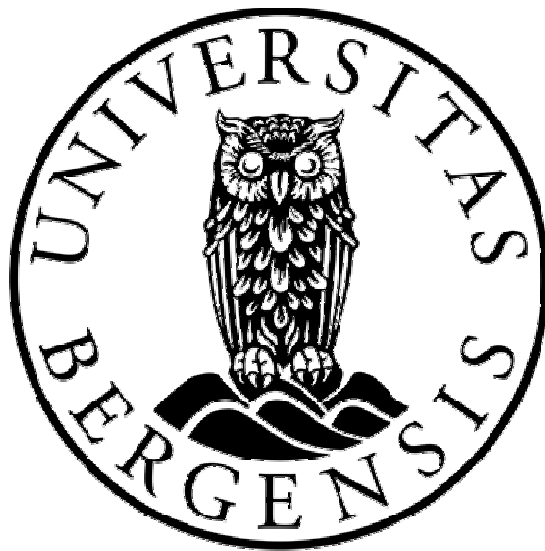


**From notochord to vertebral column: studies
on Atlantic salmon (*Salmo salar* L.)**

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Abstract

The notochord initially form a single-cell file stage of chordoblasts covered by a three layered sheath comprising a basal lamina, a main collagenous layer and an external elastic membrane. During early ontogeny, the collagenous layer of the sheath goes through changes in configuration from a circular orientation, with fibres oriented perpendicular to the long axis, and to a lamellar organization where alternating layers of parallel collagen fibres form helices, with a tangent angle of 75-80° to the long axis. The shift in collagen arrangement is taking place shortly after hatching and may reflect an adaptation of the notochord to functional demands related to stage of development. The serrated surfaces of the elastica externa encasing the notochord seem to provide an interconnection between the notochord and the myosepta, with a function related to transmission of force from the musculature.

The subsequent segmentation process leading to formation of vertebral and intervertebral regions seems to be initiated within the notochord through formation of cellular chordoblast segments. Formation of the cellular segments is closely followed by a segmental pattern of ALP activity within the chordoblast layer and formation of mineralized chordacentra within the collagenous layer of the notochord sheath. Development of the vertebral column thus seem to involve a dual segmentation where notochord derived chordacentra define the vertebral segments and the sclerotome external to the notochord provide further growth to the vertebrae.

The salmon vertebral column comprises notochord and sclerotome derived structures that tightly interconnect the vertebrae and the intervertebral regions. There are four distinct layers of bone and mineralized tissues that form the vertebral body. The two inner layers comprise the chordacentrum (mineralization of the notochord sheath) and the inner layer of the amphicoel (mineralization of the intervertebral ligament). The third layer is deposited by osteoblasts, forming the main portion of the amphicoelous centrum, and on its external surface, trabecular bone is deposited. The three inner layers have a lamellar organization, with collagen matrixes oriented perpendicular from layer to layer, while the fourth layer has a woven structure. The

layers also differ in osteocyte content. The notochord remains within the full length of the vertebral column and form the inner part of the intervertebral ligament. The sclerotomal external portion of the ligament is incorporated in the vertebrae as it is covered by bone during vertebral growth, while remaining unmineralized intervertebrally.

The present studies on salmon shows that the notochord constitutes the main axial support during embryonic and larval stages and that the enforcing rearrangement of the collagen layer of the notochord sheath seems to be important for the properties of the notochord as a fibre-wound hydrostatic skeleton. The notochord further reinforces through formation of chordacentra, generating the segmental pattern of the vertebral column, while sclerotomal tissue external to the notochord provides further growth to the vertebrae and intervertebral structures.

List of publications

Paper I

Grotmol S., Kryvi K., Nordvik K., Totland GK (2003) Notochord segmentation may lay down the pathway for the development of the vertebral bodies in the Atlantic salmon. *Anatomy and Embryology* 207: 263-272.

Paper II

Grotmol S.,* Nordvik K.,* Kryvi H., Totland GK (2005) A segmental pattern of alkaline phosphatase activity within the notochord coincides with the initial formation of the vertebral bodies. *Journal of Anatomy* 206: 427-436.

*S.G. and K.N. contributed equally to this work.

Paper III

Nordvik K., Kryvi H., Totland GK., Grotmol S (2005) The salmon vertebral body develops through mineralization of two preformed tissues that are encompassed by two layers of bone. *Journal of Anatomy* 206: 103-114.

Paper IV

Grotmol S., Kryvi K., Keynes R., Krossøy C., Nordvik K., Totland GK (2006) Stepwise enforcement of the notochord and its intersection with the myoseptum: an evolutionary path leading to development of the vertebra? *Journal of Anatomy* 209: 339-357.

