

Notochord development in Atlantic salmon (*Salmo salar* L.): exploring molecular pathways and putative mechanism of segmentation

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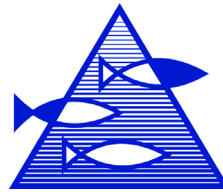
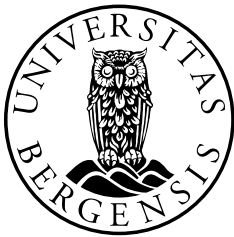
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SCIENTIFIC ENVIRONMENT

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ABSTRACT

Background

A key feature of the vertebrate body plan is the repeated compartments made up of individual vertebra, blood vessels, peripheral nerves and muscle. The vertebral column comprises a series of bony vertebral bodies with arches and intervertebral discs and joints. However, the biological mechanisms that generate this segmental pattern during embryogenesis are not fully understood. Unlike amniotes, teleosts display segmented mineralization in the notochord sheath, as the initial morphogenic step in the formation of the vertebral column. It has been hypothesized that the notochord initiates its early segmentation, and thus patterns the vertebral column in teleosts. In order to determine whether the notochord is functionally segmented, this project studied all the genes expressed in the notochord prior to and during the early stages of segmentation. Studies of this type may provide further clues to the patterning of notochord segmentation in fish and possible other vertebrates.

Methods

A micro-dissection protocol was developed to isolate the pure cellular core of the notochord, and the rest of tissues from Atlantic salmon larvae for total RNA extraction. Two DNA-sequencing technologies, EST sequencing and RNA-seq, were employed to identify the notochord-specific transcriptome in salmon. Quantitative gene expression analysis (Q-PCR) was performed on genes of interest, and spatial gene expression in the notochord was investigated by means of *in situ* hybridization. Meanwhile, TEM was used for detailed characterization of the pattern of mineralization in the collagen matrix in the notochord sheath, as well as on the outside. Synchrotron radiation based X-ray diffraction was performed in order to analyze the identity of the mineralized elements in the notochord sheath.

Results

Mineralized ring-structures of the vertebral centra were first observed in the type II collagen matrix of the notochord sheath, and slightly later in the type I collagen matrix

with sclerotomal origin. The nucleation and growth pattern of hydroxyapatite crystals were similar in both type I and type II collagen matrices. The crystals always grew as thin flakes along the long axis of the collagen fibrils, while the production of major collagen transcripts significantly decreased over time. RNA-seq of notochord samples before and during initial segmentation of notochord confirmed the down-regulation of genes involved in collagen fibrillogenesis and many other pathways during early segmentation of the notochord. At this developmental stage, *coll1a2* was expressed in a segmental pattern in one chordoblast population adjacent to the non-mineralized zone in the notochord sheath corresponding to the intervertebral region. Two genes (*vimentin*- and *elastin*-like transcripts) were found to be uniquely expressed in the notochord during early mineralization. *In-situ* results showed that the *vimentin*-like gene was expressed in the vacuolated chordocytes and in the chordoblasts lining the notochord sheath. The expression of the *elastin*-like gene was only found in the chordoblasts. A colinear expression of 71 *Hox* genes along the anterior-posterior axis of the notochord was also observed. Typical chondrogenic genes, but no osteogenic genes, were expressed in the salmon notochord, indicating a close relationship between the notochord and cartilage.

Conclusions

A few structural genes were found to be exclusively expressed in the notochord. A dramatic change of gene regulation in whole-genome scale occurs at the onset of notochord segmentation. Meanwhile, segmental expression of *coll1a2* was found in chordoblasts lining prospective non-mineralized regions of the notochord. Moreover, the initial mineralization of the vertebral body was preceded by hydroxyapatite accreting in a segmental pattern in the notochord sheath in salmon. There was both morphological and molecular evidence of a segmentation process in the developing notochord, which contributes to the segmental patterning and initial development of the vertebra and intervertebral region in Atlantic salmon.

Keywords: notochord, *coll1a2*, RNA-seq, segmentation, Atlantic salmon

ABBREVIATIONS

<i>ALP</i>	alkaline phosphatase
AP	anterior-posterior
<i>col24a1</i>	collagen alpha-1(XXIV) chain
<i>crtap</i>	cartilage-associated protein
DEG	differentially expressed gene
ECM	extracellular matrix
<i>fmod</i>	fibromodulin
KEGG	Kyoto Encyclopedia of Genes and Genomes
<i>lepre1</i>	prolyl 3-hydroxylase 1
NGS	next-generation sequencing
<i>ngs</i>	notochord granular surface
NP	nucleus pulposus
qPCR	quantitative polymerase chain reaction
QTL	quantitative trait loci
RNA-seq	RNA sequencing (massive parallel transcriptome sequencing)
RPKM	reads per kilobase per million reads
<i>Runx2</i>	Runt-related transcription factor 2
SIBLINGS	small integrin-binding ligand, N-linked glycoprotein
SNP	single nucleotide polymorphism
UTR	untranslated region

§1. INTRODUCTION

§1.1 Origin and phylogeny of the notochord

The notochord is a midline rod-like structure that appears during embryogenesis in all chordates. It is regarded as a synapomorphy of the *Chordata* phylum that comprises three subphyla: *Cephalochordata* (amphioxuses or lancelets), *Urochordata* (tunicates), and *Vertebrata* (Heimberg et al., 2010; discussion in Janvier 2010; Figure 1). In 1866, Alexander Kowalevsky bridged the link between vertebrates and invertebrates by discovering the presence of the notochord in tunicates and lancelets (reviewed by Satoh et al., 2012). In addition to possessing a notochord for at least part of their life cycle, all chordates are characterized by a dorsal hollow neural tube, pharyngeal slits, an endostyle or thyroid gland, and a postanal tail (e.g. Kardong, 2006). Recent molecular studies have indicated that urochordates and vertebrates are the closest living relatives to, and share a common ancestor with, cephalochordates (Delsuc et al., 2006; Satoh et al., 2012; Figure 1). It is likely that the success of chordates is partly due to notochord function: it supports the undulatory movement of the tail, which facilitates a unique mode of locomotion through water. Flood (1969; 1970) observed innervation and muscular properties in the amphioxus (*Branchiostoma lanceolatum*) notochord and also demonstrated that the notochord became stiffer when electrically stimulated due to muscle-like contractions inside. These contractile properties coincide with the expression of muscle-related genes in the amphioxus (*Branchiostoma belcheri*) notochord (Suzuki and Satoh, 2000; Urano et al., 2003). Therefore, the amphioxus notochord is regarded as a novel type of notochord, evolved within the cephalochordate lineage, and is therefore not referred to in the following discussion in the thesis.

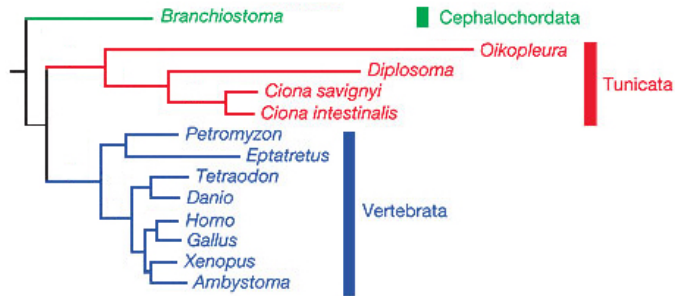


Figure 1: A phylogenetic tree of chordate evolution; modified from Delsuc et al., 2006.

The origin of vertebrates is largely defined by the evolution of the skeleton. The skeleton of jawed vertebrates is comprised of bone and cartilage, which contain mainly fibrillar type I collagen (col1) and type II collagen (col2) respectively in their extracellular matrix. Notochord and cartilage possess some properties in common, including expression of type II (*col2a1*) and type XI (*col11a1/2*) collagen (Baas et al., 2009; Boot-Handford and Tuckwell, 2003; Wada et al., 2006; Wargelius et al., 2010; Witten et al., 2010). Functionally, the notochord serves as the axial skeleton of the embryos of all chordates and is retained throughout the ontogenies of lancelets, hagfishes and some primitive actinopterygians and sarcopterygians. A vertebral column consisting of a persistent notochord and attached ossified portions of vertebrae is the primitive characteristic of the *Gnathostomata* (Arratia et al., 2001; Janvier, 2002). In teleosts, the notochord persists and fills the amphicoelous concavities in the intervertebral joints at the articular ends (Grotmol et al., 2006). During the development of mammals, the notochord is transformed, and is present only in the nucleus pulposus (NP), restricted by the outer annulus fibrosus (AF) in the intervertebral disc (Bruggeman et al., 2012; Choi et al., 2008; Pattappa et al., 2012; Risbud et al., 2010; Smits and Lefebvre, 2003).

