative and then dehydrated and embedded in paraffin. Sections (6-μm-thick) were cut and stained with haematoxylin and eosin. The sequence of the Xlndo1-morpholino antisense oligonucleotide (MO) has been published [34]. For the dorsal marginal zone assay, embryos were dissected at the early-gastrula stage (stage 10) and cultured in 1× low-calcium magnesium Riner’s supplemented with 0.2 % bovine serum albumin until stage 19.

**Immunostaining of amphioxus and Xenopus embryos**

Early-neurula-stage amphioxus embryos were fixed using 4 % paraformaldehyde in MOPS buffer at 4 °C overnight, and immunocytochemistry was performed as described [35], using a primary antibody against β-catenin (diluted 1:800 for Xenopus embryos and 1:400 for amphioxus embryos; C-2206, Sigma, St. Louis, MO, USA), a secondary Alexa Fluor 488-conjugated antibody (1:400, Invitrogen), and DAPI (1:1000, Invitrogen). Whole embryos and histological sections were imaged using a Zeiss LSM 710 confocal microscope and a Zeiss Axio Zoom V16 microscope, respectively.

**Phylogenetic tree analysis**

Phylogenetic trees were constructed with MEGA5 software [36] using the maximum-likelihood method with 1000 bootstrap reiterations. All sequences were aligned using Clustal W (http://www.clustal.org/clustal2/).

**Alignment analysis of Flrt3**

For Flrt3 protein alignment analysis, we used the multiple alignment tool in Genetyx–Mac version 16.0.7 (http://www.genetyx.co.jp/products/genetyx_mac_16/index.html).

**Accession numbers**

The sequences of the novel genes isolated were deposited in GenBank with following accession numbers. Ljgsc (KF551572), Ljdelta1 (KF564639), Ljwnt8 (KF551570), Stddk1 (KF551566), Stgsc (KF564642), Stbrachyury (KF551568), Stdelta1 (KF551567), and Stwnt8 (KF551569).

**Results and discussion**

**Genetic topography of the vertebrate dorsal mesoderm evolved through A/P expression domain shift of amphioxus mesodermal genes**

In the dorsal mesoderm of amphioxus embryos, somites and notochord are regionalized during gastrulation, and an equivalent structure of the vertebrate unsegmented head mesoderm is thought to be absent in amphioxus [7] (Fig. 1). The dorsal mesoderm of vertebrates such as *Xenopus* is formed by massive mesodermal involution during the early-gastrula stage, which is not present in amphioxus embryos and is regionalized into head mesoderm, notochord, and somites [27, 37] (Fig. 1), suggesting that genetic programs govern differences between amphioxus and vertebrate mesodermal formation. We thus examined the molecular topography of the dorsal mesoderm in amphioxus and *Xenopus*. We first compared the mid-gastrula stage of amphioxus embryos and stage 10.5 *Xenopus* embryos because the orientation of dorsal mesodermal tissue is approximately parallel to the A/P body axis in both species, and thus comparable at these stages [33] (Additional file 1: Figure S1). The key regional genes include gsc (head mesoderm), bra (notochord), delta (somite), wnt8 (somite) and dkk1 (head mesoderm, somite).

By the mid-gastrula stage, all genes examined were expressed around the blastopore and showed similar patterns in amphioxus and vertebrates (Fig. 2a and b). However, by the late-gastrula stage of *Xenopus*, the expression domains of the regional marker genes became separated anteroposteriorly, with the gsc expression domain barely overlapping with those of delta, wnt8, and bra as the head and trunk mesodermal identities became distinct (Fig. 2c–f). These dynamic shifts were also observed in basal vertebrates, such as the lamprey (*L. japonicum*) and shark (*S. torazame*) (Additional file 1: Figures S2, S3 and Table S1). During the late-gastrula stage, the presomitic mesodermal region became distinct around the blastopore, and dkk1/2/4, delta, bra, and wnt8 were co-expressed in *Xenopus* presomitic mesoderm (Fig. 2d). In amphioxus, somites were found to form directly from the tail bud, and the expression of dkk1/2/4, delta, bra, and wnt8 largely overlapped in the prospective tail bud region (Fig. 2c). These results suggest that during gastrula stages, the mesodermal genes segregate anteroposteriorly only in vertebrates, whereas in amphioxus, these genes overlap considerably (Fig. 2f).