General introduction

The notochord

The notochord is a slender rod of cells encased in a fibrous sheath that extends from the brain to the tail, defining the central axis of the embryo of all chordates. It lies beneath and parallel to the central nervous system, and dorsal to the coelom. The notochord is a derived structure of the chordate phylum which is comprised of the urochordates, the cephalochordates, and the craniates which includes the Hyperotreta (Myxinoidea) and the vertebrates. The latter group comprises the jawless Hyperoartia (Petromyzonidea) and the jawed vertebrates (Gnathostomata) that includes Chondrichthyes, Osteichthyes (Actinopterygi, including Teleostei, and Sarcopterygi, comprising Dipnoi, Latimeria and Tetrapoda; including Amphibia and Amniota).

The unique feature of the ascidian (Urochordata) notochord is that it is comprised of a fixed number of only 40 post-mitotic cells at the beginning of morphogenesis, and as the fate of the different cell lineages is well characterized at the beginning of gastrulation, tracing how the differentiated cells are organized into complex organs like the notochord is a relatively simple matter (Munro & Odell, 2002). In amphioxus (Cephalochordata), the notochord cells contain paramyosin, which through contraction may provide further stiffening of the body, and in these species, the notochord also continues anterior to the brain, which may be an adaptation to burrowing (Flood, 1975; Ruppert & Barnes, 1994). Among the vertebrates, the notochord is the main axial skeleton during the embryonic stage, and remains so into adulthood in the lamprey, some sharks, lungfish, sturgeon, Latimeria and paddlefish (Goodrich, 1930; Arratia et al. 2001), while in the rest of the vertebrates it is replaced to a greater extent by the vertebral column. The main pattern of cellular movements during notochord formation follows a similar pattern in all vertebrate clades. During gastrulation, cells migrate into the developing embryo, starting at a specific site, (known as the embryonic shield in teleosts, the dorsal blastopore lip in amphibians

and Hensen's node in avian and mammalian embryos), forming the mesoderm from which cells start to move into the midline (intercalation), comparable to when two vehicle lanes merge into a single lane. As the cells move into the midline, the tissue narrows as well as elongating dramatically (convergent extension), and in combination with cell division, this process gives rise to the notochord. In vertebrates, the notochord serves as a core around which the mesodermal cells gather to form the vertebrae. Thus the notochord is the forerunner of the vertebral column, both in evolutionary and developmental terms.

Within the vertebrate lineages of lamprey (Pasteels, 1958), chondrichthyes (Boeke, 1908), teleosts (Boeke, 1908 (Salmo), Kimmel et al. 1995 (zebrafish)), and amphibians (Hausen & Riebesell, 1991), the notochord forms a single-cell diameter stack of discoid cells, similar to that of the urochordates and the cephalochordates and in contrast to the amniotes, in which this stage has not been reported (Starck, 1979). From this initial coin stack-like stage, the notochord differentiates into a hydroskeleton with epitheloid morphology, comprising a core of vacuolated chordocytes surrounded by a layer of chordoblasts that are covered by bilaminar fibrous sheath. From this stage on, the fate of the notochord varies substantially among the vertebrates. In lampreys, the notochord is retained as the sole axial skeleton, except for the dorsal cartilages that form external to the notochord sheath. The increasing internal pressure from the vacuoles in the chordocytes on the notochord sheath provides hydrostatic pressure that stiffens the notochord. The fate of the notochord of chondrichthyes varies, and while some species retain an unconstricted notochord, the general pattern within the clade is the development of cartilaginous centra. Within the elasmobranches, paired neural and haemal arch cartilages are formed external to the notochord sheath. These cartilaginous cells may invade the external elastic membrane of the notochord, forming cartilaginous rings that are completely embedded in the fibrous sheath (chordal centra), or which spread both internal and external to the notochord sheath, forming perichordal centra (Goodrich, 1930). The cartilaginous centra may be calcified, and intervertebrally, the notochord sheath forms the intervertebral tissue of the vertebral column.

The amphibian notochord becomes surrounded by vertebrae where the centra are continuous with the neural arches, and with the haemal arches in the caudal region.

The embryonic cartilaginous vertebral elements are reduced and ossification of the vertebrae begins at an early stage of development (Goodrich, 1930). The constricted notochord sheath is not invaded by sclerotomal cells, and in some forms, it remains as a thin continuous structure after the vertebral column is formed, while in others, the notochord is reduced.

