Morphogenetic mechanisms forming the notochord rod: The turgor pressure - sheath strength model

Review

Morphogenetic Mechanisms Forming the Notochord Rod: the Turgor Pressure-Sheath Strength Model

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Running head: Counterbalancing Mechanisms in Notochord Formation

Abstract

The notochord is a defining feature of chordates. During notochord formation in vertebrates and tunicates, notochord cells display dynamic morphogenetic movement, called convergent extension, in which cells intercalate and align at the dorsal midline. However, in cephalochordates, the most basal group of chordates, the notochord is formed without convergent extension. It is simply developed from mesodermal cells at the dorsal midline. This suggests that convergent extension movement of notochord cells is a secondarily acquired developmental attribute in the common ancestor of olfactores (vertebrates + tunicates), and that the chordate ancestor innovated the notochord upon a foundation of morphogenetic mechanisms independent of cell movement. Therefore, this review focuses on biological features specific to notochord cells, which have been well studied using clawed frogs, zebrafish, and tunicates. Attributes of notochord cells, such as vacuolation, membrane trafficking, extracellular matrix formation, and apoptosis, can be understood in terms of two properties: turgor pressure of vacuoles and strength of the notochord sheath. To maintain the straight rod-like structure of the notochord, these parameters must be counterbalanced. In the future, the turgor pressure-sheath strength model, proposed in this review, will be examined in light of quantitative molecular data and mathematical simulations, illuminating the evolutionary origin of the notochord.

Keywords: chordate evolution, convergent extension, extracellular matrix, notochord, vacuolation

1. Evolutionary Origin of the Notochord

The notochord functions as the supporting organ of the chordate larval tail, composed of vacuolated inner cells, outer sheath cells, and a thick, surrounding, extracellular sheath (Stemple, 2005). Because the notochord is considered an evolutionary novelty of chordates, the origin of the notochord has been an important subject in the field of evolutionary developmental biology (Evo-Devo). To approach this question, gene expression profiles, cell-cell signaling, cell types, and morphogenetic mechanisms during embryogenesis have been heavily investigated using chordates and non-chordates (Annona, Holland, & D'Aniello, 2015; Brunet, Lauri, & Arendt, 2015; Satoh et al., 2014; Satoh, Tagawa, & Takahashi, 2012). Consequently, fundamental genes for notochord development have been identified, including those encoding transcription factors such as *brachyury*, *foxA*, and *foxD*, signaling molecules such as *chordin*, *noggin*, and *hedgehog*, and extracellular matrix (ECM) proteins such as collagen. Furthermore, many evolutionary scenarios have been proposed for the notochord, assuming its progenitors in the pre-chordate ancestor. However, it is still unclear how notochordal genes interacted to create the notochord and what cellular mechanisms underlay formation of the rod-like structure at the dorsal midline in the chordate ancestor. In this review, I focus on the second question.

2. Convergent Extension Movement in Notochord Formation

In African clawed frog (*Xenopus laevis*) and zebrafish (*Danio rerio*), it has been well documented that the notochord is formed via convergent extension movement during gastrulation (Glickman, Kimmel, Jones, & Adams, 2003; R. Keller et al., 2000; Topczewski et al., 2001; R. E. Keller, Danilchik, Gimlich, & Shih, 1985; Shindo, 2018; Tada & Heisenberg, 2012; Wallingford, Fraser, & Harland, 2002). Dorsal mesodermal cells, developing into notochord and somites, intercalate by moving toward the midline during gastrulation, resulting in anterior-posterior elongation. In mammals, unlike amphibians and teleosts, chordamesoderm initially forms at the ventral surface of the embryo, and is subsequently internalized to form the notochord. A live imaging study of mouse early embryos showed that trunk notochord also forms by convergent extension, although anterior and posterior notochord form by different morphogenetic processes (Yamanaka, Tamplin, Beckers, Gossler, & Rossant, 2007). As in *Xenopus* and zebrafish, tunicate notochord cells also intercalate from the sides to the dorsal midline (Keys, Levine, Harland, & Wallingford, 2002; Munro & Odell, 2002). These studies suggest that convergent extension movement of notochord cells predates the common ancestor of the olfactores (vertebrates + tunicates) (Fig. 1).

However, in cephalochordates (amphioxus), notochord cells probably do not employ convergent extension during gastrulation. Instead, amphioxus notochord is formed by pinching off of the dorsal archenteron roof during neurulation without obvious cell interaction (Conklin,

1932; Hatschek & Tuckey, 1893; Hirakow & Kajita, 1994). Cell labelling assays in amphioxus embryos showed that cells at the blastoporal ridge before gastrulation do not involute (Zhang, Holland, & Holland, 1997). Furthermore, when cell movement is suppressed in dorsal areas of *Xenopus* embryos, the notochord becomes wider and shorter without inhibition of differentiation (Sokol, 1996; Wallingford & Harland, 2001), which recapitulates amphioxus-like dorsal mesoderm formation (Onai, Aramaki, Inomata, Hirai, & Kuratani, 2015), suggesting that convergent extension of notochord cells emerged after the olfactores arose. In addition, convergent extension is not observed after the neurula stage (stage 20), but embryos still continue to elongate with a stretched notochord in *Xenopus*. This indicates that other morphogenetic mechanisms underlie notochord elongation, as well as convergent extension (Furukawa et al., 2019).