§1.2 Early development of the teleost notochord

The notochord originates from the dorsal organizer, a region that can induce a secondary body axis, originally identified from Spemann and Mangold's transplant

experiment in amphibians (Spemann and Mangold, 1924). During early gastrulation in teleosts, convergence and extension movements shape a part of the mesodermal tissue into an elongated midline cluster of cells that defines the primary axis of the embryo (Keller, 2002; Glickman et al., 2003; Stemple, 2005). Nodal signaling pathway is involved in the specification of the fate of the dorsal mesendoderm and induction of the mesoderm (Stemple, 2005), including a notochord fate determination along the anterior-posterior axis by expressing *gooseoid/gsc* and *floating head/flh* in prospective cells (Gritsman et al., 2000). Through further cell division and intercalation, the notochord forms as a single-cell file of disc-shaped cells – “stack of pennies” (Grotmol et al., 2006; Kimmel et al., 1995; Tong et al., 2013; Yan et al., 1995). Genetic screening of zebrafish (*Danio rerio*) mutants has identified several genes that are involved in the specification, including *bozozok/boz* and *flh*, and differentiation of the notochord, such as *no tail/ntl* and genes encoding laminins and coatomers (Amacher and Kimmel, 1998; Coutinho et al., 2004; Fekany et al., 1999; Parsons et al., 2002; Stemple et al., 1996). The notochord plays two main roles during early embryonic development. First, it serves as the midline-signaling center to the adjacent tissues. Second, it provides biomechanical support as the main embryonic axial skeleton.

§1.2.1 Notochord signals to adjacent tissues

The nature of the notochord as a signaling center is inherited from the dorsal organizer. During early embryonic development, the notochord signals to the patterning of surrounding tissues such as the central nervous system (Placzek et al., 1991; Yamada et al., 1991), the cardiovascular system (Fouquet et al., 1997), the somites (Pourquié et al., 1993), the digestive system (Kim et al., 1997), along both the dorsal-ventral (DV) axis (Cleaver and Krieg, 2001) and along the anterior-posterior (AP) axis (Pollard et al., 2006). Among the signaling molecules secreted by the notochord to induce cell differentiation and fate are the hedgehog proteins, which act in a dose-dependent manner (Ingham and Kim, 2005; Ingham and McMahon, 2001; Krauss et al., 1993). Notochord-derived Sonic hedgehog (Shh) induces the initial specification of the floor

plate in the ventral part of spinal cord, and dynamically regulates the differentiation of several neuronal progenitors (Ribes et al., 2010; Yamada et al., 1991). Shh and another hedgehog protein, Echinidna hedgehog (Ehh)/Indian hedgehog homolog b (Ihhb), act sequentially to pattern the developing somites and differentiation of slow muscle cells, mediated through the transcription factor Gli2 (Currie and Ingham, 1996; Du and Dienhart, 2001). Shh is also known to induce the differentiation of sclerotome into precursor cells of skeleton (Fan and Tessier-Lavigne, 1994).

§1.2.2 Cell differentiation determines the biomechanical properties of the embryonic notochord

In teleost embryos, inflation of the notochord combined with the restriction of expansion imposed by the sheath, builds turgidity that contributes to the biomechanics of the notochord. When it initially forms in the early embryo, the notochord possesses a cellular core that initiates the secretion of an extracellular matrix (ECM) to form the surrounding notochord sheath. The sheath consists of three distinct layers: the inner basal lamina, the mid-collagenous layer and the outer elastin membrane, the *elastica externa* (Grotmol et al., 2006; Nordvik et al., 2005; Stemple, 2005). Genetic screens of mutants and knockdown studies in zebrafish have identified genes that encode laminin $\alpha 1$, $\beta 1$, γ , and collagen XI $\alpha 1$ chain, which are essential for notochord sheath development (Baas et al., 2009; Parsons et al., 2002).

Prior to the onset of the morphogenesis of the vertebral column (around 170 d° in Atlantic salmon (*Salmo salar*)*), a process of cell differentiation with formation of large intracellular vacuoles proceeds in anterior to posterior sequence. This alters the morphology of the notochord from the “stack of pennies” state to a bi-layered epithelioid tissue (Grotmol et al., 2006). Here, two morphologically distinct cell types differentiate: 1) the vacuolated inner notochordal cells, the chordocytes; 2) the outer

* Developmental stages of salmon embryos are classified by day degrees (d°), which are defined as the sum of daily mean ambient water temperatures (°C) for each day of development (Gorodilov, 1996).

non-vacuolated epithelium-like cells, with abundant rough endoplasmic reticulum, that line the interior of notochord sheath, the chordoblasts (Grotmol et al., 2003; Yamamoto et al., 2010; Dale & Topczewski, 2011; Figure 2, 3A). Chordoblasts are responsible for secreting the ECM of the sheath (Yamamoto et al., 2010).

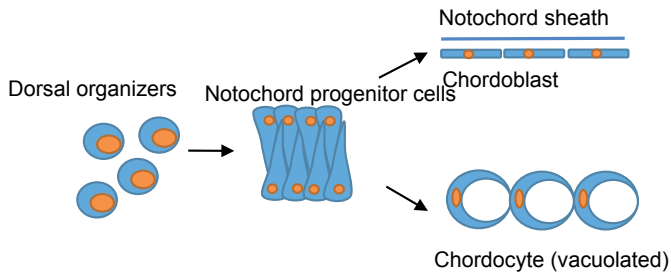


Figure 2: Notochord cell lineages in teleosts.

It has been suggested that the chordoblasts differentiate into chordocytes (Grotmol et al., 2003; Tong et al., 2013). Experimental studies in zebrafish, however, have shown that the two cell types differentiate from a single notochord precursor cell lineage (Figure 2). Here, the Jag1-mediated Notch signaling pathway determines whether cells will develop into chordoblasts or chordocytes (Figure 2; Yamamoto et al., 2010). Studies of notochord-expressed genes in zebrafish have revealed that genes encoding laminin and coatmer vesicular coat complex are important for formation of the prominent vacuole in chordocytes (Coutinho et al., 2004; Parsons et al., 2002). Furthermore, the endosomal trafficking pathway regulated by *rab32a* is linked to this process (Ellis et al., 2013). It is likely that inflation of the notochordal vacuoles is required for the elongation of the body axis during early embryonic development (Adams et al., 1990; Ellis et al., 2013; Koehl et al., 2000). In salmon, the longitudinal growth rate does not increase in the period during which the notochord inflates (discussion in Grotmol et al., 2006). Nevertheless, it is agreed that the notochord does not straighten the body, but remains highly flexible when inflated, as the embryos are kept in the spherical eggs during the inflation process (Ellis et al., 2013; Grotmol et al., 2006). The presence of vacuolated notochord-like cells in the nucleus pulposus of the intervertebral disc of amniotes (Chen et al., 2006; Hunter et al., 2004), and the

presence of chordocytes in the intervertebral joints of teleosts, suggest that the vacuolated cells may play an important role as a pre-adaptation for the development of distinct anatomical forms of intervertebral joints in different vertebrate lineages.

§1.3 Initial development of the vertebral column in teleosts

Detailed morphological studies of both Atlantic salmon and zebrafish have implied that the notochord plays a key role in vertebral column segmentation and formation in teleosts (Bensimon-Brito et al., 2012; Du et al., 2001; Fleming et al., 2004; Grotmol et al., 2003; Grotmol et al., 2005; Grotmol et al., 2006; Nordvik et al., 2005). Briefly, in Atlantic salmon, the morphogenic sequence of vertebral development is initiated during a critical window from 500 d° to 750 d° (Grotmol et al., 2006; Figure 3B). After hatching, at around 600 d°, segmental reiterated morphologically distinct bands of chordoblasts appear along the notochord axis, every second band pre-defining a zone in which mineralization within the notochord sheath will take place (Grotmol et al., 2003; Grotmol et al., 2005). The mineralized element, or chordacentrum, is the initial structure of the vertebral body and is formed within the notochord sheath (Bensimon-Brito et al., 2012; Grotmol et al., 2005; Inohaya et al., 2007; Willems et al., 2012). This type of chordacentrum is only found in actinopterygians and is not homologous to the chordacentrum in chondrichthyans, which is developed by chondrification of mesenchymal cells that invade the notochord sheath (Arratia et al., 2001). However, there is still a debate as to whether the chordoblast or differentiated sclerotomal cells induce the mineralization of the notochord sheath. In salmon, chordoblasts at the mineralization site express alkaline phosphatase (ALP), an enzyme regarded as essential in the mineralization of organic matrices, before it can be detected in sclerotomal cells located outside the chordacentrum (Grotmol et al., 2005). This observation suggests that a sub-population of chordoblasts differentiate to develop osteoblast-like properties that enable mineralization of the sheath at their locations. However, in medaka (*Oryzias latipes*), ALP activity was only detected in the sclerotomal cells outside the chordacentrum (Inohaya et al., 2007).

