Morphological and Molecular Characterization of Developing Vertebral Fusions in Atlantic Salmon (*Salmo salar*)

Morfologisk og molekylær karakterisering av utvikling
av virvelfusjoner i Atlantisk laks

Philosophiae Doctor (PhD) Thesis

Elisabeth Ytteborg

Department of Animal and Aquaculture science
Norwegian University of Life Sciences

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Elisabeth ;)

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Abbreviations

AF	Annulus fibrosus
ALP	Alkaline phosphatase
BMP	Bone morphogenetic protein
CLA	Conjugated linoleic acid
Col1a/2a/10a	Collagen type 1a/2a/10a
DHA	Docosahexaenoic acid
Dpf	Day degrees post fertilization
EPA	Eicosapentaenoic acid
FGF	Fibroblast growth factor
GH	Growth hormone
HSC	Haemopoetic stem cell
Hsp	Heat shock protein
Ihh	Indian hedgehog
ISH	In situ hybridization
IVD	Intervertebral disc
M-CSF	Macrophage colony stimulating factor
Mef2c	Myocyte enhancer factor 2c
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NP	Nucleus pulposus
OPG	Osteoprotegerin
PCNA	proliferating cell nuclear antigen
$PGE_{2,}$	Prostaglandin E2
PTHrP	Parathyroid hormone related protein
PUFA	Polyunsaturated fatty acid
QPCR	Quantitative polymerase chain reaction
RANKL	Receptor activator of nuclear factor κB ligand
Runx2	Runt-related transcription factor 2
Shh	Sonic hedge hog
Sox9	Sex determining region Y box 9
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRAP	Tartrate resistant acid phosphatase

Definitions

Acellular bone - Bone that lacks the presence of osteocytes

Arch centra – Cartilage regions in the vertebral bodies connecting the neural and haemal arches to the centra

Bone remodeling - Bone is removed (bone resorption) and new bone is added (bone formation).

Bone resorption - The process by which osteoclasts break down mineralized matrix

Cellular bone - Bone containing osteocytes

Centra - The bony structures of the vertebral bodies

Chondrocyte - Cartilage secreting cells

Chondroid bone - Tissue intermediate between bone and cartilage

Chordata - Phylum including all vertebrates

Chordoblast - Cells surrounding the chordocytes in the notochord, secrete the notochordal sheath

Chordocyte - Vacuolated cells in the notochord, make up the core of the notochord

Day degrees (d°) - The sum of daily temperature, commonly used to measure the age of fish

Differentiation - The process where a less specialized cell becomes more specialized

Ectopic bone formation - Bone forming outside the area in which it is normally expected to occur.

Embryogenesis - The process by which the embryo forms and develops from fertilization of the egg to the development of the embryo

Endochondral ossification - Bone is produced on a present cartilaginous template

Intervertebral space - The space between adjacent vertebral centra

Intramembranous ossification - Bone is formed directly without a scaffold of cartilage

Metaplasia - One differentiated cell type differentiates into another cell type, generally caused by some sort of abnormal stimulus

Mineralization - The process where an organic substance is converted to an inorganic substance

Notochord - A rod of large cells constrained by a thick extracellular sheath, functions as the central axial skeletal element of developing embryos

Ossification - Bone tissue formation

Osteoblast - Bone secreting cell

Osteoclast - Bone reabsorbing cells

Osteocyte - Osteoblasts surrounded by bone matrix

Osteoid - Newly formed bone matrix, not yet mineralized

Somite - Segmented tissue blocks that differentiate into skeletal muscle, vertebrae, and dermis

Somitogenesis - The process in which the somites are formed

Trans-chondroid ossification - Bone formation where chondrocytes trans-differentiate into bone producing cells

Trans-differentiation - When a cell transforms into a different cell type or when an already differentiated stem cell creates cells outside its already established differentiation path

Aims of the Study

The aim of the presented study was to increase the morphological and molecular understanding of how temperature induced spinal fusions develops in Atlantic salmon (*Salmo salar*). In more specific terms, this was mainly accomplished by:

- Identification of genes involved in the skeletogenesis of the spinal column (Paper I-IV)
- Comparing non-deformed fish exposed to two different temperature regimes, hence, exposing differences in risk level between the groups rather than elaborating the pathogenesis of deformed vertebrae (Paper I)
- Analyzing different stages of the fusion process using radiography, basic staining techniques and molecular methods (immunohistochemistry, quantitative real-time PCR and *in situ* hybridization) (Paper II and III)
- Establishing a method for *in vitro* studies with Atlantic salmon osteoblasts that could further be used to analyze differentiation during different culture conditions (Paper IV)