It is also possible that amphioxus secondarily lost convergent extension of notochord cells after its divergence from the olfactores. Among two hypotheses, acquisition in olfactores or secondary loss in amphioxus, I prefer the former because early embryogenesis of amphioxus appears to be prototypic in deuterostomes, for the following reasons. First, deuterostome embryos, except vertebrates, cleave radially and holoblastically into more or less equal sized blastomeres. Second, gastrulation of hemichordate embryos occurs by circumferential invagination of the vegetal endomesoderm in both direct developers (*Saccoglossus kowalevskii*) and indirect developers (*Ptychodera flava*) (Lin, Tung, Yu, & Su, 2016; Lowe et al., 2003; Rottinger & Lowe, 2012; Tagawa, Nishino, Humphreys, & Satoh, 1998), which is very similar to amphioxus gastrulation. Convergent extension has never been observed in axis elongation of hemichordate embryos. Third, convergent extension occurs in the archenteron of echinoderm gastrulae (Ettensohn, 1985; Hardin, 1989), but the process is not equivalent to that in olfactores mesoderm and ectoderm. Echinoderm archenteron elongates by cell intercalation turning a dome-shaped invagination into a long tube, whereas olfactores mesoderm and ectoderm elongate by midline-oriented cell intercalation of cell sheets, suggesting that evolutionary origins of convergent extension in olfactores notochord and sea urchin archenteron are independent. Thus, embryogenesis of the deuterostome ancestor has been often reconstructed based on features common to both amphioxus and hemichordates (Holland, 2015; Lowe, Clarke, Medeiros, Rokhsar, & Gerhart, 2015; Onai, 2018; Rottinger & Lowe, 2012).

Assuming that the deuterostome ancestor gastrulated as do amphioxus and hemichordates, convergence of mesodermal cells may not be an ancestral feature of the notochord. If so, convergent extension movement should be excluded from a discussion of possible homologies to the notochord in notochord-like organs of non-chordate animals. For example, convergent

extension of the axochord in annelid embryos, which was hypothesized as an origin of the notochord (Brunet et al., 2015; Lauri et al., 2014), may have evolved independently of the notochord. Hereafter, I describe cellular morphology that supports notochord structure, in an attempt to find more basal notochord developmental mechanisms, independent of cell movement.

3. Characteristic Features of Notochord Cells

3.1. Vacuolation

Vacuoles are found in cells of developing notochord of vertebrates and cephalochordates (Adams, Keller, & Koehl, 1990; Bancroft & Bellairs, 1976; Flood, 1975; Leeson & Leeson, 1958; Stemple, 2005; Waddington & Perry, 1962). In *Xenopus* and zebrafish, vacuolation starts at the early tailbud stage, just after completion of convergent extension (Adams et al., 1990; Yamamoto et al., 2010), indicating that vacuolation of notochord cells is one of the driving forces of axis elongation in late stage embryos. In chickens, vacuolation starts at a comparable stage (stage 14) (Flood, 1975). A zebrafish study showed that vacuoles of notochord cells are lysosome-like organelles, expressing a lysosome marker, *lamp1* (Ellis, Bagwell, & Bagnat, 2013). *lamp1* is also specifically expressed in the *Xenopus* notochord (Fig. 2A).

In mice, vacuolation has only been observed at much later stages (E15.5), when vertebrae are formed and notochord cells develop into the nucleus pulposus in intervertebral discs (Smits & Lefebvre, 2003; Wang et al., 2017). Proportionally smaller and unvacuolated notochord in mice might result from a reduced requirement of the notochord as a supporting organ, since mammalian embryos develop considerable skeletal structure before birth and do not employ tail-beating using the notochord and somites. Therefore, mammalian notochord is degenerate due to the secondary loss of vacuolation mechanisms at early embryonic stages.

In *Ciona* larvae, vacuoles are not found in notochord cells, but instead, extracellular lumens fuse so that cells form a tubular structure (Dong et al., 2009). In addition to *Ciona*, lumen formation in the notochord is commonly observed in many other, but not all, ascidians (Jiang & Smith, 2007). This process involves divergent mechanisms in tunicates, but the basic mechanism to maintain notochord structure by retaining water inside is same as vacuolation of the notochord in vertebrates. Remarkably, extracellular lumen formation is initiated after convergent extension in *Ciona* embryos, paralleling vacuolation in vertebrate notochord.

In amphioxus, notochord cells are also supported by muscle filaments (Flood, Guthrie, & Banks, 1969; Suzuki & Satoh, 2000). This implies that the ancestral notochord may have utilized both vacuoles and muscle filaments to develop sufficient strength to support the entire body.

However, a recent molecular evolutionary study demonstrated that components of muscular filaments in the amphioxus notochord originate from cephalochordate-specific gene duplication events, suggesting that muscle filaments in the notochord are an evolutionary novelty in cephalochordates, but not an ancestral feature of chordates (Inoue & Satoh, 2018). Thus, vacuoles are presumed to be the most ancient cellular mechanism to shape the rod-like structure of the notochord (Fig. 1).

Importantly, vacuoles in notochord cells need osmolytes to absorb large volumes of water. It was reported that H^+-ATPase-dependent acidification is necessary for biogenesis and maintenance of vacuoles in zebrafish notochord, but notochord vacuoles are not acidic (Ellis et al., 2013). The authors proposed a model in which alkali/H^+ exchangers on vacuolar membranes transport alkali ions into the vacuole lumen, leading to accumulation of water. They also addressed the possibility that glycosaminoglycans function as osmolytes in the notochord vacuole, but no glycosaminoglycans can be detected there. In *Ciona*, lumen expansion during notochord tubulogenesis requires Slc26 anion transporters, suggesting that anion translocation is responsible for evolutionary conservation of water accumulation in chordate notochords (Deng et al., 2013). Therefore, identification of osmolytes in the notochord vacuole could be important to understand notochord morphogenesis and its evolutionary origin. Notochord-specific expression of a putative sodium-dependent amino acid/proton antiporter gene, *slc38a8*, in *Xenopus* and zebrafish (Fig. 2A) (Shestopalov, Pitt, & Chen, 2012) and notochord-enriched expression of its homolog *slc38a1/2/3/4* in *Ciona* (Reeves, Wu, Harder, & Veeman, 2017) may offer an explanation.