Within the amniotes, most of the notochord is replaced by a vertebral column. Amniote vertebrae are characterized by a neural arch and a centrum, and an additional ventral element, the hypocentrum, in the anterior region, and the “chevron bone” posteriorly. The vertebral elements form as cartilage templates which subsequently ossify. In mammals, the only remnant of notochord tissue is in the nucleus pulposus that forms the core of the intervertebral disk (Kardong, 2002). The notochord has a wide range of fates within the actinopterygian lineage. It remains throughout life in some species, while in others it is reduced late in development. In the chondrostei (sturgeon), no vertebral centra are formed, and the notochord, with a thick notochord sheath, remains as an important structural part of the cranio-caudal axis, together with dorsal and ventral arcualian cartilages, which may ossify (Arratia et al. 2001). The notochord of leptolepis species (gars) has a thin sheath, and as the vertebrae are formed, the notochord is constricted and partly reduced.

Initially the notochord consists of a stack-of-coins like cylinder of discoid cells (chordoblasts), which deposit extracellular matrix components that form an acellular sheath. Further differentiation of the chordoblasts leads to the development of central located chordocytes, with large intracellular vacuoles, surrounded by a layer of chordoblasts. The cellular portion of the notochord is covered by a notochord sheath, comprising an inner basal lamina, a thicker collagenous layer, and an external elastic membrane (Fig. 1).

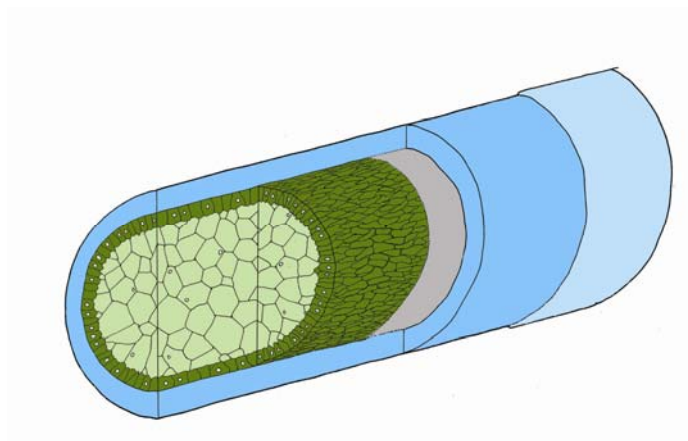


Fig 1: A schematic drawing of the components of the notochord in teleosts. It consists of a cellular part, with an inner core of vacuolated chordocytes (light green), surrounded by a layer of epitheloid chordoblasts (green), and a notochord sheath comprising an inner basal lamina (grey), a thicker collagenous layer (blue), and an external elastic membrane (light blue).

The notochord has unique structural qualities. This elongated cylindrical structure has the flexibility to support the embryo or larva during movement, for example in the undulating locomotion of swimming teleost larvae. Simultaneously, the notochord sheath provides attachment points to the segmental muscles via myosepta, while retaining the necessary stiffness for the axial muscle contractions to promote locomotion by lateral bending, and not simply buckling, of the body. The structural development of the notochord, leading to the formation of this specialized hydroskeleton, is interesting both in a developmental and an evolutionary context.

In addition to its structural role, the notochord during embryogenesis is also an important contributor of secreted factors to surrounding tissues. It secretes factors that pattern both mesodermal and ectodermal structures, and may also play a role in the development of endodermal structures (Cleaver & Krieg, 2001). The role of the notochord in patterning of the somites is known from several studies on chick and mouse, in which secretion of Sonic Hedgehog (SHH) from the notochord appears to be essential both for somite survival during the early somitogenesis and for induction of the sclerotome during later somitogenesis (Johnson et al. 1994; Marti et al. 1995; Chiang et al. 1996; Teillet et al. 1998; for a review, see Monsoro-Burq, 2005). Studies performed on chick, mouse and zebrafish also show that Noggin and SHH

synergistically induce Pax-1 expression in the developing sclerotome (McMahon et al. 1998; Monsoro-Burq, 2005).

The ventral part of the ectodermal neural tube, the floor plate, is located adjacent to the dorsal part of the notochord, and the induction of the floor plate is thought to involve Sonic Hedgehog (SHH) from the notochord, on the basis of studies of chick, Xenopus and zebrafish (Cleaver & Krieg, 2001). Within the endoderm, located immediately ventral to the notochord, induction by the notochord seems to be essential for normal development of the pancreas (chick) and the gut tube (mouse) (Cleaver & Krieg, 2001). The notochord is thus an important contributor of signals to adjacent structures and although studies on chick, mouse, Xenopus and zebrafish show that a large number of growth factors and signalling molecules, including SHH, FGF, Noggin, Chordin, the Sox family and members of the TGF- β superfamily (including TGF- β and BMP), are expressed by the notochord, though the full nature of these signals remains to be unravelled (McMahon et al. 1998; Fürthauer et al. 1999; Shamim et al. 1999; Gilbert, 2000; Cleaver & Krieg, 2001; Fawcett & Klymkowsky, 2004). Some structures, such as the gut tube of rats and the hypochord in Xenopus embryos, are dependent on the correct timing of notochord signals in order to develop normally (Cleaver & Krieg, 2001), and several genes expressed in the notochord display regionalization. These include the Hox genes in zebrafish (Prince et al. 1998), and the growth factor FGF4 in chick embryos (Shamim et al. 1999), adding further complexity to the notochord.