List of Publications

This thesis is based on the following papers:

Paper I

Ytteborg, E., Baeverfjord, G., Hjelde, K., Torgersen, J., Takle, H. Molecular pathology of vertebral deformities in hyperthermic Atlantic salmon (*Salmo salar*). BMC Physiology (In press)

Paper II

Ytteborg, E., Torgersen, J., Baeverfjord, G., Takle, H. Morphological and molecular characterization of developing vertebral fusions using a teleost model. Manuscript (submitted)

Paper III

Ytteborg, E., Torgersen, J., Pedersen M., Baeverfjord G., Hannesson K. and Takle H. Remodeling of the notochord during development of vertebral fusions in Atlantic salmon (*Salmo salar*). Manuscript

Paper IV

Ytteborg, E., Vegusdal, A., Witten, P.E., Berge, G., Takle, H., Østbye, T.K., Ruyter, B. Atlantic salmon precursor cells from white muscle differentiate into osteoblasts in vitro*:* Polyunsaturated fatty acids and temperature influence gene expression and PGE_2 production. Biochemica et Biophysica Acta, Molecular Cell Research, 1801 (2010); 127-137.

Summary

Problems with spinal disorders in Atlantic salmon (*Salmo salar*) have been increasingly in focus due to the importance of this species in the aquaculture industry. Until recently, the molecular development of spinal deformities in fish has received relatively little attention and most studies have been largely descriptive. Due to economical matters of the industry, previous deformity studies were primarily based on radiographic findings, histological staining and field studies to reveal factors inducing deformities. Consequently, few deformities have been explored beyond the level of association with particular causative factors. However, accumulated studies on intensive production regimes and incidence of deformities have been followed by more advanced studies on vertebral development and bone biology. A better understanding of cellular and molecular events during bone development in teleosts should enable us to better characterize the pathology, define particular requirements and enable us to minimize the occurrence of bone disorders.

To increase the understanding of normal and pathological bone development in Atlantic salmon, fish was exposed to two different temperature regimes from fertilization until 15g size. Fish exposed to high temperature regimes showed a markedly higher growth rate and a significant higher percentage of vertebral fusions than fish reared at low temperatures. The major aim of paper I, II and III was to study the long term effect of hyperthermic conditions upon bone development in Atlantic salmon with focus on pathological development of spinal fusions. Analyzing non-deformed vertebrae from the two temperature regimes (paper I) revealed that the increased risk of developing vertebral fusions was linked to a down-regulated transcription of genes involved in production and mineralization of extracellular matrix components. Furthermore, morphological changes in the arch centra identified chondrocytes with a distorted maturation pattern and an increased zone of hypertrophic chondrocytes. The data presented indicate that production of both bone and cartilage is disrupted when fast growth is promoted. Paper II and III were devoted to characterize developing spinal fusions, hence describe typical hallmarks in the fusion process. An intermediate and a terminal stage of the fusion process were studied at a morphological level using radiography and histology and at a gene expressional level using quantitative real-time PCR, *in situ* hybridization and immunohistochemistry. In paper II the focus was directed towards bone and cartilage formation in the centra, whereas in paper III the notochord was analyzed. The development of vertebral fusions is a dynamic process, but as suggested in this thesis, the process

may be summarized as four major events. First, disorganized and proliferating cells were prominent at the vertebral growth zones and in the notochord. The marked border between the osteoblast growth zones and the chondrocytic arches became less distinct, as proliferating cells and chondrocytes blended through an intermediate zone (paper II). Second, *in situ* hybridization visualized that proliferating cells in the intermediate zone co-expressed mixed signals of chondrogenic and osteogenic markers, suggesting a metaplastic shift in these cells (paper II). A similar shift also occurred in the notochord where proliferating chordoblasts changed transcription profile to be more osteogenic (paper II and III). Third, as the pathology progressed, the notochordal sheath stretched and a thinner and more fragmented elastic membrane was detected (paper III). Immunohistochemistry further revealed that the structural organization in the notochordal sheath was altered upon development of vertebral fusions. Fourth, ectopic bone formation was found in the arch centra and in the intervertebral regions (paper II). The formation of ectopic bone indicated that the metaplastic shift in proliferating cells led to cells capable of producing mineralized matrix.