3.2. Membrane Trafficking

To form vacuoles, cellular membranes must be rearranged. Therefore, intracellular membrane trafficking systems are important for notochord development. In zebrafish, late endosomal trafficking, regulated by the vacuole-specific Rab32a, is required for notochord vacuole formation (Ellis et al., 2013). Retrograde transport from the trans-Golgi network to the cis-Golgi network and endoplasmic reticulum is also required for notochord formation, as shown by mutant phenotypes of three coatomer subunits, *copa*, *copb1*, and *copb2* (Coutinho et al., 2004). Expression levels of these genes are transiently elevated in chordamesoderm prior to notochord differentiation (Thisse et al., 2001; Thisse & Thisse, 2004; Coutinho et al., 2004). Similarly, genes related to secretory pathways are activated in the developing *Xenopus* notochord (Tanegashima, Zhao, Rebbert, & Dawid, 2009). Among them, a subunit of the coatomer complex, *copz1*, is specifically expressed in *Xenopus* notochord (Fig. 2A). In *Ciona* embryos, expression levels of *copb* genes (*COPB* and *COPB'*) are transiently elevated in notochord precursor cells, whereas *COPA* expression is detected ubiquitously with high levels in the sensory vesicle (Kugler et al.,

2008), suggesting that enrichment of some coatomer complex proteins was necessary for notochord formation in ancestral olfactores. It is also known that expression of *rcn3*, which encodes a calcium-binding protein localized to the lumen of endoplasmic reticulum, is restricted to the notochord in *Xenopus* and zebrafish (Fig. 2A). Rcn3 may regulate membrane trafficking and osmolyte transport from endoplasmic reticulum to the Golgi network for vacuole formation, a possibility that should be examined in the future.

The contribution of endocytosis to growing vacuoles was also investigated in zebrafish embryos (Ellis et al., 2013). Embryos mosaically expressing a dominant-negative form of dynamin, an endocytosis and Golgi complex exit regulator, or Rab5c, an early endosome regulator, formed vacuoles almost normally. To detect endocytosis more directly, dissociated notochord cells were cultured with the endocytic tracer, FM4-64, for tracking internalized membranes. However, the dye never labelled vacuole membranes. These data indicate that notochord vacuoles do not receive internalized membranes or soluble cargo via endocytosis. Thus, endocytosis and early endosomal trafficking do not contribute to vacuole formation and maintenance in notochord cells.

Caveola formation is also characteristic of vertebrate notochord cells. Caveolae are stable flask-shaped structures on plasma membranes coated with caveolin and cavin proteins, which are involved in endocytosis and mechanosensing, via interactions with actin filaments (Echarri & Del Pozo, 2015; Parton & Simons, 2007). In zebrafish, *caveolin* genes, *cav1* and *cav3*, are strongly expressed in notochord (Nixon et al., 2007; Nixon et al., 2005). Similarly, *Xenopus*, chick, and mouse notochord cells also express *caveolin* (Nixon et al., 2007) (Fig. 2A). A loss of function study in zebrafish showed that disruption of caveolae led to undulation of the notochord (Nixon et al., 2007). In addition, when zebrafish embryos were exposed to genistein, a caveola inhibitor, notochord deformities occurred (Schiller et al., 2013). As mentioned above, endocytosis does not contribute to vacuole formation (Ellis et al., 2013). Therefore, caveola formation on plasma membranes of vacuolated cells is important not for endocytosis, but for mechanosensing during notochord morphogenesis. Recent studies of zebrafish notochord caveolae showed that they resist mechanical stress produced by tail-beating (Garcia et al., 2017; Lim et al., 2017). Without caveolae, notochords were easily damaged by locomotion. Therefore, the main role of caveolae in the notochord is mechanoprotection to maintain stable structures by interacting with the actin skeleton and other filaments (e.g. intermediate filaments in the zebrafish notochord (Nixon et al., 2007)). In *Ciona*, *cav1/3* expression is mainly enriched in mesenchyme and muscle (Reeves et al., 2017), but misexpressed caveolin protein localized to notochord lumens (Deng et al., 2013). It is possible that caveolae have an evolutionarily conserved role of maintaining notochord stability.

Importantly, in zebrafish, collapse of vacuolated cells triggers invasion of sheath cells to the inner area and invading cells trans-differentiate into vacuolated cells (Garcia et al., 2017). Evolutionary conservation of this self-restorative system should be addressed using other models.