The concept of segmentation

Repeated series of similar structures along a body, or duplicated sections in a structure, represent segments, and the process that divides a body into repeated sections, is called segmentation (Kardong, 2002). Segmentation is common among animals, occurring both in invertebrates, most prominently in annelids and arthropods, and within the vertebrate lineage (Ruppert & Barnes, 1994).

Segmentation in invertebrates such as annelids often involves a division of the epithelium and a replication of a number of structures, such as nerves, muscles, blood-vessels, reproductive and excretory organs, all being present within each

segment, while in vertebrates, the segmental form of organization is mostly present in the trunk and the embryonic hindbrain (Ruppert and Barnes, 1994; Stern, 1990).

In adult vertebrates, segmentation is manifested in structures such as the body musculature, the central and the peripheral nervous system, the branching of the blood vessels and the vertebral column. This segmental organization is achieved during the embryonic stages, and the most obvious segmental structures that appear during these stages are the somites, which arise from the paraxial mesoderm, developing sequentially in a cranial-caudal direction. The somites form in pairs as epitheloid blocks of cells, laterally flanking the neural tube and the notochord. As the somites develop, they become further differentiated. The ventromedial part of the somite forms the sclerotome, and the dorsolateral portion forms the dermamyotome. The sclerotome loses its epitheloid character and becomes mesenchymal, contributing to the formation of vertebral structures. The dermamyotome portion of the somites gives rise to the segmental muscles of the body wall, and to the dermis.

The segmental muscles forming from the dermamyotome in zebrafish are segmentally innervated by motor neurons (Westerfield et al. 1986), and several studies on chicks have indicated a role for the somites in the segmental patterning of the peripheral nervous system (Keynes & Stern, 1984; Kelly Kuan et al. 2004). These studies have shown that the spinal axons grow through, and the neural crest cells (which form the dorsal root ganglia) migrate through, the anterior part of the sclerotomal portion of the somite, so that the segmentation of the spinal axons and their ganglia is related to the metameric pattern of the vertebral column (Kelly Kuan et al. 2004). In zebrafish, the sclerotome makes up a relatively smaller portion of the somite, compared to the avian and mammalian sclerotome, and the relationship between the sclerotomal part of the somites and the peripheral nervous system in zebrafish is less clear (Morin-Kensicki & Eisen, 1997).

The classical theory of the segmental patterning of the vertebral column was presented by Remak (1855; reviewed by Brand-Saberi and Christ, 2000). This theory proposed that the sclerotomes are divided into cranial and caudal halves, where the anterior half of one sclerotome fuses with the posterior half of the adjacent sclerotome, leading to the formation of resegmented compartments, which

subsequently form the vertebrae (Fig. 2). In this way, each muscle segment spans two vertebrae. This implies that each resegmented sclerotomal compartment only contributes to the formation of one vertebra.

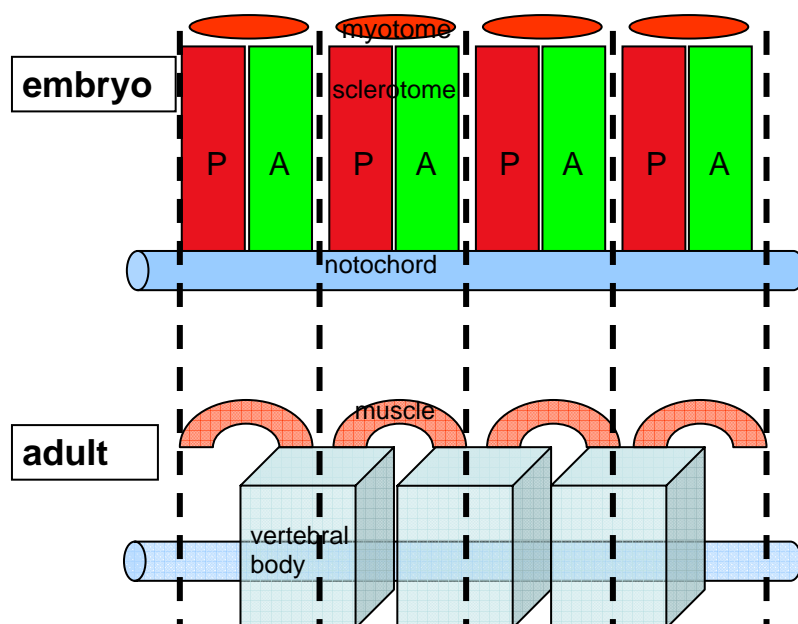


Fig. 2: According to the ‘resegmentation’ model, the vertebral segments are derived from the somites through sclerotome division, where each sclerotome is divided into an anterior and posterior part, forming resegmented compartments from which the vertebrae are formed. Anterior sclerotome-half (A), posterior sclerotome-half (P).