The major aim of paper IV was to develop an *in vitro* system for Atlantic salmon osteoblasts so that problems related to bone development could be more specifically targeted. Unspecialized primary cells from Atlantic salmon white muscle differentiate in osteogenic medium to osteoblasts-like cells. The cells changed their morphology from elongated to become more cobblestone, started expressing osteoblast specific markers and stained for Alkaline phosphatase during the differentiation period. The differentiated cells were further used to study the effects of two factors that influence bone formation in Atlantic salmon under commercial farming conditions; elevated temperature and polyunsaturated fatty acid composition. The *in vitro* response showed resemblances with *in vivo* findings, supporting that we had succeeded in differentiating the precursor cells to become osteoblast like cells.

Through the work presented in this thesis we have added knowledge to both normal and pathological development of the Atlantic salmon vertebrae. Most markers for bone and cartilage development had not previously been described in Atlantic salmon. The defined markers can be used to investigate how the progression of skeletogenesis is modulated by a variety of factors and reveals the potential use of gene transcription profiling as a prognostic approach in aquaculture. Moreover, Atlantic salmon has shown to be comparable to mammalian models used in revealing the complex pathology involved in the development of spinal malformation.

Sammendrag

Problemer med deformerte ryggvirvler hos Atlantisk laks (Salmo salar) har vært stadig mer i fokus på grunn av dens betydning i oppdrettsnæringen. Inntil nylig har den molekylære utvikling av ryggdeformiteter i fisk fått relativt lite oppmerksomhet og de fleste studier har i stor grad vært deskriptive. Basert på økonomiske hensyn har tidligere studier primært vært basert på å avdekke spesifikke utløsende faktorer som induserer deformiteter. Til dette har man benyttet forskingsverktøy som røntgen, histologi og feltstudier. Studier av intensive produksjonsregimer og forekomst av misdannelser har etter hvert blitt fulgt av mer avanserte studier av ryggvirvelutvikling og benbiologi. En bedre forståelse av cellulære og molekylære mekanismer i benutviklingen hos teleoster vil gi oss et bedre grunnlag til å forstå patologien, bidra til å definere spesielle behov og dermed minske forekomsten av skjelettdeformiteter.

Målet med artikkel I, II og III var å studere den langsiktige effekten av hyperthermi på benutvikling hos Atlantisk laks med fokus på patologisk utvikling av fusjoner. Det ble gjennomført et eksperimentelt forsøk hvor fisk ble eksponert for to ulike temperaturregimer fra befruktning frem til 15g størrelse. Fisk utsatt for høy temperatur viste en langt høyere vekstrate og en betydelig høyere andel av vertebrale fusjoner enn fisk som hadde blitt utsatt for lave temperaturer. Ved å analysere ikke-deformerte virvler fra de to temperaturregimene viste det seg at den økte risikoen for å utvikle fusjoner var knyttet til en nedregulert transkripsjon av gener involvert i produksjon og mineralisering av ekstracellulær matriks (artikkel I). Det ble også funnet at rask vekst forstyrrer produksjon av både ben- og bruskdannelse. Morfologiske analyser av bruskområdene viste at chondrocytene hadde et forandret modningsmønster og en økt sone av hypertrofe chondrocyter. Dataene som presenteres viser at rask vekst forstyrrer produksjon av både ben- og bruskdannelse. I artikkel II og III ble typiske utviklingstrekk i fusjonsprosessen beskrevet. Et intermediært og et terminalt stadium av fusjonsprosessen ble studert morfologisk ved hjelp av røntgenundersøkelser og histologi og på et molekylært nivå ved hjelp av kvantitativ real-time PCR, *in situ* hybridisering og immunohistokjemi. I artikkel II var fokuset rettet mot ben- og bruskdannelse i virvlene, mens i artikkel III ble notokorden analysert. Utviklingen av vertebrale fusjoner er en dynamisk prosess, men som foreslått i denne avhandlingen kan prosessen oppsummeres i fire store hovedmomenter. For det første observerte vi uorganiserte og prolifererende celler i vekstsonene på toppen av

virvlene. Grensen mellom osteoblast- og chondrocytområdene ble mindre tydelig ettersom de prolifererende cellene blandet seg og dannet en overgangssone (artikkel II). For det andre viste *in situ* hybridisering at disse prolifererende cellene uttrykte både chondrogene og osteogene genmarkører, noe som tyder på et metaplastisk skifte i disse cellene (artikkel II). Et tilsvarende skifte oppstod også i notokorden der prolifererende chordoblaster forandret transkripsjonsprofil til en mer osteogen fenotype (artikkel II og III). For det tredje ble notokordskjeden strukket og den elastiske membranen som omgir notokorden ble tynnere og mer fragmentert ettersom patologien utviklet seg (artikkel III). Immunohistokjemi avdekket videre at den strukturelle oppbyggingen av notokordskjeden ble forandret under utviklingen av fusjoner. For det fjerde ble ektopisk bendannelse oppdaget i bruskområder og i intervertebrale områder (artikkel II). Dannelsen av ektopisk ben indikerte at det metaplastiske skiftet i prolifererende celler førte til celler som var i stand til å mineralisere matriks.