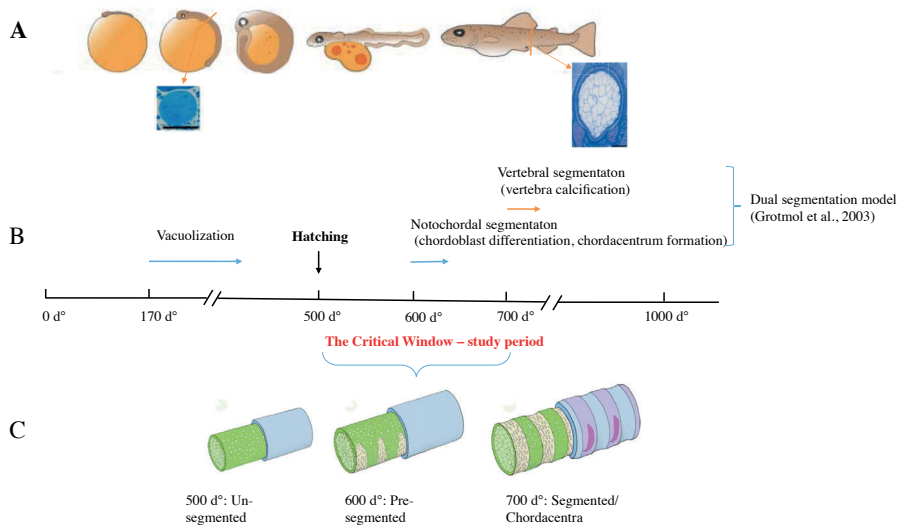


Figure 3: Notochord development in Atlantic salmon embryo and larva: (A) Ontogeny of Atlantic salmon from fertilization to yolk-sac larva; transverse sections of notochord are shown; (B) Notochord development and vertebral segmentation along developmental stages by day degrees (d°); (C) After hatching, metameric bands of chordoblasts change axis and induce a shift in the collagen-winding architecture (Grotmol et al., 2003; Grotmol et al., 2006); The segmentation proceeds until around 750 d°, when mineralized ring structures (chordocentra) form in the notochord sheath in a segmental pattern (Adopted from Paper I).

Clusters of sclerotomal cells differentiate successively on the surface of the chordacentrum, and deposit peri-notochordal bone (perichordal centrum/autocentrum) through direct ossification, using the notochord as a scaffold (Fleming et al., 2004; Grotmol et al., 2003; Inohaya et al., 2007; Nordvik et al., 2005; Wargelius et al., 2005; Figure 4A, B). The formation of vertebral centra through direct ossification, without a cartilage intermediate in teleost, is different from that of higher vertebrates (Arratia et al., 2001; de Azevedo et al., 2012; Inohaya et al., 2007). Here, at least three populations of sclerotomal cells are evident outside the notochord in medaka, zebrafish and salmon during early peri-notochordal ossification. One type of cells (Class I), scattered outside the chordacentrum and presumably the same cells express ALP and *col10a1*, but not *osterix* and *osteocalcin* (Inohaya et al., 2007; Renn et al., 2013; Spoorendonk et al., 2008). These cells are not mature osteoblasts, but they may contribute to the centrum mineralization since they show ALP activity (Grotmol et al., 2005; Inohaya et al., 2007). Majority of the sclerotomal cells (Class II) are clustered in

the intervertebral region and differentiate into fibroblasts (Inohaya et al., 2007; Nordvik et al., 2005; Figure 4B). These cells do not have osteoblast properties and not involved in mineralization process, but they are responsible for excretion of ECM outside the notochord sheath. A population of mature osteoblasts (Class III) expressing *osterix* were observed at the anterior and posterior rims of the autocentrum (Inohaya et al., 2007; Spoorendonk et al., 2008). These cells have been indicated to have sclerotome origin and suggested to be differentiated from the nearby Class II cells in the intervertebral region (Inohaya et al., 2007). Accordingly, these *osterix*-positive osteoblasts secrete the bony matrix in the future head skeleton, vertebral arches and anterior and posterior rims of vertebral centra. However, they only appear after the notochord sheath has been mineralized, and are therefore not involved in the chordacentrum formation, although they are required for autocentrum formation in transgenic medaka (Renn and Winkler, 2009; Willems et al., 2012).

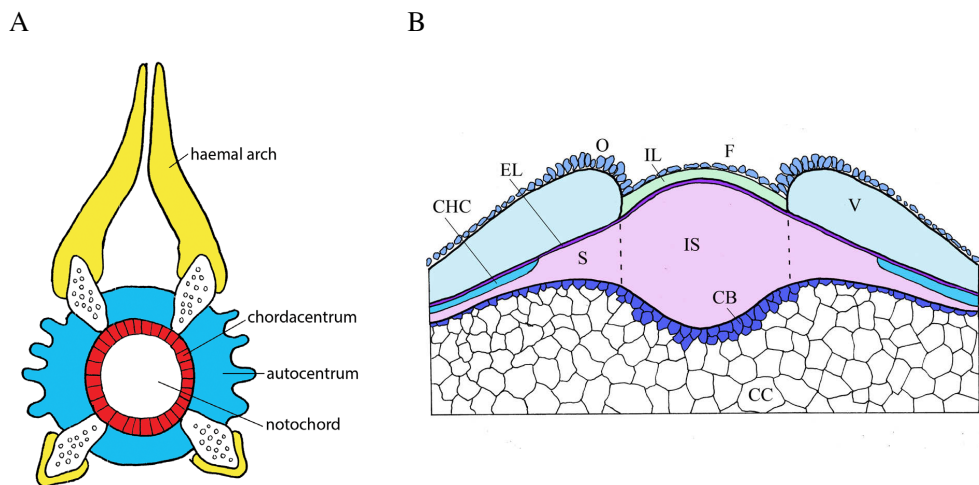


Figure 4: The notochord and surrounding vertebral structures. A: skeletal elements of a teleost vertebra (vertebral body and arches) from a transverse view; modified from Nordvik et al., 2005. B: osteoblast, fibroblast and chordoblast distribution, outside and inside the notochord sheath, respectively, in the intervertebral region. CHC: chordacentrum; EL: elastic membrane; O: osteoblasts (Class III); IL: intervertebral ligament; F: fibroblasts (Class II cells in Inohaya et al., 2007); V: vertebral centra (autocentrum); S: notochord sheath; IS: intervertebral region of the sheath; CB: chordoblasts; CC: chordocytes.

§1.4 Proposed hypotheses for vertebral segmentation in teleosts

Segmental patterning of the vertebral column in amniotes is widely accepted to be originate from the somites, as described in the ‘resegmentation’ model (Christ et al., 2004; Christ et al., 2007; Senthinathan et al., 2012). That is, each vertebra develops strictly in an inherent pattern by recombination of the posterior half of one somite pair and the anterior half of the next (Christ et al., 2004). For teleosts, a similar model has been proposed: the ‘leaky resegmentation’ model, in which sclerotomal cells from one somite contribute to two adjacent vertebrae in a manner that does not show strict AP polarity (Morin-Kensicki et al., 2002). The model is based on observations in the *fsr* zebrafish mutant, which found disrupted somite borders and malformed sclerotome-derived structures such as neural and haemal arches (Fleming et al., 2004; van Eeden et al., 1998), but normal vertebral body patterning (Fleming et al., 2004). Combined with the observations on the development of the chordacentrum in salmon and zebrafish (Fleming et al., 2004; Grotmol et al., 2003), a dual segmentation model has been proposed for vertebral column development in teleosts (Fleming et al., 2004; Grotmol et al., 2003; Figure 3B). Accordingly, both chordoblasts and sclerotome-derived cells contribute to vertebral centrum development: the chordoblasts, through the formation of the chordacentrum and nucleation of the vertebra, while sclerotome-derived cells form the arches and peri-notochordal bone. However, just how regulatory pathways and interacting molecules mediate interactions between notochord cells, chondrocytes and osteoblasts; how segmental patterning is imposed and conveyed; as well as what is the nature of the mechanisms underlying control of the mineralization of notochord sheath, are questions that remain to be elucidated. A better understanding the molecular pathways involved in the morphogenic processes that shape the teleost notochord would be valuable, as it would provide information about vertebral development, and potentially, might offer clues regarding the evolution of chordates and vertebrates.

Regional expression of Hox genes has been linked to the morphologies of distinct regions of vertebral column, in both teleosts and mammals (Bird and Mabee, 2003;

Mallo et al., 2010; Morin-Kensicki et al., 2002; Wellik, 2007). Hox genes encode transcriptional regulatory proteins that specify vertebral identity along the AP axis in vertebrates (Mallo et al., 2010; Wellik, 2007). Interestingly, a typical collinear expression of four *Hox* genes was found in zebrafish notochord, providing the first evidence of a regulated AP regionalization of the notochord (Prince et al., 1998). Genome duplications have given rise to four Hox clusters (HoxA, HoxB, HoxC, HoxD) in most vertebrates. Hox genes within a cluster that share sequence similarities are called paralogous genes. As a result of teleost specific genome duplication, zebrafish and medaka maintain seven clusters (Amores et al., 1998; Kurosawa et al., 2006), fugu (*Takifugu rubripes*) keeps eight clusters (Amores et al., 2004), while 13 clusters are found in Atlantic salmon (118 genes) due to the additional genome duplication and gene losses in salmonids (Mungpakdee et al., 2008). A global survey of *Hox* gene expression in the notochord may provide novel insights into the AP regionalization in this axial organ.