3.3. Extracellular Matrix 1: Collagenous Fibrils

To support the larval tail of chordates, the extracellular matrix (ECM)-based notochord sheath must be formed. In vertebrate, tunicate, and cephalochordate notochord sheath, thick layers of collagenous fibrils are observed (Adams et al., 1990; Bancroft & Bellairs, 1976; Cloney, 1969; Flood, 1975; Leeson & Leeson, 1958; Miyamoto & Crowther, 1985; Waddington & Perry, 1962). Among fibril-forming collagen genes, *col2a1,* which encodes the collagen type II alpha-1 chain, has been well documented as a notochord-enriched gene. In vertebrates, *col2a1* is strongly expressed in notochord, but also in other tissues surrounding the notochord, such as floor plate, somites, and hypochord (Cheah, Lau, Au, & Tam, 1991; Su, Suzuki, Bieker, Solursh, & Ramirez, 1991; Swiderski & Solursh, 1992; Yan, Hatta, Riggleman, & Postlethwait, 1995). A study using *Col2a1*-null mice showed that *Col2al* is required for removal of the notochord, so as to give rise to formation of intervertebral discs (Aszodi, Chan, Hunziker, Bateman, & Fassler, 1998). In *Ciona*, a *col2a1* ortholog (*CiFCol1*) is expressed in notochord and surrounding tissues, from neurulae to larvae (Reeves et al., 2017; Wada, Okuyama, Satoh, & Zhang, 2006). In amphioxus, expression of a *col2a1* ortholog (*ColA*) is restricted to the notochord in late neurulae, and to the neural tube and notochord in early larvae (Meulemans & Bronner-Fraser, 2007; Wada et al., 2006). These findings indicate that clade A fibrillar collagen (an ortholog group including Col2a1) has been a fundamental component of the notochord sheath since chordates arose (Fig. 1).

Fibrillar collagens in other clades (B and C) are also enriched in the notochord. Among Clade B genes, restricted expression of *col11a1* in the notochord was observed in early zebrafish embryos (Baas, Malbouyres, Haftek-Terreau, Le Guellec, & Ruggiero, 2009). Among clade C genes, expression of *col27a1* genes (*col27a1a* and *col27a1b*) is restricted to zebrafish notochord, and morphants showed buckling of the notochord (Christiansen, Lang, Pace, & Parichy, 2009). In *Ciona*, expression of a clade B gene (*CiFCol2*) and two clade C genes (*CiFcol3* and *CiFcol4*) is also observed in the notochord (Wada et al., 2006). Because amphioxus secondarily lost clade B and C genes, it is not clear whether the chordate ancestor utilized all clades of fibrillar collagens to form the notochord, or whether the olfactores ancestor added clade B and C fibrillar collagens to reinforce the notochord. As mentioned above, amphioxus notochord is unique in containing muscle fibers, possibly due to insufficient strength of its ECM sheath, which lacks clade B and C fibrillar collagens.

A type IX collagen gene, *col9a2*, which encodes a fibril-associated collagen, is specifically expressed in zebrafish notochord sheath cells (Garcia et al., 2017). During spine formation, *col9a2* expression in sheath cells shows segmented patterns along the anteroposterior axis. Spines are formed in *col9a2*-negative regions, which express *entpd5a*, whereas *col9a2*-positive regions develop into intervertebral discs (Wopat et al., 2018). Type IX collagen expression is also observed in other vertebrate notochords (Hayashi et al., 1992; Pollet et al., 2005; Swiderski & Solursh, 1992) (Fig. 2A). In *Ciona*, a type IX collagen gene (*Ci-COL9-1A*) is also expressed in the notochord, and suppressed by Hox10 in endoderm strand (Kawai et al., 2015), suggesting that type IX collagen participation in notochord sheath formation may have predated the divergence of vertebrates and tunicates. In contrast to type IX collagens, type X collagens, which form hexagonal networks, are secreted in segments where vertebrae form (Linsenmayer, Gibney, & Schmid, 1986; Renn, Buttner, To, Chan, & Winkler, 2013). Mutually exclusive expression patterns of type IX and X collagens during notochord segmentation correspond to cartilage-like tissues that form intervertebral discs and mineralized bone to form vertebrae, respectively. Notochord segmentation has never been reported in non-vertebrate chordates at either molecular or morphological levels. Grafting experiments using chick embryos showed that notochord does not contain intrinsic segmentation patterns, but that it attracts sclerotome cells from somites, in which segmentation patterns are intrinsically determined (Ward, Pang, Evans, & Stern, 2018). Taken together, notochord segmentation is probably specific to vertebrates to form vertebrae (Fig. 1).

Another short-chain collagen gene that forms hexagonal networks, *col8a1a*, is also enriched in the notochord, floor plate, and hypochord of zebrafish embryos, and loss of *col8a1a* function causes malformation of the notochord and vertebrae (Gansner & Gitlin, 2008; Gray et al., 2014; Shestopalov et al., 2012). In *Xenopus*, *col8a1* expression was also detected in the floor plate and hypochord of later-stage embryos (Parain et al., 2012). Thus, many types of fibrillar and non-fibrillar collagens are expressed and function cooperatively to produce sufficient notochord sheath stiffness.

Lysyl oxidase is a copper-dependent amine oxidase that catalyzes cross-linking of collagen and elastin fibers in the ECM. In *Xenopus* and zebrafish, lysyl oxidase genes, *lox*, *loxl1*, *loxl2*, *loxl3*, and *loxl5*, are specifically expressed in notochord (Fig. 2A) (Gansner, Mendelsohn, Hultman, Johnson, & Gitlin, 2007; Geach & Dale, 2005). Loss of function assays of lysyl oxidase genes and chemical inhibitor (β-aminoproprionitrile) treatment resulted in distortion of the notochord (Gansner et al., 2007; Geach & Dale, 2005). These data suggest that ECM stiffness is maintained by lysyl oxidase activity. In *Ciona* embryos, two lysyl oxidase genes, *lox1* and *lox4*,

are expressed in notochord (Kugler et al., 2008), suggesting conservative roles of these enzymes to cross-link ECM layers of the notochord sheath.