Det primære målet i artikkel IV var å utvikle et *in vitro* system for osteoblaster fra Atlantisk laks slik at man lettere kan studere spesifikke problemer tilknyttet benutvikling. Uspesialiserte primærceller fra laksens hvite anaerobe muskler ble dyrket i osteogent medium til osteoblast-lignende celler. I løpet av differensieringsperioden forandret cellene morfologi fra en avlang form til å bli mer firkantet, de uttrykte osteoblast-spesifikke markører og farget for alkalisk fosfatase. Videre ble de differensierte cellene brukt til å studere effekten av to faktorer som påvirker bendannelse hos oppdrettslaks; høy temperatur og sammensetningen av flerumettede fettsyrer. *In vitro* responsen viste likheter med in vivo funn og støtter opp om at vi har lykkes i å differensiere forløperceller til osteoblastlignende celler.

Gjennom det arbeidet som presenteres i denne avhandlingen har vi fått en bedre kunnskap om den normale og den patologiske utvikling av ryggvirvlene hos Atlantisk laks. De fleste markører for ben- og bruskutvikling har ikke vært beskrevet tidligere i laksefisk. De definerte markørene kan brukes til å undersøke hvordan utviklingen av ryggraden påvirkes av en rekke faktorer og viser potensialet for bruk av gentranskripsjon som en prognostisk tilnærming i akvakultur. Videre har Atlantisk laks vist seg å være sammenlignbar med pattedyrmodeller som brukes til å undersøke den komplekse patologien involvert i utviklingen av ryggmisdannelser.

General Introduction

Atlantic salmon (*Salmo salar*) makes up 85% of the total sale of Norwegian farmed fish, with a production of more than 850 000 tons (Directorate of Fisheries, 2009). Compared to 425 000 tons in 1999, the amount of produced fish has been more than doubled the last 10 years. To keep up with the growing demand, the aquaculture industry is constantly searching for new strategies to improve the rearing conditions and reduce production time and cost. Elevated temperature during the fresh water period was commonly used in the 90`ies to speed up developmental rate. However, an increasing number of fish developing skeletal abnormalities was observed (Figure 1). Recommendations limiting temperatures to safe levels, ≤8°C during egg rearing and ≤12°C after fist feeding, led to substantial reductions in skeletal malformations [1,2]. However, in the last two years, the start feeding temperature has been increased again, due to the stakeholders demand for reduced production time. Further, the growing need of replacing fish meal in commercial fish feeds have come into focus and deformities related to feed ingredient replacements, malnutrition and mineral deficiency are investigated. Cultured salmon is bred for rapid growth, and the industry will aim at obtaining the optimal growth rate by optimizing both diets and environmental factors accordingly. It is therefore important to completely understand the molecular and cellular events in bone development in salmon in order to deal with upcoming questions.

Figure 1. Deformed (top) and non deformed Atlantic salmon and corresponding radiographic pictures. Photo: Grete Baeverford, Nofima.

The spinal column

The vertebral column is the defining feature of all vertebrates, composed of an alternating pattern of vertebral bodies (centra) and intervertebral regions. While centra give support and strength to the organism, intervertebral regions provide flexibility. The notochord is found in embryos of all chordates, being well conserved between species as the forerunner of the spinal column. However, whereas only remnants of the notochord exist in the mammalian intervertebral disc (IVD) between

adjacent vertebrae [3], the notochord persists throughout all life stages and throughout the entire length of the fully developed vertebral column in many teleosts including Atlantic salmon. The segmented pattern of the spine is established during embryogenesis when the precursors of the vertebrae, the somites, are formed. The somites form in pairs as epitheloid blocks of cells, which further develops into sclerotomes and myotomes (review [4]). The sclerotome loses its epitheloid character and becomes mesenchymal, contributing to the formation of vertebral structures. The Atlantic salmon vertebrae consists of approximately 58 vertebral bodies with neural and heamal arches protruding from the top and bottom of the centrum, respectively [5].