**Interference of mesodermal involution promotes an amphioxus developmental mode in Xenopus embryos**

Just after the stage in which mesodermal gene expression arrangements were similar in amphioxus and *Xenopus*, the dorsal mesoderm in *Xenopus* spread anteriorly to the blastocoel to form the head mesoderm (Fig. 3a and b).
However, in amphioxus, the relative increase in mesodermal size was much lower than that in vertebrates (Fig. 3c). This suggests that, in vertebrates, the dynamic mesodermal gene shift is achieved by increasing the mesoderm, which is primarily dictated by mesodermal cell movements (e.g. involution, convergence and extension) (Fig. 3c). During gastrulation, mesodermal involution is controlled by convergence and extension of the dorsal axial mesoderm in vertebrates [38, 39]. To determine whether mesodermal involution is essential for the mesodermal gene shift, we suppressed convergent extension by inhibiting the Wnt planar cell polarity (PCP) signalling pathway, a key signal transduction pathway in convergence and extension [40, 41]. For the loss of function study of Wnt/PCP signalling, we injected Xldd1 (a dominant-negative form of Xldsh; [42, 43]) mRNA into Xenopus embryos [26].

In the dye only-injected control embryos, labelled cells migrated anteriorly and expressed gsc but not delta-2 or bra (Fig. 3d, e, g and i). In Xldd1 mRNA-injected embryos, however, labelled cells did not migrate anteriorly, but remained close to the blastopore, and gsc, delta-2, and bra were not separated anteroposteriorly as observed in the control embryos during the late-gastrula stage (Fig. 3f, h and j). Additionally, the size of the dorsal mesoderm was much smaller in the Xldd1 mRNA–injected embryos compared with control embryos (Fig. 3e–j), indicating that the developmental sequences of the dorsal mesoderm were somewhat similar to those in amphioxus. Microinjection of XlshidelDEP mRNA, a mutant dsh that specifically inhibits the Wnt/PCP signalling pathway [44], indicated that the effect of Xldd1 injection resulted from suppression of the Wnt/PCP signalling pathway (Additional file 1: Figure S4A–F).

Fig. 1 Comparison of amphioxus and vertebrate early development. a Adult female amphioxus (Branchiostoma japonicum). b Adult female shark (Scyliorhinus torazame). c, d Comparison of dorsal mesoderm formation between amphioxus (c) and Xenopus (d) embryos. The notochord progenitor is labelled in pink, with the somite in green and head mesoderm in red. nc notochord, s somite, hm head mesoderm.
Fig. 2 (See legend on next page.)
Overlapping head and trunk marker gene expression was also detected based on the results of the dorsal marginal zone assay, suggesting that the effect of Xldd1 injection was not attributable to differences in the amount of yolk, but more likely to the loss of mesodermal cell movement (Additional file 1: Figure S4I–L).

These results are consistent with morphological changes observed at the tail bud stage in Xldd1-injected Xenopus embryos assimilated to an amphioxus-like condition. Specifically, the anterior-most somite, normally appearing just posterior to the anterior end of the notochord, was extended anteriorly into the prechordal domain (Fig. 3k–p). In these embryos, the somite marker myoD was expressed normally, similar to mrf1 in amphioxus embryos (Fig. 3q–s). Interestingly, ectopic expression of tbx1, a head mesoderm marker in vertebrates [10], was also detected in the Xldd1-injected Xenopus somites, similar to amphioxus tbx1/10, a somite marker in amphioxus [45], expression (Fig. 3t–w and Additional file 1: Figure S4G and H). These results suggest that the vertebrate-specific mesodermal involution during gastrulation is likely responsible for the A/P distinction between head and trunk mesoderm, which does not occur in amphioxus.

### A/P patterning in the dorsal mesoderm by different Wnt/β-catenin-signalling input is a vertebrate novelty

Previous functional studies in vertebrates have shown that the dorsal mesoderm is regionalized by a Wnt/β-catenin-signalling gradient along the A/P axis during early embryogenesis [46]. The failure of A/P segregation of mesodermal regional gene expression in Xldd1 mRNA-injected embryos suggests that Wnt/β-catenin-signalling pathway control of these downstream genes is compromised in this context. We examined the nuclear localization of β-catenin, a downstream factor in the Wnt/β-catenin signalling pathway, in Xldd1 mRNA-injected embryos. In the control embryos, nuclear localization of β-catenin was observed in the posterior dorsal mesodermal cells, but not in the anterior region (Fig. 4a–c). In Xldd1 mRNA-injected embryos, however, β-catenin localized to the nucleus in some cells, but there was no clear A/P difference in the degree of nuclear localization (Fig. 4d–f). The lack of obvious A/P difference in β-catenin nuclear localization was also observed in the amphioxus dorsal mesoderm (Fig. 4g–l).