The resegmentation theory has been supported by several cell lineage studies on avian embryos (Aoyama & Asamoto, 2000; Huang et al. 2000; Christ et al. 2000; 2004), while somite lineage studies on chick and zebrafish have challenged this theory, as they have shown that cells derived from single somite halves contribute to vertebral formation in a non-lineage-restricted manner, thus contributing to the formation of more than one vertebra (Stern & Keynes, 1987; Morin-Kensicki et al. 2002). The prospect of an alternative mechanism of vertebral segmentation is intriguing, and the close physical relationship between the notochord and the surrounding sclerotome, as well as the inductive influence of the notochord on a number of adjacent structures, including the sclerotome, make the notochord a highly interesting candidate for a key role in the segmentation process.

Bone formation in the vertebral column

As the teleost vertebrae are formed, the notochord is constricted in that area, but proliferates in the intervertebral region, thus remaining as part of the vertebral column, interconnecting the vertebrae and keeping them separated by filling the amphicoelous concavities at the articulating ends. In the intervertebral regions, the notochord sheath contributes to the inner part of the ligament (Goodrich, 1930; Arratia et al. 2001).

The development of the vertebral column involves the formation of bone and/or cartilagenous structures. Cartilage is composed of a matrix of collagenous fibres and ground substance, as well as cartilaginous cells (chondrocytes). The dorsal and ventral arcualia form as cartilaginous processes, and in the teleost lineage, the cartilage is covered by bone during embryonic development.

Bone is a specialized connective tissue consisting of mineralized extracellular matrix and cells. Bone matrix (osteoid), mainly composed of collagen I, is deposited by osteoblasts and subsequently mineralized. The mineralization of the osteoid involves deposition of hydroxyapatite in close relation to the collagen fibres. It is regulated by the osteoblasts by mechanisms still not completely understood (Marks and Odgren, 2002), although alkaline phosphatase (ALP) appears to be involved in the process (Whyte, 2002). ALP is a known marker of preosteoblasts and osteoblasts in mammals (Whyte, 2002) as well as teleosts (Witten, 1997). The enzyme is present in a number of tissues such as liver, intestines, kidney and brain, and the different ALP isoenzymes are tissue specifically expressed, working primarily on cell surfaces. One of the isoforms is ubiquitous, although especially abundant in hepatic, renal and skeletal tissues; the latter in which ALP is thought to play an important role in mineralization of organic matrixes (for review see Whyte, 1994).

During bone growth, osteoblasts may be trapped in the matrix, forming osteocytes (Franz-Odenaal et al. 2006). The structure and composition of the bone in vertebral bodies have been studied in several teleost species, displaying differences in bone

cell content (Witten, 1997; Witten et al. 2000). Primitive teleost species generally have cellular bone, where osteocytes are embedded in the calcium phosphate matrix; while more advanced teleosts have bone completely lacking osteocytes, thereby only constituting matrix (Moss, 1961). Osteoclasts, the third type of bone cells, are bone-reabsorbing cells, involved in remodelling of both acellular and cellular bone (Sire et al. 1990; Witten and Villwock, 1997). Studies on chick and mouse have shown that they are derived from hemopoietic precursors, and have thus a different origin than the mesenchymal osteoblasts and osteocytes (for review see Suda et al. 1992).

Different types of bone can be defined according to their embryonic developmental pattern. Bone formation arising within a cartilaginous template is defined as endochondral bone. The centre of ossification is inside the cartilage, which is subsequently completely replaced by bone. This form of ossification is present in formation of the amniote vertebrae. Intramembranous ossification involves a direct transformation of mesenchyme into osteoblasts, and the bone is formed without the presence of a cartilaginous template (Karaplis, 2002). Examples of intramembranous bone are dermal bone, which forms directly through ossification of mesenchyme, and sesamoid bones, appearing directly within tendons, derived from connective tissue, possibly as a response to mechanical stress (Kardong, 2002). A third mode of ossification is perichondral and periosteal bone formation, where osteoblasts differentiate in the connective tissue covering the cartilage (perichondrium) or the bone (periosteum), depositing bone directly on the surface of the pre-existing structure (Kardong, 2002), as bone deposition external to the cartilage arcualia.