The notochord

The notochord is a flexible, rod-shaped body of cells derived from the mesoderm, which defines the primitive axis of chordate embryos [6]. In the early gastrula of teleosts, notochordal cells (the chordoblasts) arise from the chordamesoderm during the formation of the embryonic shield, which is comparable to the mammalian dorsal organizer [7]. Within the chordamesoderm, cells move towards the dorsal midline, align, divide and give rise to the notochord [8-11]. The mature notochord of Atlantic salmon consists of a core of chordocytes, a layer of chordoblasts, an acellular fibrous sheath and an outer elastic membrane (Figure 2) [12].

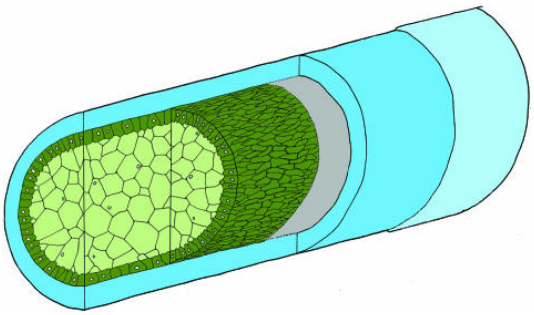

Figure 2. Schematic view of the main structural components of the notochord in Atlantic salmon. The chordocytes (light green) a monolayer of chordoblasts (green), basal lamina (grey), notochordal sheath (blue) and external elastic membrane (light blue). Revised from Grotmol et al. 2005 [17].

The chordoblasts continue to divide throughout life in accordance with sustained notochordal growth [12]. They further maturate into chordocytes, containing large fluid filled vacuoles which functional role is to maintain internal hydrostatic pressure [8,10,13]. Moreover, the chordoblasts produce the basal membrane and extracellular matrix (ECM) components of the notochordal sheath, which in both mammals and teleosts, like Atlantic salmon, has been shown to consist of mainly collagen (col) type 2 [14-16]. The acellular sheath and the elastic membrane surrounding

the teleost notochord restricts expansion of the vacuolated chordocytes [17], thus generating the hydroskeletal properties [18]. In Atlantic salmon, the notochord is absolute essential for locomotion and stiffening of the larvae since cartilage and bone develop post hatch (around 350-500 day degrees post fertilization, dpf) [7]. In addition to its structural role, the notochord secrete factors to surrounding tissues and contribute to vertebral patterning during embryogenesis [19,20]. The role of the notochord in patterning of the somites is known from several studies from chicken, mouse and zebrafish, 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 (review [21]). In vertebrate species with limited growth, such as humans, the notochord ceases its regulating role for vertebral development as part of the normal ontogeny, followed by the transformation of notochordal tissue into cartilage [22,23]. In Atlantic salmon, however, the notochord should fulfill its regulating role for vertebral body differentiation throughout life, since salmon and other fish species do not stop growing. In salmon and zebrafish, the notochord directly initiates vertebral development by forming the initial structural elements of the vertebral body, the mineralized chordacentrum [17,20].

The vertebrae

Atlantic salmon spinal column is formed directly in bone, in contrasts to the formation of the vertebrae of avian and mammalian species, which are first formed in cartilage [24,25]. Grotmol et al. [12,13,17,26] have described four distinct layers of bone and mineralized tissue that form the vertebral bodies in Atlantic salmon. The different layers are shown in Figure 3.

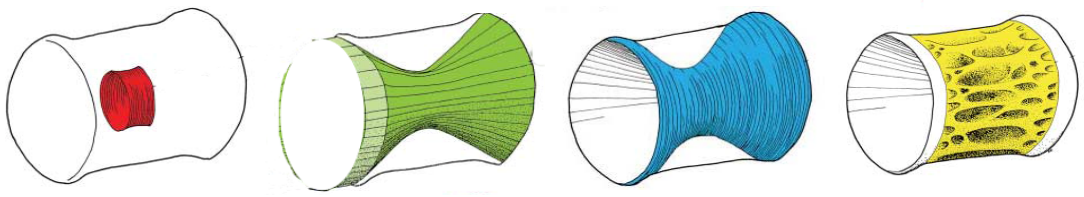

Figure 3. Schematic illustrations of the layers within the vertebral body of Atlantic salmon. Layer 1 (chordacentrum, red) forms through mineralization of the notochordal sheath; Layer 2 (intervertebral ligament, green) a continuous, thin layer encases the entire notochord with vertebral part of the layer (green) and a part participating in forming the intervertebral ligament (light green); Layer 3 (amphicoel, blue) the compact bone; Layer 4 (arcosentrum, yellow) the cancellous bone of the trabeculae. Revised from Nordvik et al. 2004 [13].