3.4. Extracellular Matrix 2: Proteoglycans

Proteoglycans are also important components of the notochord sheath ECM. It was reported that some proteoglycan genes are enriched in the notochord. Prolyl 3-hydroxylase 1, P3H1, also called Leprecan, is specifically expressed in the developing notochord of *Ciona* and *Xenopus* (Hotta et al., 2000) (Fig. 2A). Functional analysis using *Ciona* embryos showed that Leprecan is essential for notochord morphogenesis by hydroxylating collagens in endoplasmic reticulum with partner proteins such as CRTAP and CYPB, which are also expressed in the notochord (Dunn & Di Gregorio, 2009).

Among glycosaminoglycans, highly sulfated keratan sulfate (HSKS) has been used as a molecular marker of the notochord during early development. Immunostaining using monoclonal antibodies against HSKS (e.g. MZ-15 and 5D4) results in very specific labeling of the notochord and otic vesicles in *Xenopus* and zebrafish larvae (Ellis et al., 2013; Geach & Dale, 2005; Smith & Watt, 1985) (Fig. 2B). Enzymes involved in tissue-specific enrichment of HSKS have never been reported, but expression of some enzymes must be restricted to the notochord. As mentioned above, no glycosaminoglycans are present in vacuoles of zebrafish notochord, indicating that HSKS proteoglycans are not osmolytes for vacuolation (Ellis et al., 2013). One possible function of HSKS in collagen fibrils of the notochord sheath may be to distribute the applied load under mechanical stress, as in articular cartilage (Roughley & Mort, 2014).

In *Ciona*, 6-O-sulfotransferease genes, *Ci-C6ST-like1*, *Ci-C6ST-like7*, an uronyl 2-O-sulfotransferase gene, *Ci-UST-like1*, and a Ci-β1,4-Galactosyltransferase gene, *Ci-β4GalT*, are specifically expressed in the notochord (Hotta et al., 2000; Katikala et al., 2013; Tetsukawa, Nakamura, & Fujiwara, 2010). Although enrichment of HSKS in the *Ciona* notochord has not been examined, those notochord-specific enzymes must produce highly sulfated glycosaminoglycans in the notochord. Further investigation of HSKS and other glycosaminoglycans in tunicate and amphioxus notochord should reveal the ancestral state of glycosaminoglycans around the notochord.

3.5. Extracellular Matrix 3: Laminins and Fibronectins

Enrichment of other ECM components, such as laminin and fibronectin, has been reported in vertebrates and tunicates. Laminins are heterotrimeric proteins of laminin α, β, and γ chains, and are key components of the basement membrane. Studies using zebrafish mutants and morphants

of laminin genes (*lama1*, *lama4*, *lama5*, *lamb1*, and *lamc1*) showed that formation of either laminin 111, 411, or 511 is essential for normal development of the notochord (Parsons et al., 2002; Pollard et al., 2006). Enrichment of laminin 111 in the notochord was also reported in *Xenopus* embryos (Fey & Hausen, 1990). Functional analyses of dystroglycan, a transmembrane laminin receptor, using *Xenopus* embryos demonstrated that interactions between dystroglycan and laminin are required for notochord vacuole differentiation and maintenance of the cortical actin cytoskeleton network during vacuolation (Buisson et al., 2014). *Ciona* laminin genes, *lama1*, *lama3/4/5*, *lamb1*, *lamc1*, are also specifically expressed in the notochord (Katikala et al., 2013; Kugler et al., 2008; Veeman et al., 2008), and a mutant of *lama3/4/5* showed a short-tailed phenotype due to loss of the morphological boundary around the developing notochord (Veeman et al., 2008). Enrichment of laminin 111 proteins was also observed around notochord, somites, neural tube, gut, and vessels in amphioxus larvae (Pascual-Anaya et al., 2013), suggesting that laminin 111 maintains the morphological boundaries around not only the notochord, but also other tissues. It is worth examining expression patterns of laminin genes (especially *lama3/4/5*) in amphioxus to investigate evolutionary conservation of notochord-specific laminin components.

Fibronectin, which binds to integrin $\alpha_5\beta_1$, is also enriched in vertebrate and *Ciona* notochords (Gawantka et al., 1998; Koshida et al., 2005; Kugler et al., 2008). Functional analyses using *Xenopus*, zebrafish, and *Ciona* embryos showed that fibronectin is essential for intercalation of notochord cells during convergent extension movement (Davidson, Marsden, Keller, & Desimone, 2006; Segade, Cota, Famiglietti, Cha, & Davidson, 2016; Trinh & Stainier, 2004). Importantly, fibronectin is also enriched in paraxial mesoderm (somites) of *Xenopus* and zebrafish embryos, which participate in convergent extension together with axial mesoderm (notochord), whereas fibronectin expression is highly specific to the notochord in *Ciona* embryos, possibly because only notochord cells intercalate to the midline in *Ciona* embryos. It was reported that genomes of amphioxus, echinoderms, and protostomes lack the fibronectin-like gene (Tucker & Chiquet-Ehrismann, 2009). Taken together, the olfactores ancestor must have innovated fibronectins for convergent extension of mesodermal cells. In other words, fibronectins are not likely to have been ancestral components of the notochord sheath ECM (Fig. 1). Alternatively, amphioxus might have secondarily lost fibronectin together with convergent extension.