The teleost vertebral column is composed of amphicoelous (hour-glass shaped) vertebrae and intervertebral ligaments, which consist of both notochord- and somite-derived tissues (Laerm, 1976) and in some actinopterygians, including some teleosts, the vertebral bodies are initiated by the formation of mineralized or calcified rings within the notochord sheath, called chordacentra (Goodrich, 1930; François, 1966). Following the formation of chordacentra, bone is deposited external to the notochord, and the two layers of bone combine to form the vertebral body. In other actinopterygian species the vertebral centra form solely from bone deposited external to the notochord (Arratia et al. 2001). The paired dorsal and ventral arcualian cartilages, which are covered with bone during embryonic stages, are not involved in

formation of the vertebral centra in actinopterygians, but the bone covering them may fuse to the centrum. These different patterns of formation observed in the development of vertebrae within the actinopterygian lineages may provide information on their phylogenetic relationship, and a detailed description of the structural components comprising the salmon vertebrae would contribute to this knowledge.

The presence of chordacentra in several actinopterygians, such as the Atlantic salmon (François, 1966) displays the development of segmental structures within the notochord. The mechanisms by which the chordacentra develop within the notochord sheath are unclear, and the possibility of these structures being derived from the notochord itself, is fascinating and is what stimulated the studies described here.

Aims of the study

Both evolutionary and developmentally, the notochord is a precursor to the vertebral column and the developmental fate of the notochord varies within different vertebrate lineages. The present study aims to elucidate the morphogenesis in the development from notochord to vertebral column in the Atlantic salmon. The study will comprise the structural development of the notochord during early ontogeny, both before and during the initial formation of the vertebral bodies. Furthermore, the role of the notochord in the segmentation process will be investigated and possible osteoblast like properties in the chordoblast layer of the notochord will be studied. A further objective is to provide a detailed morphological description of the structural components involved in the formation and growth of the vertebral and intervertebral regions.

General discussion

Why study the salmon notochord?

The Atlantic salmon was chosen for the study, as it has certain advantages over smaller, more rapidly developing teleosts such as the zebrafish. The salmon notochord is large and the chordoblasts are prominent. In comparison, the notochord of the zebrafish has a similar general structure except for the chordoblast layer, which consists of thin squamous cells that are difficult to identify using light microscopy methods. In addition, early-stage development in salmon is slow, facilitating detailed examination of developmental events that in other species may be closely related in time.

The chordoblasts – a weaver of collagen

The notochord functions as an axial skeleton for the developing embryo and produces signals that pattern surrounding tissues. Initially, the salmon notochord comprises a single-cell diameter column of discoid chordoblasts surrounded by a sheath consisting of a basal lamina, a collagenous layer and an external elastic membrane (Paper IV). In different species, it has been shown that the collagenous layer is made up of type II collagen (Linsenmayer et al. 1973; Sandell 1994; Domowicz et al. 1995; Gotz et al. 1995; Ng et al. 1997; Hayes et al. 2001; Paper IV). Initially, in salmon, the collagen fibrils have a circular orientation (perpendicular to the body axis) (Paper IV), and this arrangement probably reflects the morphology of the discoid chordoblasts, which are ideally suited for depositing circumferential collagen fibrils. This pattern of fibril winding probably provides higher flexibility than alternative configurations, thus allowing tight curling of the embryo inside the egg. Following the single-cell diameter column stage, two cell types differentiate, a process that results in the formation of a core of vacuolated cells surrounded by a monolayer of non-vacuolated cells (Paper IV). In the present study, we have denoted the vacuolated notochord cells as chordocytes and the non-vacuolated cells as chordoblasts,

reflecting their respective features. The chordocytes are differentiated from chordoblasts, with the functional role of maintaining internal hydrostatic pressure, while the chordoblasts secrete the matrix of the sheath, and divide to sustain notochord growth.

The chordoblast layer passes through several changes in morphology, both before and during formation of the vertebral column (Papers I, IV). The salmon notochord displays changes in the organization of the collagenous sheath, from a circumferential orientation and to a lamellar structure in which the helical geometry changes between layers (Paper IV). The organization with alternating lamellar layers probably reflects a gradual enforcement of the notochord sheath, to prepare for increased activity after hatching. When the lamellae are formed, the chordoblasts have a spindle-shaped morphology and a specific axial orientation, and formation of the lamellar structure may be linked to the successive shifts in cell architecture within the chordoblast layer. Thus, the deposition of collagen may be cyclic, in a process whereby a layer of fibrils is deposited in one direction before there is a change in chordoblast orientation and a new layer of fibrils is deposited, alternating the fibril winding pattern.