The two inner layers comprise the chordacentrum (mineralization of the notochord sheath) and the inner layer of the amphicoel (mineralization of the intervertebral ligament), whereas the two outer layers are deposited by osteoblasts, forming the main portion of the amphicoel (autocentrum) and the cancellous bone of the trabeculae (arcocentrum). Throughout the early yolk-sac stage the notochord is un-segmented with a uniform notochordal sheath of even thickness surrounded by the sclerotome consisting of undifferentiated mesenchymal stem cells (MSCs) [17,26]. The MSCs are situated on the external elastic membrane only interrupted by the neural and haemal arch cartilages, which are formed around 300dpf. In teleosts in general, the development and ossification of the neural and haemal arches precede those of the vertebral bodies [27-29]. In Atlantic salmon and other teleosts, the segmentation process leading to formation of vertebral and intervertebral regions starts with the formation of the chordacentra, where matrix in the outer half of the notochordal sheath becomes mineralized [20,24,26,30]. This mineralization process starts at approximately 680dpf in the region beneath the dorsal fin along the ventral midline on the inside of the elastic membrane [17]. From here it proceeds towards the dorsal side in a bilateral symmetrical manner finally forming a complete cylinder as ring-shaped acellular mineralized zones. Differentiation of sclerotomal MSCs into osteoblasts (bone forming cells) occur outside the chordacentra [31] and it is believed that the chordacentra regulates this differentiation process [17]. The second layer, the intervertebral ligaments, is deposited by fibroblasts on the outside of the chordacentrum and elastic membrane of the notochord. In the region of the chordacentrum, osteoblasts condense outside the intervertebral ligament and deposit osteoid (bone matrix) with concentric collagen fibres orientated circularly. In Atlantic salmon, deposition of osteoid from these osteoblasts starts around 800dpf [17]. Mineralization of the intervertebral ligament is initiated when the external intervertebral ligament is covered with this osteoid [13]. Together, the mineralized ligament and the autocentrum develop into the compact bone of the vertebral amphicoel. Denser osteoblast populations are located along the cranial and caudal rims of each vertebral body, leading to the biconid hour-glass shaped vertebra [30]. At approximately 1000dpf, formation of the arcosentrum starts through deposition of the cancellous bone of the trabeculae. As the arcosentrum grow through the activity of osteoblasts located along the distal ridges of the cancellous bone, the trabeculae becomes more branched and filled with adipose tissue [12,13]. After finishing shaping the amphicoel, the Atlantic salmon vertebrae continue to grow throughout life [13]. An overview of the mature vertebrae of Atlantic salmon is shown in Figure 4.

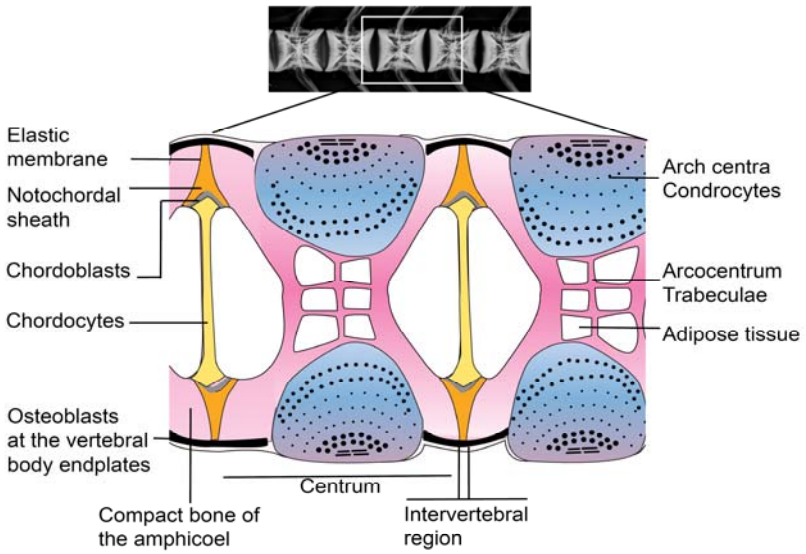

Figure 4. Overview of the mature salmon vertebra.