3.6. Cell Proliferation and Apoptosis

Cell number is also important for organizing the size and shape of the organ. In mouse, *Xenopus*, and zebrafish embryos, notochord cells are relatively quiescent (Bellomo, Lander, Harragan, & Brown, 1996; Mendieta-Serrano, Schnabel, Lomeli, & Salas-Vidal, 2013; Saka & Smith, 2001). In *Xenopus* notochord and somites, a cyclin-dependent kinase inhibitor gene, $p27^{XIC1}$, is strongly

expressed to repress cell proliferation (Hardcastle & Papalopulu, 2000). Cell division of notochord and muscle cells is also arrested at later stages of *Ciona* embryos (Nakamura, Terai, Okubo, Hotta, & Oka, 2012). It was also shown that proliferating cells decline progressively in notochord and somites of amphioxus embryos (Holland & Holland, 2006). This indicates that cell cycle arrest is an ancestral feature of chordate dorsal mesoderm (notochord and somites) and that cell division does not contribute strongly to notochord morphogenesis during larval tail formation (Fig. 1).

Apoptosis is also involved in proper formation of the notochord. In *Xenopus*, apoptotic cells gradually increase in the notochord after neurulation, and inhibition of apoptosis by Bcl2 overexpression severely disrupted notochord structure (Malikova, Van Stry, & Symes, 2007). A kinked notochord phenotype developed after the onset of vacuolation. In zebrafish, apoptosis of notochord cells was also observed after neurulation and increased at the stage when notochord cells vacuolate and swell (Cole & Ross, 2001). In *Ciona*, swimming larvae do not show apoptotic features, but during subsequent metamorphosis and tail regression, many apoptotic cells are found in notochord, muscles, tunic, and epidermis (Chambon et al., 2002). Because the sessile ascidian life style is a derived feature, apoptosis of notochord cells during metamorphosis may also be related to specific ascidian biological attributes. In contrast to the olfactores, the developing notochord does not undergo apoptosis in amphioxus embryos (Holland & Holland, 2006). A reasonable explanation for the difference between amphioxus and gnathostome vertebrates is that amphioxus maintains the notochord as a functional supporting organ throughout life, but gnathostome vertebrates replace the notochord with a vertebral column. Thus, the need for notochord cells differs significantly between amphioxus and gnathostomes. Alternatively, it is possible that insufficient vacuolation of amphioxus notochord fails to induce apoptosis of notochord cells. The linked timing of apoptosis and vacuolation in vertebrates may reflect apoptosis induction by mechanical stresses produced by vacuoles. The number of notochord cells may be modulated by mechanisms such as the Hippo signaling pathway to control organ size (Varelas, 2014; Yu, Zhao, & Guan, 2015). In fact, a zebrafish mutant of Hippo signaling pathway components ($yap^{-/-}$; $taz^{+/-}$) showed an undulating notochord phenotype, suggesting that levels of Hippo signaling control notochord morphogenesis (Astone et al., 2018).

4. Turgor Pressure-Sheath Strength Model in Notochord Formation

Integrating the many notochord cell characteristics discussed in section 3, I propose a model for self-organized morphological programs of the notochord that are independent of cell movement. I call it the “turgor pressure-sheath strength” model (Fig. 3). Under this model, each parameter is counterbalanced by the other, such that notochord formation is self-organizing. Notochord cells

may monitor internal and external osmotic/hydrostatic pressure via the cytoskeleton, cell adhesion proteins, and ECMs, and modulate cell numbers and sheath strength via apoptosis and secretion pathways, respectively. To maintain the straight rod-like structure necessary as a supporting organ, mechanical stresses produced by locomotion and environment are also transduced into this system. Although turgor pressure is thought to be absent in mammalian notochords because they are not vacuolated, molecular mechanisms underlying cell survival, ECM formation, and mechanical stress response in nucleus pulposus can be applied to this model (Merceron et al., 2014; Smits & Lefebvre, 2003).

As discussed in section 2, convergent extension movement of notochord cells is a possible novelty in olfactores to narrow the notochord rod but not to regulate tissue differentiation. In fact, rod-shaped notochord is formed in *Xenopus* embryos in which cell movement is inhibited by misexpression of a dominant-negative form of Dishevelled (Onai, Aramaki, Inomata, Hirai, & Kuratani, 2015; Sokol, 1996; Wallingford & Harland, 2001). Because vacuolation initiates after completion of convergent extension (Adams, Keller, & Koehl, 1990; Buisson et al., 2014; Furukawa et al., 2019), "turgor pressure-sheath strength" regulation and cell movement are temporally separated. A recent simulation study using a physical model packing water-absorbing polymer beads in a silicone tube demonstrates that organization of the vertebrate notochord is governed by the density of vacuolated cells and the aspect ratio of the notochord rod (Norman et al., 2018). This further indicates independency of cell movement in notochord formation after vacuolation. Thus, vacuolated cells are self-organized in the notochord sheath.

Other evidence that supports the "turgor pressure-sheath strength" model has been reported. For example, coatomer- and laminin-depleted zebrafish mutants showed high levels of apoptosis and less vacuolation (Coutinho et al., 2004; Parsons et al., 2002). Therefore, membrane trafficking and basement membrane formation affect apoptosis and vacuolation pathways. Contemporaneous initiation of vacuolation and apoptosis in vertebrate notochords (Cole & Ross, 2001; Malikova et al., 2007) imply coordinated regulatory mechanisms. Transdifferentiation of sheath cells into vacuolated cells in zebrafish (Garcia et al., 2017) may be part of the self-organizing system of the notochord. However, molecular dynamics and interactions of regulatory/structural proteins have not been verified.