The present study shows that the salmon notochord is a highly dynamic organ, continuously developing its structural morphology from the time of formation until the vertebrae are fully formed. Current understanding of the functional properties of the notochord is based on a different interpretation of the structure of the sheath (Adams et al. 1990; Koehl et al. 2000), and the changes in the fibril winding pattern have not been taken into account. How this structural complexity of the notochord relates to the mechanical properties and their underlying physics is intriguing and needs further elucidation. Such future studies may modify current understanding of the contribution of the notochord to embryonic elongation and straightening, in addition to its function during swimming (for further discussion see Paper IV). Many questions thus remain to be answered; in amniote embryos, for instance, a single-cell file stage has not been observed (Gotz et al. 1995; Zavala & Vázquez-Nin 2000), and the initial orientation of the collagen of the sheath has not been thoroughly elucidated. In contrast to species that hatch in water, land-living vertebrates need skeletons with the ability to bear weight at birth or hatching. Hence, vertebral development occurs

during embryogenesis, and in this process partial embryonic straightening may be a prerequisite. Lack of discoid chordoblasts in these species may possibly reflect a different mode of notochord sheath formation with a different configuration of fibril winding that promotes notochord straightening to a greater degree.

What is the origin of the segmental pattern of the salmon vertebral column?

Our studies of the notochord during initial development of the vertebrae, presented in Papers I and II, indicate that the segmental pattern of the column is derived from the notochord, a mechanism of patterning that has not been described previously.

Our view is thus inconsistent with the resegmentation hypothesis that states that the vertebral column is mainly a somite-derived structure, both in patterning and cellular origin (Papers I, II). As predicted by this hypothesis, in amniote vertebrate embryos the region of the somite that forms vertebrae, the sclerotome, has been shown to be polarized into anterior and posterior halves, and this polarity is critical in positioning the early elements of the segmented peripheral nervous system correctly in relation to the developing vertebral column (Keynes & Stern, 1984, 1988; Kelly Kuan et al., 2004). Somite cell lineage studies, based on the construction of chick-quail chimaeras by surgical grafting of half-somites from quail to chick embryos, are also broadly consistent with resegmentation in confirming that vertebral bodies arise from cells of adjacent somites (eg. Goldstein & Kalcheim, 1992; Huang et al., 2000).

However, cell lineage studies have not provided conclusive results confirming the resegmentation model, which thus remains controversial (Verbout, 1976; Fleming et al., 2004; Papers I, III), and two lines of evidence in particular have led us to question it. First, somite cell lineage studies in both the chick embryo (Stern & Keynes, 1987) and zebrafish embryo (Morin-Kensicki et al., 2002) have shown that cells derived from one half-sclerotome can contribute daughter cells to two consecutive vertebrae, a result that is not consistent with resegmentation according to strict lineage-restriction criteria. An alternative possibility is that the notochord, the midline structure around which vertebral bodies develop, and which is known to contribute the central portion of the intervertebral disc (nucleus pulposus), plays a key role in vertebral patterning.

In the present study we made a detailed morphological study of the development of the notochord and vertebral bodies in the Atlantic salmon (Papers I, II, III), and showed that in this species vertebral body formation is preceded by a change in the axial orientation of segmentally-repeated groups of notochord cells (chordoblasts), and a segmental expression of alkaline phosphatase (ALP) by these cells. Coincident with this differentiation of the notochord into segments, the initial components of the vertebral bodies, the chordacentra, form as segmented calcified rings within the acellular notochord sheath, a pattern that suggests that they are generated by the notochord. At this early stage of vertebral development no osteoblasts are present around the notochord. Rather, the segmental expression of ALP by the notochord suggests that notochord cells themselves develop osteoblast-like properties, and that these cells govern the mineralization of the chordacentra, thus nucleating the formation of vertebrae. ALP is a known marker of preosteoblasts and osteoblasts (Whyte, 2002) and in sea bream, an increased ALP activity in osteoblasts is observed in relation to the mineralization of the extracellular matrix (Pombinho et al. 2004). The chordoblasts may thus be involved in both the formation of the notochord sheath and in its subsequent mineralization. Together, these findings lend support to the concept that the notochord plays a major role in initiating the development of the vertebral column through segmental differentiation of the notochord sheath into vertebral bodies and intervertebral regions, a process in which the somites do not participate.

Consistent with our observations in the salmon, it has been shown that vertebral bodies in another teleost, the zebrafish, arise as segmented peri-notochordal rings via secretion of so-called bone matrix from the notochord rather than the somites (Fleming et al., 2004). The early zebrafish vertebral bodies do not form via an intermediate cartilaginous stage, nor do they contain osteoblasts. Critically, their formation can be prevented by laser ablation of single notochord cells at defined, segmentally reiterated positions *in vivo*, strongly suggesting that the notochord determines vertebral segmental patterning. The analysis of *fused somites* mutant zebrafish embryos, in which sclerotome polarization is disrupted, has also been informative; while segmentation of the sclerotome-derived vertebral neural arches is abnormal in the mutant form, vertebral body segmentation proceeds normally. This

shows that segmental patterning of the vertebral arches can be dissociated genetically from that of the vertebral bodies, and supports the possibility that vertebral body patterning is determined independently of the somites (van Eeden et al., 1996; Fleming et al., 2004).