Consistent with this result, a previous functional study showed that Wnt/β-catenin signalling had no role in the A/P patterning of the dorsal mesoderm during the gastrula stages [23]. These findings suggest that anterior low and posterior high Wnt/β-catenin-signalling input is important in the segregation of regional marker genes of the dorsal mesoderm along the A/P axis that evolved in vertebrates.

### Evolution of head mesoderm in vertebrates

In this study, we propose that the vertebrate dorsal mesoderm evolved as an entirely novel pattern associated with a new mechanism of mesoderm specification. As was first described by Ernst Haeckel [47], amphioxus gastrulation involves a simple invagination similar to that in cnidarians, with little change in the spatial relationships between the ectoderm and mesendoderm during development. Unlike in vertebrates, the dorsal mesoderm in amphioxus co-expresses both vertebrate head and trunk mesoderm marker genes (Fig. 5). Thus, the amphioxus dorsal mesoderm remains unspecified along the A/P axis due to the absence of a vertebrate-specific developmental program (Fig. 5). Vertebrate mesoderm, however, is uniquely polarized along the A/P axis into the head and trunk mesoderm based on mesodermal patterning mediated by vertebrate-specific cell movement. By the end of gastrulation, the head and trunk mesodermal identities are specified by anteroposteriorly dislocated expression of regional marker genes. This patterning mechanism also controls A/P patterning of the overlying neuroectoderm. In vertebrates, the A/P regional identity of the CNS is organized by vertical signals from the underlying dorsal mesoderm containing A/P pattern information [48]. In amphioxus, based on the topography of regional markers, the CNS is patterned into domains largely comparable to the fore-/mid-/hindbrain and the spinal cord in vertebrate embryos [5]. However, three major signalling centres (anterior neural ridge, zona limitans intrathalamica, and midbrain–hindbrain boundary) are absent in the amphioxus CNS [5, 49]. Given that our current study indicated that the A/P regional identity of the vertebrate dorsal mesoderm is fundamentally different from that of amphioxus (Fig. 5a), the three major signalling centres in the neuroectoderm of vertebrate embryos may have evolved through a reorganization of the dorsal mesoderm in an amphioxus-like chordate ancestor.
Fig. 3 (See legend on next page.)
Evolutionary reorganization of the entire dorsal mesoderm as described above would imply that individual amphioxus somites are not homologous to any specific region of the vertebrate head mesoderm. Our scenario instead favours the novel nature of the vertebrate mesoderm generated by modification in mesodermal patterning dynamics. From the perspective of vertebrate mesodermal specification, the segmented mesoderm in amphioxus appears as an intermediate between the head mesoderm and trunk somites, and vertebrate somites do not represent primitive traits, but rather derived traits established by removal of head mesoderm-like properties from ancestral somites. This scenario of vertebrate head evolution correlates with the observation that peripheral nerves in amphioxus possess traits of both cranial and spinal nerves [50, 51]. The mesodermal developmental pattern is shared among chordates only in the early gastrulae, in which the mesoderm is not yet antero-posteriorly polarized, possibly representing a plesiomorphic state (Fig. 5). The A/P patterning of mesodermal identities through the different Wnt/β-catenin-signalling input takes place only in vertebrates later in the developmental process; along with unique changes in cell movement, this can be considered a synapomorphic developmental trait for this animal subphylum. As proposed by Haeckel, this indicates that the vertebrate body plan is established by recapitulating an amphioxus-like ancestral pattern (plesiomorphy) during the early-gastrula
stage and engaging a novel pattern (synapomorphy) during the later stages. The comparison of mesodermal gene expression of vertebrates, amphioxus, and hemichordates (as an out-group taxon) suggests that the amphioxus mesoderm has an intermediate nature, possibly representing a plesiomorphic state for deuterostomes (http://www.ibiology.org/ibioseminars/evolution-ecology/marc-w-kirschner-part-3.html). This characterization of the evolutionary sequence of developmental dynamics in chordates also provides insight into the potential origins of mesoderm and mesodermal segments in bilaterians.