5. Future Perspectives

To examine the turgor pressure-sheath strength model in greater detail, quantitative and theoretical approaches will be required. For example, by tracking fluorescently labeled protein components of notochord cells under conditions in which a particular cell activity is ablated,

molecular dynamics and interactions between secretion pathways (e.g. vacuole proteins, ion-transporters, caveolins, coatomers, and ECMs) can be investigated quantitatively. Direct measurement of turgor pressure in notochord cells and its dynamics during development and under stress could yield absolute measurements of forces experienced in the notochord. Based on such biophysical information, stress experiments could be performed to examine the influence of mechanical stresses on notochord cells. During these experiments, cell proliferation and apoptosis should be monitored. For such purposes, *in vitro* culture systems of notochord cells will be beneficial. To integrate all quantitative data regarding notochord cell morphogenesis, mathematical/theoretical modeling and simulation should be employed.

Notochord cells in gnathostome vertebrates eventually develop into the nucleus pulposus in adult inter vertebral discs (Choi, Cohn, & Harfe, 2008; Linsenmayer et al., 1986; Risbud & Shapiro, 2011; Swiderski & Solursh, 1992). Some zebrafish mutants with abnormal notochord formation develop disordered spines (Ellis et al., 2013; Wopat et al., 2018). As mentioned in section 3.3, segmentation of the notochord is likely to be a vertebrate-specific developmental mechanism to form vertebrae, but notochord-derived cells in adult intervertebral discs should share cellular properties with notochord cells, because they still express some notochord genes, such as *brachyury* (Risbud & Shapiro, 2011). Thus, self-organizing morphological mechanisms of the notochord could be applied to human diseases. For example, damage to the nucleus pulposus leads to intervertebral disc degeneration and back pain, and on rare occasions, notochord remnant cells transform into malignant tumors called chordomas (Choi et al., 2008). Because my model includes physical stresses, deformation, and cell proliferation interactively, causes and remedies of degeneration and tumorigenesis of notochord remnant cells may be explained, providing insights into drug discoveries for these diseases.

Finally, to address the original Evo-Devo question of notochord origins, it is important to examine cellular/molecular mechanisms of amphioxus notochord formation, because of the basal phylogenetic position of this taxon among chordates. If some components of vertebrate notochord cells commonly function in amphioxus notochord, those genes should be ancestral components of the notochord. In addition, little is known about notochord development in amniotes, chondrichthyans, and cyclostomes. It is still possible that some components of amphibian/teleost notochord cells are not shared in those taxa. Comparisons of gene regulatory networks and morphological mechanisms of the notochord among chordate lineages, should ultimately explain the developmental origin of the notochord.

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Figure Legends

Figure 1. The notochord evolved in various steps in chordate lineages.

Presumed evolutionary events are designated on a phylogenetic tree of three chordate lineages, cephalochordates, tunicates, and vertebrates. Black bars indicate the phylogenetic positions when new notochord developmental systems were acquired. Open bars indicate the phylogenetic positions when new cellular components were acquired for the notochord. As mentioned in section 2, convergent extension movement of notochord cells likely originated with the olfactores. Other features are mentioned in section 3.

Figure 2. Notochord-specific expression of cellular components in *Xenopus* tadpoles

(A) Whole-mount *in situ* hybridization images of notochord-specific genes in *Xenopus* tadpoles. All images are taken from Xenbase (Bowes et al., 2010; Karimi et al., 2018). Stage and source: *lamp1*, *p3h1*, *rcn3*, *cav1* – NF stage 29 and 30 Copyright © CNRS UMR 8080, Nicolas Pollet et al., 2005 (Pollet et al., 2005); *slc38a8* and *copz1* – NF stage 28 Copyright © Papalopulu Lab, Nancy Papalopulu; *col9a3* – NF stage 28 Copyright © CNRS UMR 8080, Nicolas Pollet et al., 2005 (Pollet et al., 2005); *loxl1* – NF stage 29 and 30 Copyright © 2005 Image reproduced with permission of the publisher and the copyright holder (Geach & Dale, 2005).

(B) A whole-mount immunostaining image of highly sulfated keratan sulfate (HSKS) in a *Xenopus* tadpole (Yasuoka, unpublished). Notochord and otic vesicles are specifically stained with a monoclonal antibody (5D4).

Figure 3. The turgor pressure-sheath strength model

A schematic of the self-organizing system of notochord formation, in which many cellular activities such as vacuolation, ECM formation, and apoptosis influence one another to maintain notochord structure.