Our findings on vertebral patterning in the salmon model raise the intriguing possibility that the notochord plays a key, perhaps ancient, role in the segmental patterning of the vertebral column. Contrary to the widely-held view of the notochord as an unsegmented structure, they imply that the teleost notochord is the expression of an occult genetic programme, coordinated with somite development that drives vertebral body segmentation. It is possible, however, that notochord/vertebral body segmentation has arisen independently in actinopterygian (ray-finned) fishes, including teleosts, and does not apply to vertebrates of the sarcopterygian (lobe-finned) lineage within which tetrapods have evolved. There are experimental findings reported in the literature that are nonetheless consistent with the possibility of notochord segmentation in tetrapod embryos. In particular, surgical removal of a several-segment length of the trunk notochord in early amphibian embryos (Kitchin, 1949) and chick embryos (Strudel, 1955) has been shown to generate an unsegmented mass of hyaline cartilage in the body midline, ventral to the spinal cord, a site that would normally be occupied by segmented vertebrae and intervertebral discs. We have confirmed Strudel's observation in the chick embryo. We therefore believe that further detailed investigation of notochord segmentation in amniote vertebrates is crucial. It should also be noted that the descriptive details of vertebral development in avian and mammalian embryos are essentially similar, so the underlying molecular genetic mechanisms of vertebral patterning are likely to be fundamentally equivalent in chick, mouse and, by extension, human development.

Development of the vertebrae

In salmon, the vertebral body comprises four mineralized or ossified layers. Formation of the different layers seems to involve several patterns of mineralization and bone deposition, forming a vertebral body where matrix structure and cellularity

differ among the individual layers (Paper III). The chordacentrum forms the initial layer by a mineralization of type II collagen in the notochord sheath.

In zebrafish, secretion of bone matrix from notochord cells during the formation of vertebral bodies has been described by Fleming et al. (2004), and chordoblasts that express ALP activity have been observed in salmon prior to chordacentrum formation (Paper II). Interestingly, a recent study of zebrafish has demonstrated the presence of Bone Gla Protein (Bgp) in developing vertebrae (Gavaia et al. 2006), parallel in time with the formation of chordacentra within the notochord sheath itself (Fleming et al. 2004). This indicates that Bgp is present in the mineralized chordacentra in zebrafish. Bgp is a bone-specific Ca^{2+} -binding protein, found in bone from bony fish to mammals (Pinto et al. 2001). The precise role of Bgp in bone mineralization, especially in non-mammalian vertebrates, is a matter of debate. In mammals it seems to interact directly with bone hydroxyapatite, and it is regarded as a specific osteoblast marker as it is secreted by osteoblasts (Hauschka et al. 1989). Whether Bgp is expressed alongside ALP during chordacentrum development in salmon remains to be elucidated.

The main part of the amphicoel and the trabecular bone are the two layers of the vertebral body that are formed by direct deposition of bone by osteoblasts (Paper III). The macroscopic difference and difference in cellularity between the amphicoel and the trabecular bone may indicate that the bony matter of the two structures is deposited by two different cell populations. This notion is supported by cell-culture studies on sea bream, in which the presence of two bone-derived cell lines derived from the vertebrae has been described (Pombinho et al. 2004). In salmon, the osteocyte number is ten times as high in the arcocentrum as in the autocentrum (Paper III), indicating that the arcocentrum-forming osteoblasts are more likely to differentiate into osteocytes. Moreover, the osteoblasts involved in autocentrum formation are circularly aligned around the notochord, depositing collagen fibres perpendicular to the anteroposterior axis, while the osteoblasts associated with the arcocentrum deposit osteoid with a spongy structure (Paper III).

The salmon vertebral body displays an elegant form of development, with an open structure that allows the vertebral body to grow without the need for remodelling. This

growth pattern may explain why no osteoclasts could be observed during the formation of the vertebral body of a cichlid (Witten & Villwock, 1997).

Main conclusion

A major result of the present study indicate that the salmon notochord itself develops into a functional vertebral column through stepwise enforcement of its sheath; the chordacentra representing primitive vertebra and the non-mineralized sheath the intervertebral ligament. In an evolutionary context, the appearance of the chordacentrum may represent the pinnacle of notochord development as the sole axial skeleton, and our interpretation is that it has formed a structural core and a segmental pattern, around which the more advanced vertebral structures have evolved.

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