Possible mechanism of mesodermal involution unique to vertebrates

In this study, we showed that inhibition of mesodermal involution in vertebrate embryos recapitulated amphioxus development (Fig. 3). The lack of mesodermal involution and likely convergent extension in amphioxus gastrulation indicates that the developmental program for mesodermal involution in vertebrates is absent in amphioxus. Disruption of cadherin-mediated cell–cell adhesion is essential during mesodermal involution and convergent extension in vertebrates, and fibronectin leucine-rich-repeat transmembrane 3 (Flrt3) and a small GTPase (Rnd1) control C-cadherin degradation [26, 34, 52, 53]. A BLAST search revealed a homologue of rnd1 in amphioxus, but not of flrt3 (Additional file 1: Figure S5A and B). In amphioxus, rnd1 expression was observed around the blastopore (Additional file 1: Figure S6A–H). However, overexpression of Bfrnd1 mRNA could not rescue the loss of endogenous rnd1 in Xenopus (Additional file 1: Figure S6L–M). These findings suggest that involvement of the cadherin degradation system in mesodermal involution as well

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**Fig. 5** Vertebrate head mesoderm evolved through polarization of ancestral mesodermal patterning. a In amphioxus and vertebrates, the dorsal mesoderm has two groups of genes that encode positional value (Group 1, orange; Group 2, light blue). At stage I (early-to mid-gastrula stages), group 1 and group 2 are co-expressed both in amphioxus and vertebrate dorsal mesoderm. At stage II (late-gastrula stage), group 1 genes shift anteriorly and group 2 genes shift posteriorly in vertebrates, whereas the amphioxus mesoderm retains the overlapped condition. Numbers indicate positional value of the dorsal mesoderm along the A/P axis. c chordate, a amphioxus, v vertebrates. b The dorsal mesoderm of vertebrates evolved by the reorganization of mesodermal genes under the control of Wnt/β-catenin-signalling input. Notch/Delta signalling regulates somitogenesis both in amphioxus and vertebrates. s somite, nt notochord, tb tail bud, psm presomitic mesoderm, cnh chordoneural hinge, m mesenchyme, hm head mesoderm. hairy genes are key components that regulate somite formation during development [16]. pitx2 and en2 are expressed in head mesoderm of vertebrate embryos [10].
as convergence and extension may have emerged in the vertebrate lineage.

Conclusions
Our findings indicate that the A/P patterning of the vertebrate dorsal mesoderm evolved from an amphioxus-like ancestral mesoderm through A/P polarization of mesodermal specification to divide into the unsegmented head mesoderm anteriorly and the segmented trunk somites posteriorly. Vertebrate head mesoderm is thus an evolutionary novelty.

Additional file

Additional file 1: Figure S1. Dorsal mesoderm formation in chordates. Figure S2. Phylogenetic trees of dorsal mesoderm genes. Figure S3. Dorsal mesodermal gene expression in lamprey (L. japonicum) and shark (S. torazame) embryos. Figure S4. Suppression of the Wnt/PCP-signaling pathway in Xenopus embryos. Figure S5. Flrt3 evolved in the vertebrate lineage. Figure S6. Flrt3-Rnd1 system is essential for mesoderm formation in vertebrates. Table S1. Summary of expression pattern of genes in Figure S2. (DOCX 8727 kb)

Competing interests
The authors declare they have no competing interests.

Authors' contributions
TO and SK wrote the paper. TO and TH performed the experiments. TO, TA and HI designed the experiments. All authors read and approved the final manuscript.

Acknowledgements
We thank Linda Holland and Nick Holland of the University of California, San Diego, CA, USA for help with the collection of amphioxus and for discussion. We also thank Yasuhashi Henmi of Kumamoto University, Japan and Kinya Yasaki of Hiroshima University, Japan for helping with amphioxus collection and Shigeiho Kuraku of RIKEN, Japan for preparing the phylogenetic trees. This research was supported by a KAKENHI Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science (grant number 24702227).

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Received: 9 June 2015 Accepted: 22 September 2015
Published online: 09 November 2015

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