§1.5 The Atlantic salmon as a model for notochord development

Atlantic salmon was selected for this study because of its inherent advantages, such as 1) a large notochord that facilitates detailed microscopic examination and microdissection, which in turn produces sufficient tissue to allow molecular studies at DNA, RNA and protein levels to be carried out; 2) its relatively slow development compared to other teleost models, permits more accurate notochord collection for stage-specific transcriptome sequencing and gene expression assays. However, due to their long generation time, non-transparent eggs and incomplete genomic information, it is relatively difficult to apply modern genetic manipulations such as gene knockout, knockdown and transgenic technologies to the embryos for functional studies, compared to classic models such as the medaka, zebrafish and the African clawed frog (*Xenopus laevis*). Atlantic salmon being an economically important aquaculture species, eggs at any desired stage can be obtained throughout the production season from nearby fish farms.

§1.6 Notochord-specific genes for studying vertebra development in teleosts

A number of studies have used transgenic zebrafish and medaka fluorescence-based specific gene expression technology *in vivo* to study notochord and vertebral development (Bensimon-Brito et al., 2012; Haga et al., 2009; Renn et al., 2006; Renn et al., 2013; Willems et al., 2012). This advanced technology makes it possible to locate cells (e.g. osteoblasts) by targeting the promoters of genes that display cell-specific expression in tissues (e.g. *osterix* and *twhh*). However, this is dependent on the availability of genomic information about the specific marker genes and their promoter regions, in addition to the specific patterns of expression. Specific genetic marker for notochord cells are availability to only a limited degree, except for widely used *shh* and *ntl* (homologous to Brachyury in mouse), *flh* (homologous to Noto in mouse) (Choi et al., 2008; McCann et al., 2012; Schulte-Merker et al., 1994), and previously-mentioned genes that have been identified through zebrafish mutant screens (Stemple, 2005). It is essential to identify additional notochord-specific genes, especially those expressed at early segmentation of the notochord, since they may shed light on differentiation and functions of the notochord.

§1.7 Transcript identification using cDNA library/EST sequencing

An expressed sequence tag (EST) is a DNA sequence that corresponds to a short piece of RNA. Complementary DNA (cDNA) is synthesized from RNA using reverse transcriptase. A cDNA library can be produced from all transcribed RNA from specific tissues at desired developmental stages. Fragments of cDNA that correspond to a part of transcribed gene can be cloned into vectors and further transformed into *E.coli*. Plasmids with cDNA fragment inserts can then be isolated from *E.coli* colonies and sequenced with Sanger technology (Sanger and Coulson, 1975) to produce a set of ESTs (Adams et al., 1991; Figure 5). EST sequencing has been widely used as the classic method of identifying and quantifying gene transcripts expressed by a specific cell line or tissue. Variations of semi-automated tag-based methods have been developed to improve the depth, including serial analysis of gene expression (SAGE; Velculescu et al., 1995) and massively parallel signature sequencing (MPSS; Brenner

et al., 2000). Modifications of cDNA library preparation protocols, include size selection, gene selection at 5' or 3' ends, 5' and 3' UTR selection, as well as amplification of rare transcripts and identification of splice variants and differentially regulated genes. With a normalization step during cDNA library preparation, the abundances of cDNAs within a library are equalized, thus reducing the common transcripts and increasing the possibility of novel gene discovery (Soares et al., 1994). With an EST of approximately 500-800 base pairs (bp) transcript length, putative orthologs can be assigned and gene functions predicted reliably through a reciprocal BLAST search for homologous genes identified in model species such as zebrafish, mouse and human (Woods et al., 2000). Using a physical map of a genome, sequences of ESTs can be mapped to the specific chromosome locations and facilitate comparative genomic analysis (Woods et al., 2000). EST sequences obtained by Sanger sequencing also produce accurate long sequences. EST libraries can be used to annotate the draft genome and genome assembly scaffolds produced by high-throughput sequencing methods such as RNA-seq, while EST libraries can also help in SNP identification. ESTs can also be used to design probes for hybridization-based high-throughput technology such as microarrays. In small-scale projects, EST sequencing based on conventional Sanger technology is still the method of choice, but this is likely to be replaced in the near future by the high-throughput transcriptome-sequencing technology (RNA-seq).

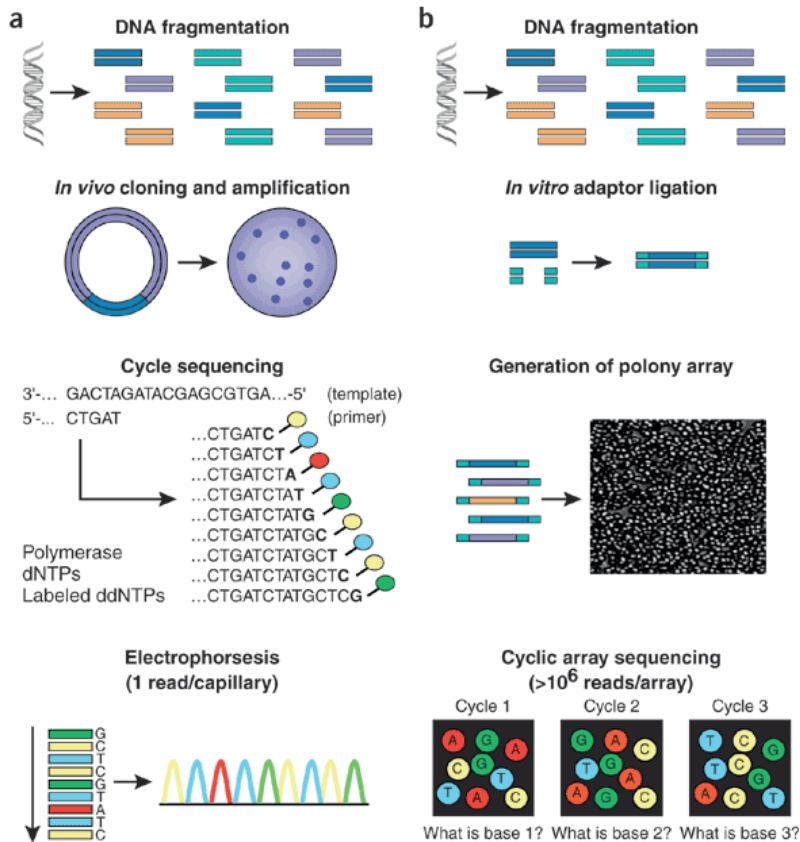


Figure 5: Workflows of two transcriptome-sequencing technologies: (a) Sanger-based sequencing and (b) Next-generation sequencing (Adopted from Shendure and Ji, 2008).

§1.8 Gene expression profiling by high-throughput transcriptome sequencing (RNA-seq)

The advent of massive parallel DNA sequencing technology (commonly referred to Next-Generation DNA Sequencing or NGS) has revolutionized the field of DNA sequencing (reviewed in Shendure et al., 2004; Shendure & Ji, 2008). NGS is a cost-effective approach and provides cyclic-array technology based on polymerase colony technology and fluorescent imaging-based data collection (Mitra and Church, 1999; Mitra et al., 2003; Shendure and Ji, 2008; Figure 5). Compared to conventional capillary-based Sanger-sequencing technologies, NGS does not require manual cloning and bacteria colony picking, thus offering a greater degree of parallelism and

automation. Although details differ among commercialized platforms (e.g. SOLiD by Applied Biosystems; 454 pyrosequencing by Roche Applied Science; Solexa by Illumina), the principle workflows of NGS platforms are similar. Library preparation is performed by random fragmentation of DNA, followed by *in vitro* adaptor ligation at both ends of the fragments. After amplification of these short sequences immobilized in clusters, alternating cycles of enzymatic reactions and image-based sequence acquisition are processed (Figure 5). Hundred of millions of short-reads can be generated in a single run of sequencing machines. With an available reference genome assembly, these short-reads can then be directly aligned to the species genome. In the course of the past decade, reference genomes of several teleost species have been published: fugu (Aparicio et al., 2002), spotted green puffer (*Tetraodon nigroviridis*; Jaillon et al., 2004), medaka (Kasahara et al., 2007), three-spined stickleback (*Gasterosteus aculeatus*; Jones et al., 2012), zebrafish (Zv9 assembly; Howe et al., 2013), cod (*Gadus morhua*; Star et al., 2011), while sequencing projects for more species, including Atlantic salmon (Davidson et al., 2010), are in progress. Applications of NGS include genome resequencing (e.g. polymorphism discovery in individual genomes), metagenomic sequencing (environmental microbial community), transcriptome sequencing or RNA-seq (quantification of global gene expression), sequencing of bisulfite-treated DNA (DNA methylation) and ChIP-seq (protein-DNA interactions) (Shendure and Ji, 2008). RNA-seq offers the possibility of whole-transcriptome profiling. RNA-seq can also detect alternative splicing, non-coding RNA, and contributes to the discovery of novel transcripts. With the rapid development of bioinformatics tools, it can also be used to assemble the genome of novel organisms or to build *de novo* transcriptome assembly if genomic information is absent. It has clear advantages over previous gene expression analysis methods such as qPCR and microarray, in terms of scale and its lack of need for pre-defined genomic coordinates, respectively (Wang et al., 2009). Compared to the less sensitive microarray, RNA-seq is highly reproducible and provides both greater accuracy and better coverage of the transcriptome (Marioni et al., 2008; Wang et al., 2009). In RNA-seq analysis, gene expression is digitally measured in terms of counts of reads (commonly referred as RPKM), and thus scales linearly even at extreme values in

RNA-seq, whereas microarrays are limited to detecting fluorescence signals (Marioni et al., 2008; Mortazavi et al., 2008). Furthermore, RNA-seq also provides information about splicing variants and UTR regions, which cannot be readily detected by microarray (Mortazavi et al., 2008). RNA-seq has been used to detect transcriptional changes during early development in model teleost species (zebrafish: Vesterlund et al., 2011) and to identify muscle-specific transcripts in non-model species (Trout: Palstra et al., 2013). However, most genomic and statistical tools for RNA-seq analysis have been developed for model species and for non-model species such as Atlantic salmon they are few in number. In aquaculture species, microarrays have been successfully utilized and these are still very useful for genotyping and SNP analyses, gene expression associate with development, reproduction, pathology and disease, as well as for QTL mapping (Liu et al., 2012). Besides the commonly perceived imperfection of drawing biological inferences from the mRNA level, such as mRNA stability and turnover, inconsistency between gene expression and protein level, it is also important to note that gene expression is highly tissue/cell specific (Brawand et al., 2011; Wolf, 2013).