Skeletal deformities

Deformities in the spinal column have been observed in a diverse array of vertebrates and a number of causatives have been suggested. Spinal disorders are a major concern for human health and often related to painful conditions [32]. Spinal lesions observed in wild animals, such as brown bear, sandtiger shark and smallmouth bass are occasionally found and often reflect environmental problems [33-36]. Deformities in domesticated animals like chicken, broilers, pigs and farmed fish are recognized as a reoccurring problem in intensive production system and represent both ethical and economical challenges for the industry [37-41]. Fish with spinal deformities, such as salmon, trout, cod, halibut, sea bass and sea bream, do not swim efficiently, are less capable of acquiring food, are at a greater risk of predation, are more susceptible to physiological imbalance and are down-graded at slaughter [42]. Witten et al. [43] recently published a survey on commonly observed vertebral malformations in Atlantic salmon which included different grades and combinations of platyspondyly (compressions), ankylosis (fusions), lordosis (V-shaped vertebral column), kyphosis (/\-shaped vertebral column) and scoliosis (S-shaped vertebral column). Histological characterization of compressions and fusions have described shape alterations of vertebral body endplates, reduced intervertebral space, transformation of intervertebral notochord tissue into cartilage, mineralization of the intervertebral cartilage and replacement of intervertebral cartilage by bone [44-46]. Fusion, compression and chondrogenic transformation of skeletal tissue have also been reported from lordosis and kyphosis [47].

Histological examinations of teleosts have further indicated cellular plasticity (like metaplastic shifts and trans-differentiation) and development of intermediate tissues as pathological events [44,46,48,49]. However, most deformity studies in teleosts have been largely descriptive and primarily performed to reveal factors contributing to increased occurrence of skeletal deformities, e.g. genetics, infections, fast growth, light regimes, vaccination, water current and quality, pollution, malnutrition and elevated temperatures [37,50-58].

Temperature

Temperature studies showing increased risk of developing vertebral malformations have been carried out for teleost species like sea bass, sea bream, rainbow trout, cod, halibut and salmon [2,59-61]. However, most temperature studies have focused on teratogenic effects upon heat shock during embryonic development and few studies have focused on the long term effect. In several vertebrates, including Atlantic salmon, the teratogenic effect of temperature is dependent on the developmental stage of the embryo and on the processes taking place [62]. For example, heat shock during somitogenesis is commonly related to segmentation failure [63-65] and these may develop into spinal fusions later in ontogeny [64]. At the cellular level, heat shock may affect the highly ordered sequence of cell proliferation, differentiation, migration, apoptosis and gene expression that characterizes embryonic development [62]. Osteoblasts and chondrocytes (cartilage forming cells) are cell types producing large quantities of ECM and may therefore be particularly sensitive to elevated temperatures, due to reduced normal protein synthesis [66,67]. However, in mammalian osteoblast cultures, heat shock seems to be dosage dependent and may have both positive and negative effects on osteoblastogenesis [68,69]. Exposure to mild heat shock may stimulate osteogenic differentiation and enhance mineralization, whereas long-term treatment may result in inhibited proliferation and reduced mineralization [68-70]. Mammalian chondrocytes are shown to be more thermoresistant than osteoblasts [71], but elevated temperatures may interrupt their normal differentiation pattern and delay endochondral bone formation [66]. Mammalian osteoclasts (calcified matrix resorbing cells) are also temperature sensitive and hypothermic conditions may stimulate their activity [72].

Studies have shown that Atlantic salmon is sensitive to elevated temperatures throughout the entire freshwater period, as elevated temperature regimes before and after start-feeding have independently shown to induce vertebral malformations [73,74]. Moreover, temperature induced

vertebral pathology initiated in the freshwater period may continue to develop during the seawater rearing [2]. Whereas deformities induced late in the development by elevated temperatures may possibly be linked to faster growth and the impact this might have on the natural maturation and ontogeny of the vertebral bodies.

Fast growth

Conditions that accompany fast growth in farmed animals, e.g. light and feeding regimes, elevated temperatures and breeding, are linked to increased numbers of spinal deformities [39,40,75]. Fast growing Atlantic salmon has been shown to develop “soft” (low mineralized) bone compared to fish with lower growth rates [76] and to have an increased risk of developing vertebral deformities [77,78]. In fast growing Atlantic salmon, elevated muscle mass exercise pressure on under-calcified bone that increases the mechanical pressure, which might trigger formation of intermediate tissues and malformations [44]. Comparative studies have been performed in commercially farmed chicken, which are the product of long-term selective breeding for high growth rates [79]. Fast growing chicken have weaker bone structures and increased rates of skeletal abnormalities than slower growing broilers, which reduces the bone's ability to adapt to the higher loads induced by the increasing body weight [80]. In Atlantic salmon, however, high genetic growth rates has not been correlated to increased rates of deformities [53]. At the cellular level, a general trade-off between proliferation and differentiation has been suggested as a cause for delayed skeletal development in fast growing species of birds [80,81]. It has further been suggested that during rapid growth the time required for bone matrix to be produced and mineralized may be reduced to a critical level [82]; hence development of a “soft” bone phenotype. This causative relation has been suggested for fast growing under-yearling Atlantic salmon smolt that has a higher incidence of vertebral deformities than slower growing yearling smolt [76]. Fast growing chickens are also characterized by disturbed chondrocytic maturation [83], where cartilage do not mature enough to ossify [84]. Overall, both bone and cartilage formation is disturbed during fast growth and may equally contribute to weakened skeletal structures. It is however possible that malformations linked to both elevated temperature and fast growth could be related to secondary effects, such as alterations of nutritional preferences. Limited vitamin C make for example rainbow trout more sensitive to elevated temperature [85] and elevated dietary mineral content may reduce the prevalence of vertebral deformities in fast growing Atlantic salmon [86].