Figure 1

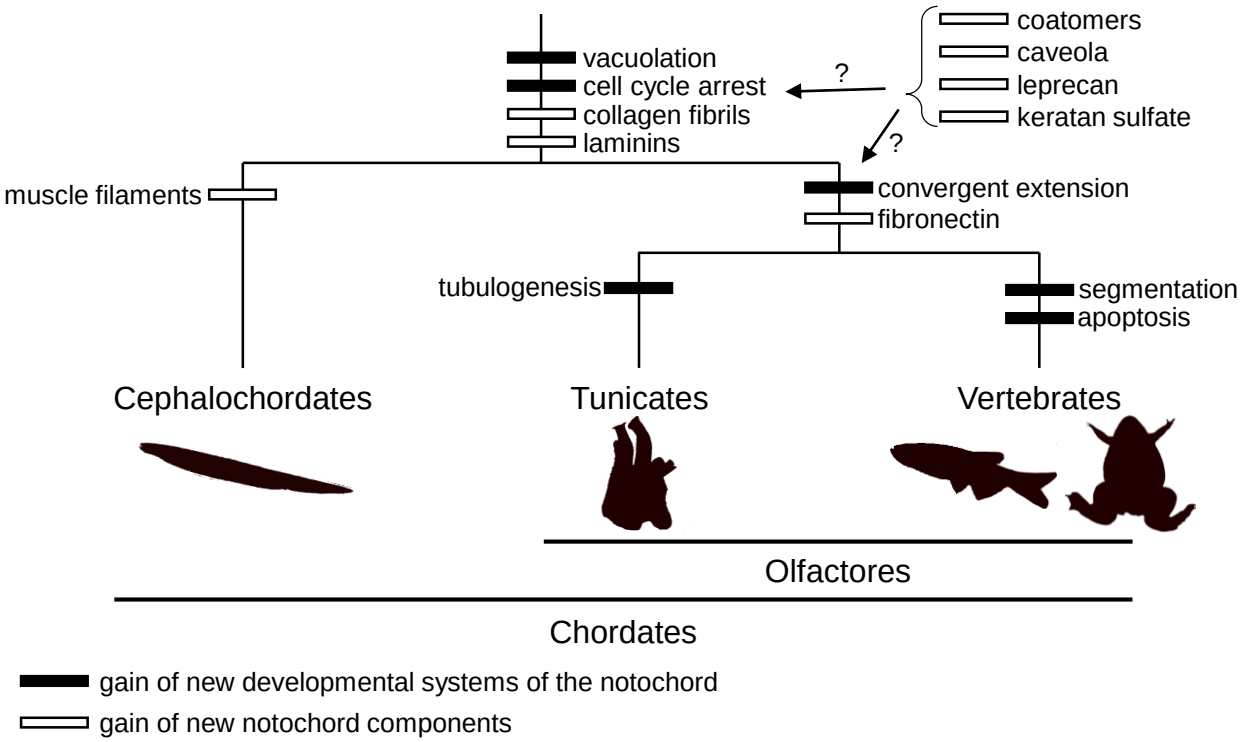
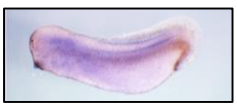
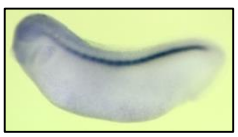

coatomers
caveola
leprecan
keratan sulfate
?
?
vacuolation
cell cycle arrest
collagen fibrils
laminins
muscle filaments
convergent extension
fibronectin
tubulogenesis
segmentation
apoptosis
Cephalochordates
Tunicates
Vertebrates
Olfactores
Chordates
gain of new developmental systems of the notochord
gain of new notochord components

Figure 2

A

gene name	subcellular localization	expression in *Xenopus*
lamp1	vacuole	
slc38a8	cell membrane	
copz1	Golgi	
rcn3	ER	
cav1	cell membrane	
col9a3	ECMs	
p3h1/leprecan	ECMs	
loxl1	cytosol	

B

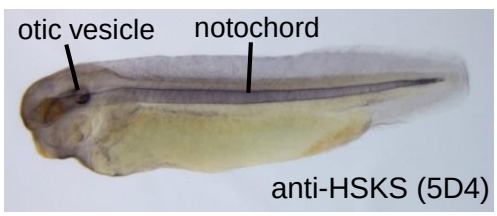
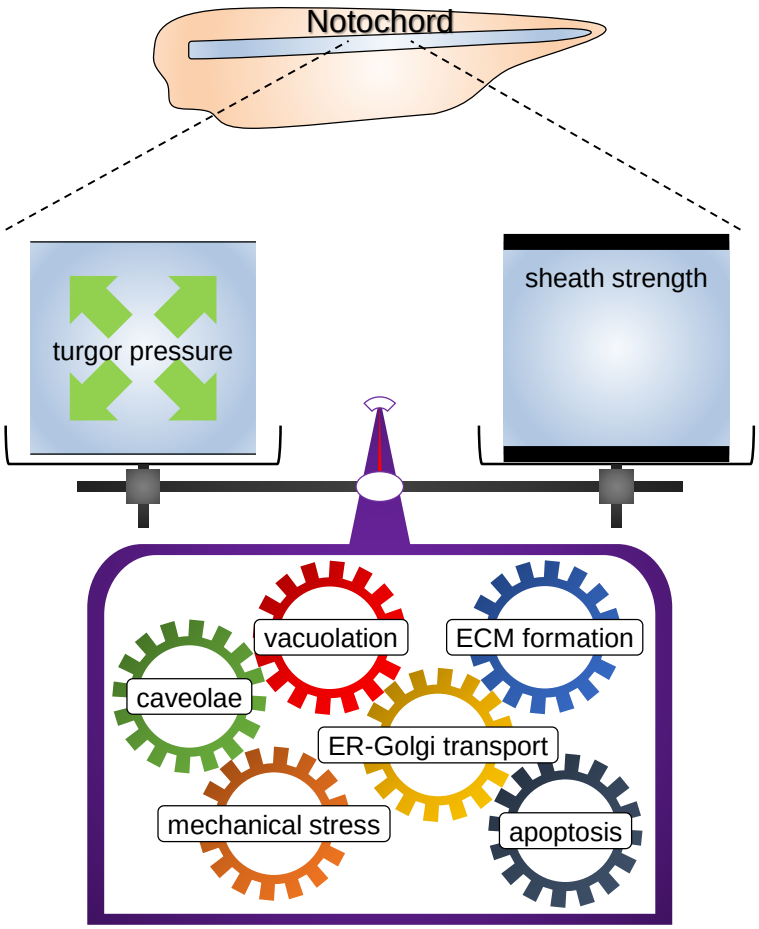


Figure 3


Notochord
turgor pressure
sheath strength
vacuolation
ECM formation
caveolae
ER-Golgi transport
mechanical stress
apoptosis