§2. OBJECTIVES OF THIS STUDY

The notochord is known to be a major signaling center during embryogenesis. Recent studies have confirmed that in mammals, the nucleus pulposus within the intervertebral disc arises from signals originating in the embryonic notochord. Thereby, mammalian diseases such as intervertebral disc degeneration (IDD) and lower back pain have been linked to the notochord and more knowledge about growth and development of this tissue might shed light on the etiology of these diseases. The Atlantic salmon is an economically important aquaculture species. Bone deformities are common problems in the aquaculture industry and have been shown to have notochordal origin. A better understanding of the genetic network, including the active signaling pathways that regulate cell lineage and differentiation could provide a basis to address these pathological problems. The morphological development of chordoblasts and the mineralization of the notochord sheath in Atlantic salmon shed light on a possible role of the notochord in laying down the segmental patterns prior to vertebra formation. The primary objective of this study was to gain a better understanding of the segmentation process in teleosts by defining its origins. The following objectives were pursued and are described in the present papers:

- 1) To identify and characterize stage-specific genes that are uniquely expressed in the notochord before and during notochord segmentation and mineralization.
- 2) To spatially and temporally quantify gene expression at whole-transcriptome level and identify the functions of differentially expressed genes and pathways underlying notochord segmentation.
- 3) To examine the initial mineralization pattern in the notochord sheath and provide clues for the mechanism of biomineralization.

§3. LIST OF PUBLICATIONS

Paper I:

Sagstad, A., Grotmol, S., Kryvi, H., Krossøy, C., Totland, G. K., Malde, K., **Wang, S.**, Hansen, T., Wargelius, A., 2011. Identification of *vimentin*- and *elastin*-like transcripts specifically expressed in developing notochord of Atlantic salmon (*Salmo salar* L.). *Cell and Tissue Research* 346, 191-202.

Paper II:

Wang, S., Furmanek, T., Kryvi, H., Krossøy, C., Totland, G. K., Grotmol, S., Wargelius, A., 2014. Transcriptome sequencing of Atlantic salmon (*Salmo salar* L.) notochord prior to development of the vertebrae provides clues to regulation of positional fate, chordoblast lineage and mineralization. (*Submitted manuscript*)

Paper III:

Wang, S., Kryvi, H., Grotmol, S., Wargelius, A., Krossøy, C., Epple, M., Neues, F., Furmanek, T., Totland, G., 2013. Mineralization of the vertebral bodies in Atlantic salmon (*Salmo salar* L.) is initiated segmentally in the form of hydroxyapatite crystal accretions in the notochord sheath. *Journal of Anatomy* 223, 159-170.

§4. SUMMARY OF RESULTS

Paper I:

This paper describes the genes identified in a normalized cDNA library from micro-dissected Atlantic salmon notochord before and during early segmentation of the notochord. From a total of 1968 Sanger-sequenced clones, 1911 ESTs with an average length of 706 bp were reported. Among these, 170 ESTs were predicted to be involved in the process of development with Gene Ontology (GO) and subjected to gene expression analysis. Twenty-two of these 170 transcripts were predominantly expressed in the notochord from 490 d° to 810 d°, as compared to the rest of tissues in salmon larva. Quantitative real-time PCR were designed to detect differential expression of these 22 genes during ontogeny: ten genes were up-regulated at specific stages from 650 d° to 760 d° and one was down-regulated from 720 d°, when early mineralization of the sheath commenced. Two genes (*vimentin*- and *elastin*-like transcripts) were specifically expressed in the salmon notochord. The *vimentin*-like gene was expressed in both vacuolated chordocytes and epithelium-like chordoblasts. This gene expression is co-localized with the densely packed intermediate filaments in the chordocyte cytoplasm. The *elastin*-like gene was only expressed in chordoblasts.

Paper II:

Paper II demonstrates the notochord-specific transcriptome prior to notochord mineralization in Atlantic salmon. Notochord samples were collected at three developmental stages (noted as T1: 510 ±20 d°; T2: 610 ±20 d°; T3: 710 ±20 d°). In each stage, notochords were further cut into three parts along the longitudinal axis (noted as anterior, mid and posterior). Nine RNA libraries were prepared for RNA-seq to detect differential gene expression spatially and temporally. Around 22 million pair-ended raw reads were collected from each library and mapped to the draft salmon genome assembly. A total of 66569 transcripts were identified, of which 55775 were annotated. Colinear expressions of 71 salmon *Hox* genes were detected in the notochord. Key chondrogenic genes were expressed in the notochord (*sox9*, *sox5*, *sox6*,

tgfb3, *ihhb* and *col2a1*), but not osteogenic transcription factors (*runx2/cbfa1*, *osterix*), suggesting a chondrogenic-like lineage of notochord cells. Differentially expressed genes (DEGs) were picked up by NOIseq and further grouped with KEGG ID in KEGG pathways. Many KEGG pathways were down-regulated from 610 d° to 710 d°, implying a terminal differentiation of notochord and shut-down of protein synthesis at the time of notochord segmentation. *Col11a2* was segmentally expressed in one population of chordoblasts lining prospective non-mineralizing regions along the notochord.

Paper III:

Paper III characterizes the initial mineralization pattern of the vertebrae as hydroxyapatite crystal accretion in the notochord sheath. Mineralization starts in separate elements, which fuse to form complete ring structures in a segmental pattern within the notochord sheath. Apatite crystals nucleate and grow along the long axis of collagen fibers and always parallel to the fiber, first in the collagen type II matrix in the notochord sheath and then in the sclerotome-derived collagen type I matrix in a similar manner. The collagen matrices provide support to the growth of apatite from plate-like structure to the dendritic shape. The chordacentrum was confirmed to contain hydroxyapatite using synchrotron-based X-ray diffraction. Quantitative PCR was performed to confirm that the major collagen type of notochord sheath is type II collagen (*col2a1*), but not type I collagen (*colla2*). Both *col2a1* and *colla1* were down-regulated from 610 d° to 710 d°. In addition, expressions of four key genes (*shh*, *ihhb*, *pth1r* and *tgfb1*) were detected in the notochord from 510 d° to 710 d°. An up-regulation of *pth1r* and *tgfb1* was shown from 610 d° to 710 d°.

§5. DISCUSSION

§5.1 The dynamic notochord transcriptome reveals regulation of transcripts along the anterior-posterior axis and over time.

A total of 66569 putative genes were expressed in the salmon notochord (**Paper II**). Of these, 2470 and 3147 DEGs were detected along the AP axis and over time (510-710 d°), respectively, and the full DEG lists can be accessed from <https://marineseq.imr.no/salmon/shouw/Appendix/>. Since a normalized cDNA library strategy was adopted in **Paper I**, the distribution of transcripts did not reflect abundance in the notochord transcriptome. However in **Paper I**, notochord-specific genes were identified among the 1911 EST sequenced, probably due to the technique that enriched for scarce transcripts. In **Paper II**, pathway analysis performed on DEGs in the notochord during the early segmentation phase found only few pathways that were regulated from 510 d° to 610 d°. Likewise in **Paper I** only one of the 22 genes studied in detail showed differential expression at similar stages. However at 700 d°, many pathways were regulated, such as those related to cell signaling and interactions, cell growth, and protein synthesis (610 d° to 710 d°) (**Paper II**). Similarly, the majority of genes studied in detail in **Paper I** displayed differential expression around 700 d°. We also found that genes showing polarity expressions along the AP axis were predominantly expressed at the posterior end of the notochord (**Paper II**). The difference along the AP axis could be attributed to the fact that the notochord arises from three distinct morphogenetic origins along the AP axis, as has been shown in mouse (Yamanaka et al., 2007). The difference along the AP axis could also be linked to spatial-temporal regulation of development mediated through *Hox* gene expression. We demonstrated the spatial-temporal expression of 71 *Hox* genes along the AP axis in the salmon notochord (**Paper II**). This suggests that the morphologically uniform notochord is functionally subdivided into regions along the AP axis, possibly related to notochord segmentation. Similarly, a global expression pattern of *Hox* genes has only been investigated in another fish, the European eel (*Anguilla anguilla*). In that study, however, the whole embryo was studied (Henkel et al., 2012). Some similarities were identified between the salmon notochord and the embryo axis in eel, such as high

expression levels for genes in the HoxB cluster and paralogue groups 5-9, suggesting a coordinated function that marks the polarity identity along the AP axis in teleost fish (**Paper II**, Henkel et al., 2012). Taken together, **Papers I, II, III** not only confirm the existence of a dynamic transcriptome in the notochord, but also infer biological meaning related to positional fate (*Hox* genes), cell lineage determination (expression of cartilage-type molecules, §5.2), segmentation (segmented expression of *coll1a2*, §5.3), structural uniqueness (*vimentin* and *elastin*, §5.4) and mineralization processes (mineral crystal accretion in notochord, §5.5). The following sections of the discussion will address these features.

§5.2 A close link between chondrocytes and notochordal cells

This series of studies provides two pieces of molecular evidences in support of the hypothesis that chondrocytes are closely related to notochord cells (Zhang and Cohn, 2006). First, salmon notochord expresses predominantly the major collagen of cartilage (*col2a1*) rather than the major collagen of bone (*colla2*) (**Paper III**; Figure 6). This is consistent with previous studies of collagen type II expression in the notochord both at mRNA and protein levels in gnathostomes (Yan et al., 1995; Zhao et al., 1997). Collagen type II was shown to be essential during the transformation of notochord into NP, as in *Col2a1*-null mice notochord persisted as a rod-like structure (Aszódi et al., 1998). Collagen type II belongs to the clade A collagen gene family that consists of the fibrillar collagen type I, II, III and V. An ancestral fibrillar collagen (ColA) gene is expressed in notochord of stem-group chordates (lancelets and tunicates) (Wada et al., 2006; Zhang and Cohn, 2006). It has further been suggested that the duplication and diversification of the ancestral ColA gene may underlie the evolutionary origin of vertebrate skeletal tissues (Zhang and Cohn, 2006). It is noteworthy that the clade A collagen gene family is physically linked with Hox gene clusters, and thus share its duplication history with vertebrates and both gene families may have facilitated the diversification of vertebrate connective tissue types (Bailey et al., 1997; Morvan-Dubois et al., 2003; Zhang and Cohn, 2006). Expression of another fibrillar, cartilage-type collagen gene *coll1a1* was found in the notochord (**Papers I,**

II, III; Figure 6). Furthermore, when we examine all ECM genes annotated with GO (including the collagen gene family) from the RNA-seq gene list, none of the collagen genes encoding bone-type collagens (I, V) were highly expressed in the notochord (**Paper II**; Figure 6). Because of the importance of ECM genes in the notochord, all ECM genes from **Paper II** were clustered based on their expression patterns over time (510-710 d°), shown in Appendix Figure 1. This reveals a possible co-regulation mechanism for the ECM genes within a cluster (e.g. *col2a1* and *coll1a1*) (**Papers II, III**; Figure 6).

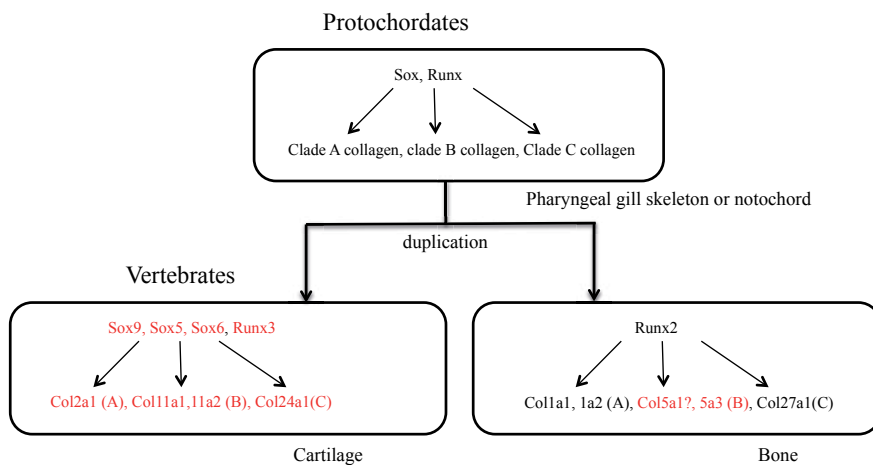


Figure 6: Cartilage-type collagen genes and regulatory gene expressed (red colored) from RNA-seq in the Atlantic salmon notochord; modified from Wada, 2010.

A second finding of our study supports the notion of closeness between cartilage and notochord. It was found that many key regulatory genes of chondrogenic pathways, but not osteogenic pathways, are expressed in the notochord (**Paper II**). For example, *Sox9*, *sox5* and *sox6* were found in the salmon notochord (**Paper II**; Figure 6). It is known that *Sox9* drives *col2a1* expression (Lefebvre et al., 1997; Yan et al., 2005). *Sox5* and *Sox6* function as *cis*-acting elements, as they increase the binding efficiency of *Sox9* to its recognition sites. *Sox9*, *Sox5* and *Sox6* are known as the chondrogenic Sox trio, because they can up-regulate cartilage matrix gene expression (Han and

Lefebvre, 2008). On the basis of a knockout study in mice, Sox5 and Sox6 were shown to mediate notochord sheath formation and are required for notochord cell survival (Smits and Lefebvre, 2003). In mammals, Sox9 directly binds to Runx2 and inhibits its role during skeletogenesis, while in zebrafish one ortholog of Sox9, *sox9b* retains this dominant role (Yamashita et al., 2009; Yan et al., 2005; Zhou et al., 2006). One *sox9b* transcript was detected in our RNA-seq data, displaying a decreasing trend over time, which may correlate with the down-regulation of collagen transcripts over time. Interestingly, expression of the transcriptional regulatory genes of fibrillar collagens such as *SoxD*, *SoxE* and *Runt* were found in the notochord in amphioxus and lamprey (Hecht et al., 2008; Meulemans and Bronner-Fraser, 2007; Ohtani et al., 2008; Wada, 2010; Figure 6). However, we could not detect putative transcripts annotated as osteogenic marker genes in the notochord, such as *runx2 (cbfa1)*, *osterix (transcription factor sp7)*, and *osteocalcin (bpg)* in our RNA-seq gene list. Nor was *runx2* detected by qPCR in salmon notochord (**Paper II**). Likewise, no expressions of typical osteogenic marker genes (*osteocalcin*, *colla*) have been detected in the notochord in salmon at a later stage of development (Krossøy et al., 2009; Ytteborg et al., 2010a; Ytteborg et al., 2010b) nor in medaka (Inohaya et al., 2007; Renn et al., 2006). To summarize, notochord cells resemble immature chondrocytes in that they express cartilage matrix genes, such as *col2a1*, *coll1a1*, *coll1a2*, and are potentially regulated by chondrogenic markers such as those in the Sox gene family (**Papers I, II, III**; Figure 6). Also, *runx1* and *runx3*, which are known to be involved in chondrocyte maturation, are expressed in the salmon notochord (**Paper II**). Interestingly, *runx2* transcription was observed in the notochord cells in deformed fused vertebrae in salmon, which indicates a state shift of these cells from a chondrogenic to an osteogenic state, accompanied by mineralization of the intervertebral space under pathological conditions (Ytteborg et al., 2010a). Co-expression of Runt and Hedgehog genes in mouse and lancelet is found in cartilage-related tissues (including notochord), and was also demonstrated here in salmon notochord. This co-expression indicates that a conserved molecular pathway involving these genes is of relevance for vertebrate cartilage evolution (Hecht et al., 2008; **Papers II, III**; Figure 6).

§5.3 Segmentation in the chordoblast layer in the salmon notochord

A segmental expression of *col11a2* mRNA was found in the chordoblasts, demarcating the future non-mineralized intervertebral region in salmon notochord at 700 d° (**Paper II**). This provides supplementary evidence of the previously observed segmental pattern of ALP enzymatic activities in chordoblasts, which further strengthens the notion that the notochord derives a segmental pattern from itself (Grotmol et al., 2003; Grotmol et al., 2005; **Paper II**; Figure 7). However, it has also been shown in medaka that sclerotomal cells outside the notochord sheath display *col10a1* transcription segmentally, in a way that resembles the pattern of chordoblast populations during notochord sheath mineralization (Bensimon-Brito et al., 2012; Grotmol et al., 2005; Renn et al., 2013; **Papers II, III**; Figure 7). These observations indicate a degree of crosstalk via unknown mechanisms between these two cell types during the segmental patterning of the vertebral centra. Type XI collagen is the key regulator of type II collagen fibrillogenesis in cartilaginous matrix. As previously demonstrated in mice, *Col11a1* and *col2a1* transcripts show co-localized expression, prominent in the vertebrae, in the osteoblasts of the trabecular bone and chondroid tissue of the intervertebral disc (Li et al., 1995; Yoshioka et al., 1995), the co-expression has now also been observed in the salmon notochord (**Papers II, III**; Appendix Figure 1). Notably, *col11a2* transcript shares high sequence similarity to *col5a1* (Wargelius et al., 2010), therefore manual blast were performed in the RNA-seq study to confirm that the gene is most likely to be *col11a2*. A coordinated down-regulation of *col2a1*, *col11a1*, *col11a2* and other ECM transcripts is observed from 610 d° to 710 d° (**Papers II, II**; Appendix Figure 1).

Interestingly, *col11a1* is expressed throughout the chordoblast layer, whereas *col11a2* expression is segmental and restricted to the chordoblast population of the prospective intervertebral region (Wargelius et al., 2010; **Paper II**; Figure 7). This raises the possibility of a regulatory role of *col11a2* in the collagen matrix in the non-mineralized notochord sheath in salmon. We may speculate that one population of chordoblasts sustains active production of non-mineralizing ECM components such as

col11a2, while another population of chordoblasts differentiate and start to produce mineralizing ECM. This speculation tends to be supported by the observed enlargement of amphicoelous concavities in the intervertebral region and confined narrow funnel in the middle of the vertebra (intravertebral canal; **Paper III**). Interestingly in medaka, mesenchymal cells in the intervertebral region differentiate into *osterix*-positive osteoblasts strengthens the importance of the intervertebral region as a growth center, in a later stage during peri-notochordal bone deposition (Inohaya et al., 2007). It has been observed that conditional ablation of *osterix*-positive osteoblasts led to a loss of intervertebral region, resulting in the fusion of centra in transgenic medaka (Willems et al., 2012). It has also been indicated that *Wnt4b* signals from the floor plate cells outside the notochord are essential for the maintenance of the non-mineralized intervertebral ligament in medaka (Inohaya et al., 2010). The cellular mechanism patterns the coincident segmentation of cell populations in the intervertebral region should be further investigated to elucidate the origin of segmentation signals.

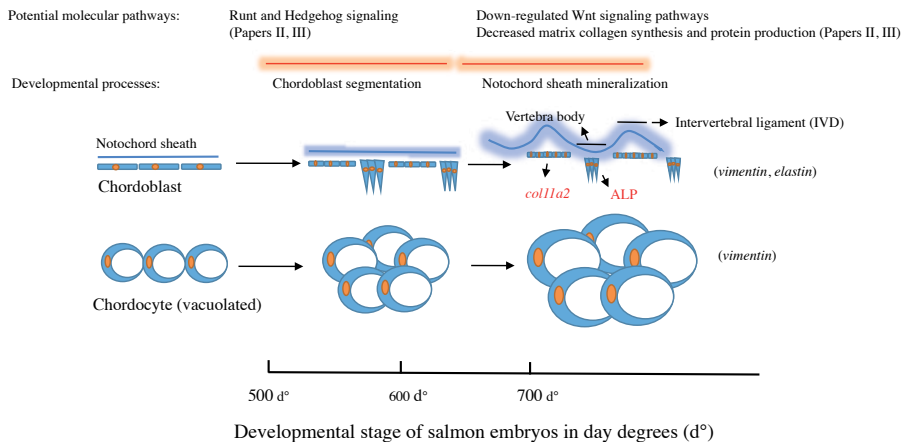


Figure 7: Chordoblast segmentation and potential regulatory pathways in Atlantic salmon. Chordoblasts are differentiated into two populations in a metameric pattern at around 600 d°: One population displays alkaline phosphatase (ALP) activity at prospective mineralization sites, while the other expresses *col11a2* transcripts in prospective non-mineralized intervertebral regions. A segmented notochord can be observed after the initiation of mineralization in the notochord sheath, around 700 d° and onward. Potential molecular pathways related to the segmentation of notochord are shown.

Potential regulatory pathways during notochord segmentation and mineralization are listed in Figure 7, and more in **Paper II**. Both Hedgehog and TGF- β pathways have recently been shown to play important roles during transformation from notochord into NP in the intervertebral disc in mice (Choi et al., 2012; Dahia et al., 2012; Jin et al., 2011; Maier et al., 2013). It was shown that the notochord sheath is essential for the formation of NP in mice (Choi and Harfe, 2011). These findings link the functioning of embryonic and adult notochord and also suggest that normal patterning of the intervertebral region is needed to obtain a normal vertebral column. Further studies on signaling pathways found to be regulated around 700 d^o, especially the Wnt signaling pathway, might reveal which cells and cell communications are essential for the segmental patterning of notochord and prospective vertebral column (**Paper II**; Inohaya et al., 2010).

§5.4 Characterization of notochord-specific proteins

Functional studies in zebrafish confirm that ECM components are essential for the maintenance of notochord cells, and knockdown of these transcripts may lead to distinct distortion of the notochord and shortening of the body length (Corallo et al., 2013; Tong et al., 2013). It has also been shown that a number of structural genes have unique expressions in the notochord such as *emilin3*, *ngs*, *calymmin* in zebrafish (Cerdà et al., 2002; Corallo et al., 2013; Tong et al., 2013), and *vimentin*- and *elastin*-like transcripts in salmon (**Paper I**). The *vimentin*-like transcript has been shown to be predominantly and highly expressed in both chordoblasts and chordocytes in the salmon notochord (**Paper I**; Figure 7). It may play a similar role as *ngs* in zebrafish, because both genes are potentially translated into novel types of intermediate filament family proteins in the notochord (Tong et al., 2013). The *elastin*-like transcript is expressed exclusively in the chordoblasts, suggesting that it may be assembled in elastic membrane in the notochord sheath, and may operate as a ‘force transmitter’ between the myosepta and the notochord (Grotmol et al., 2006; **Paper I**). A recent study demonstrated the essential role of another intermediate filament protein, ECM

glycoprotein Emilin3, expressed solely in the notochord in zebrafish (Tong et al., 2013) and the transcripts have now also been found in the salmon notochord (**Paper II**).

§5.5 Mineralization in the notochord sheath

Paper III displays a dynamic teleost-specific mineralization process that forms chordacentra in the notochord sheath. This work also confirms that the chordacentrum is the initial bony structure that forms as the innermost part of the vertebral centra. Unlike the chordacentra formation described in zebrafish (Bensimon-Brito et al., 2012; Du et al., 2001; Fleming et al., 2004) and in medaka (Inohaya et al., 2007), salmon chordacentra initiate in the ventral region as three separate bony elements and do not occur in an anterior to posterior sequence along the body axis. However, chordacentra display a mineralizing pattern from a basoventral origin, similar to the caudal fin centra formation in zebrafish, at the insert of the previously formed haemal arch (Bensimon-Brito et al., 2010; Bensimon-Brito et al., 2012). With the aid of detailed observations with transmission electron microscopy (TEM), it was shown that mineralization in the chordacentra starts with thin plate-like hydroxyapatite crystals that grow on the surface of parallel-organized collagen fibrils in the morphologically homogenous type II collagen matrix of the notochord sheath before occurring in the type I collagen matrix external to the notochord. This mineralization sequence is similar to what was observed in medaka (Inohaya et al., 2007). An *in vitro* study by Wang and co-authors (2012), showed that collagen fibrils can initiate and orient the growth of hydroxyapatite minerals in the absence of any other vertebrate ECM molecules of calcifying tissue. **Paper III** shows that hydroxyapatite can be initiated in both type I and type II collagen matrices. A down-regulation of collagen fibrillogenesis-related genes: *col2a1*, *coll1a1*, *col24a1* and other genes involved in the synthesis, folding and assembly of collagen helix (*fmod*, *crtap*, *lepre1*) takes place from 610 d° to 710 d°, when the hydroxyapatite is nucleated (**Papers II, III**; Cabral et al., 2007; Goldberg et al., 2006), suggesting that there is a close relationship between collagen fiber structure and hydroxyapatite initiation and/or growth. Different fiber diameters and angles in lamellar fibril layers in the notochord sheath have been

reported in both *Xenopus* (Adams et al., 1990) and Atlantic salmon (Grotmol et al., 2006; Nordvik et al., 2005). Interestingly, both studies provided morphological evidence of a dynamic change of the fiber composition and fiber angle in the sheath matrix as part of enforcement of the tissue. The pattern of collagen winding corresponds to the alternative banding pattern of chordablasts and suggests an inductive role of chordoblast cell population in mineralization of the notochord sheath. However, it is also possible that putative *osterix*-negative osteoblasts (Class I) lying on top of the prospective chordacentrum (Renn et al., 2013; Spoorendonk et al., 2008) contribute to the mineralization of the collagen I matrix outside the notochord in a similar manner as do chordoblasts, based on their growth pattern of hydroxyapatite crystals (**Paper III**). These *osterix*-negative osteoblasts displayed *coll10a1* transcription prior to mineralization of the notochord sheath in transgenic medaka (Renn et al., 2013). Furthermore, hydroxyapatite is known to display osteoinductivity and it has been demonstrated that hydroxyapatite exhibited the ability to induce expression of osteo-specific genes, including alkaline phosphatase, type I collagen, osteocalcin except *Runx2* in mouse stem cells *in vitro* (Lin et al., 2009). It is possible that hydroxyapatite mediates cell communication between osteoblasts and chordoblast populations along the notochord.

No expressions of genes encoding organic matrix proteins/SIBLINGS (osteocalcin, osteoponin, bone sialoprotein/bsp, phosphophoryn/pp) were found in the RNA-seq gene list (**Paper II**). Instead, the osteogenesis inhibition gene *fetub* was down-regulated from 510 d° to 710 d°. *Fetub* encodes a Fetuin-B protein, which belongs to the TGF- β cytokine-antagonist fetuin family (Binkert, 1999; Demetriou et al., 1996; Olivier et al., 2000). Together with the counter-acting up-regulation of *tgfb1*, this is a strong indication of an induced TGF- β signaling from 510 d° to 710 d° in the notochord, which may lead to hydroxyapatite nucleation in the notochord sheath.

§5.6 Methods of studying notochord-specific transcriptome composition in a non-model species

In many organisms, the embryonic notochord is small and difficult to be extracted alone. The large size of salmon embryos makes it possible to isolate notochord samples through micro-dissection prior to the stage when notochord sheath mineralizes. The isolation protocol employed in all three papers is detailed in **Paper I**. Moreover, as shown in **Paper II**, the isolated notochord contains two cell types, i.e., epithelium-like chordoblasts and vacuolated chordocytes. Current technology precludes the possibility of separating these two cell lineages, which would be required to obtain their separate transcriptomes. Two sequencing-based methods were used to identify expressed transcripts in the notochord: RNA-seq and EST sequencing from a normalized cDNA library. RNA-seq produces much higher throughput than EST sequencing (**Papers I, II**). The average lengths of transcripts identified by the two methods are quite similar, at around 800 bp. However it is likely that the sequence integrity is higher in the EST sequences obtained from Sanger sequencing since the whole sequence is the result of one sequencing reaction, while RNA-seq is a combination of several sequences.

Previous studies have published various RNA-seq workflows, but these cannot be directly applied to the salmon species because of the un-annotated salmon reference genome and a lack of bioinformatics tools, such as genome viewer and pathway visualization tools. A validated RNA-seq data analysis pipeline using a preliminary large contig assembly of Atlantic salmon is outlined in Supplementary Figure 2 in **Paper II**. In summary, around 21 million 100bp pair-end raw reads were collected from each notochord library. Quality checks were performed at different levels, including total RNA, polyA+ RNA selection and raw reads quality, before direct alignment of reads to the draft salmon genome assembly, which had been annotated by the gene prediction software Augustus (Stanke and Morgenstern, 2005). Although the length of UTR regions differ between tissues (Ramsköld et al., 2009), we used a condition under which we predicted the UTR to be >1000 bp downstream from the

predicted coding regions. Based on this condition, 32% reads were predicted to be UTR regions (**Paper II**). However, these reads were excluded from the gene expression level analysis, as previous studies have shown that excluding these UTR-reads yields more accurate gene expression estimation (Ramsköld et al., 2009, **Paper II**). Due to the fragmentation of the current draft genome assembly (contig N50 = 9342), many variants of the same gene or paralogous genes are present in a mix in the genome. No attempts were made to identify splice variants, although different expression patterns were observed in genes annotated with the same gene symbol from Swissprot. We attempted to fuse genes based on gene symbol, but we noticed that fused gene expression in RNA-seq could not correlate to qPCR analysis. Therefore no fusion of transcripts annotated with the same gene symbol was performed. The lack of replicates in the RNA-seq study limited the analysis for detecting DEGs to NOIseq (Tarazona et al., 2011) as other available options require sample replicates, such as DESeq (Anders and Huber, 2010), edgeR (Robinson et al., 2010), and baySeq (Hardcastle and Kelly, 2010). By summing the gene expression values from three libraries (either spatially or temporally), the sensitivity of NOIseq in terms of finding DEGs was increased, which was important for downstream KEGG pathway analysis. Among the 22 genes subjected to independent qPCR validation, 13 genes were DEGs, according to NOIseq (>1.5 fold change). Twelve of them were confirmed by qPCR, except for *fos* which displayed large variation between replicates. This indicates that the identification of DEGs using NOIseq in our pipeline was highly reliable, and clustering these DEGs in KEGG analysis reflects a valid snapshot of the gene regulation in the salmon notochord. An online tool for gene enrichment analysis was tested (DAVID, Huang et al. 2009) and concluded similar pathways as presented in **Paper II**. Attempts to test other gene enrichment analysis methods can be done in further RNA-seq studies. Moreover, since one of the aims of this study was to identify notochord-specific genes, *in situ* hybridization was employed to confirm the spatial expressions of candidate notochord-specific genes (*elastin*-like and *vimentin*-like transcripts in **Paper I**; *coll1a2* in **Paper II**).

§6. CONCLUSIONS AND FUTURE PERSPECTIVES

§6.1 The dynamic notochord transcriptome in Atlantic salmon

Two transcriptome-sequencing technologies were successfully applied to characterize a dynamic notochord-specific transcriptome during the notochord segmentation and early mineralization stages in Atlantic salmon (500-750 d°) and also between anterior and posterior segments of the notochord. Clustering differentially expressed genes in KEGG analysis demonstrated down-regulation of pathways associated with ECM, cell division, metabolism and development at onset of notochord mineralization (around 700 d°). These results reveal a critical phase of a functional change in the notochord prior to formation of vertebral body formation in Atlantic salmon. Moreover, in the analysis of anterior-posterior polarity in the transcriptome, 71 *Hox* genes were expressed in a collinear fashion along the salmon notochord. Clustering of these *Hox* genes revealed a pattern that could be correlated to the initial segmentation of the notochord along the AP axis. Furthermore, global transcriptome analysis revealed expression of chondrogenic factors and cartilage-type collagens in the notochord, but no osteogenic factors. These results suggest that the teleost notochord is more closely related to chondrogenic than osteogenic tissue.

This study has also demonstrated for the first time the existence of a segmented gene expression within the early notochord, thus showing that the teleost notochord can be segmented prior to mineralization. The segmented gene expression of *coll1a2* was exhibited in one chordoblast population lining the future non-mineralized region of the notochord. The notochord is also a highly specialized tissue, and to display its specific phenotype it probably expresses a number of unique molecules. Two structural genes (*vimentin*- and *elastin*-like transcripts) were specifically expressed in the notochord. The *vimentin*-like gene was expressed in both vacuolated chordocytes and epithelium-like chordoblasts. The *elastin*-like gene was only expressed in chordoblasts.

§6.2 Mineralization in the notochord sheath

The segmented mineralized rings that form in the notochord were also studied in detail. It was shown that mineralization of the rings took place through hydroxyapatite nucleation along the long axis of collagen fibers and always parallel with the fiber. Moreover, the first mineral nucleation always appeared in the collagen type II matrix in the notochord sheath.

§6.3 Future perspectives

In the present study, we provide for the first time molecular evidence that the notochord is a segmented tissue prior to early mineralization of the notochord sheath. Findings in this study are in agreement with previous histological studies of the notochord sheath in salmon at these stages (Grotmol et al., 2006). The findings support the hypothesis that notochord segmentation precedes the vertebral column in teleosts. Results from this study point to several future directions in the studies of notochord development and vertebrate skeletogenesis:

- 1) In-situ hybridization and functional studies of notochord-specific and other ECM-related genes (e.g. *coll1a2*, *coll1a1*, *col2a1*, *fmod*, *tgfb1-3*) identified in this study should be carried out in experimental model species such as zebrafish and mouse. These candidate genes may display segmental expression as demonstrated with *coll1a2* in salmon notochord and *fmod* in intervertebral disc in mice, and have potential roles in the regulations of the development of intervertebral disc (Sohn et al., 2010). The authors also showed that *Tgfb2* is a regulator of intervertebral disc development. Likewise, *tgfb1*, *tgfb2*, *tgfb3* may have a function role in notochord morphogenesis in salmon.
- 2) Transgenic medaka or zebrafish reporter lines should be generated and used to study the transcription status of *coll1a2* in the teleost notochord *in vivo*. These tools will be instrumental for us to investigate how the segmental pattern occurs, and compare with the expression of *coll10a1* (Renn et al., 2013).

- 3) Future RNA-seq studies can be performed on the rest of tissues without the notochord together with the data generated in this study, to identify more genes that are uniquely expressed in the notochord in Atlantic salmon. RNA-seq studies can also be done with dissected notochord/NP at a later stage in salmon or in other model species such as spotted gar and mouse at similar stage (E9.0-E12.5 in mouse). A laser-dissection technology has been demonstrated for this purpose (Sohn et al., 2010).
- 4) Cultured cell lines of chondrocyte and osteoblast, which are closely related to mineralization of ECM, were generated (Conceição et al., 2013). Based on these cell lines, a set of ECM genes involved in mineralization were identified by anti-mineralogenic activity of vanadate (Tiago et al., 2008; Tiago et al., 2011). It will be interesting to find out if similar genes and pathways are expressed in the chordoblasts during the mineralization of the notochord sheath. These cell lines can also be used for further in vitro studies on potential roles of hydroxyapatite, TGF- β and Fetuin in mineralization.

§7. REFERENCE

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