Nutrition

Due to the growing need of replacing fish meal in commercial fish feeds, the use of vegetable enriched diets has increased during the latest years. However, an increased risk of developing skeletal malformations is linked to replacing feed ingredients. Nutritional imbalances, including minerals such as phosphorous, magnesium and zinc, vitamins like A, C, or D and phospholipids may contribute to an increased frequency of skeletal abnormalities (review [87]). Helland et al. [48] suggested that high levels of phytic acid in vegetable feed ingredients may impair the availability of essential minerals and lead to increased occurrence of hyperdense vertebrae. In teleosts, calcium and phosphorous are the main constituents of the mineral fraction in bone. Phosphorus is mainly absorbed through the diet, and deficiency may cause abnormally soft and malformed bones in salmonids [88,89]. Vitamin D_3 is involved in the regulation of mineral homeostasis [90,91], whereas Vitamin C is important for collagen formation [85,92]. In both mammals and teleosts, high doses of vitamin A have shown teratogenic effects similar to those induced by elevated temperatures [73,93,94]. Furthermore, the diets used for farmed salmon have traditionally been based on fish oils rich in polyunsaturated fatty acids (PUFAs) of the n−3 type, such as Docosahexaenoic (DHA) and Eicosapentaenoic acid (EPA) and replacing fish oils with vegetable oils will reduce their concentration in the feed [95,96]. Lipid nutrition has an important function in bone biology, as demonstrated in several vertebrate species [97,98]. Whereas n-3 PUFAs have beneficial effects on bone mineral density, increased n−6/n−3 fatty acid ratios may reduce bone formation in both mammals and Atlantic salmon [97,99-102]. Recent studies on sea bass larvae have shown that the ratios of dietary PUFAs are directly related to vertebral malformations [103]. Overall, in situations with altered dietary mineral supply, growth may be maintained at the expense of normal bone development. A better understanding of cellular and molecular events during bone development in teleosts should enable us to better characterize the pathology, define particular requirements and enable us to minimize the occurrence of bone disorders.

Cellular and molecular regulation of bone remodeling

Osteoblasts and chondrocytes are both derived from the MSC lineage [104], whereas osteoclasts are derived from haemopoietic stem cells (HSC) [105]. MSCs may also give rise to adipocytes and myocytes [104] and HSCs to blood and lymphoid lineages [106,107]. The key genetic factors regulating lineage determination and differentiation of MSC and HSC are conserved among

vertebrates at the molecular level in both sequence and expression pattern [108-111]. A complex set of molecular pathways, in particular members of the Wnt family, bone morphogenetic proteins (Bmps) and other members of the transformin growth factor ß (TGF-ß) superfamily, the hedgehog proteins (indian and sonic), fibroblast growth factors (FGFs) and cytokines activate key transcription factors involved in bone and cartilage development [112-115]. Bone formation may further occur via two basic mechanisms: MSC either differentiate directly into bone producing osteoblasts (intramembranous ossification) or by first forming a cartilaginous template secreted by chondrocytes which is later replaced by bone (endochondral ossification) [112]. In teleosts like Atlantic salmon, compact bone of the amphicoel and trabeculae is formed directly through intramembranous ossification, whereas the arch centra are modeled through endochondral ossification [116].

Osteoblasts differentiation, bone formation and mineralization

The differentiation of MSC into mature osteoblasts involves several phases, which may be divided into three subsequent stages; commitment, extracellular matrix production and mineralization. Each stage is characterized by expression of distinct osteoblast markers regulated by major signaling pathways. The key markers involved in osteogenesis are shown in Figure 5.

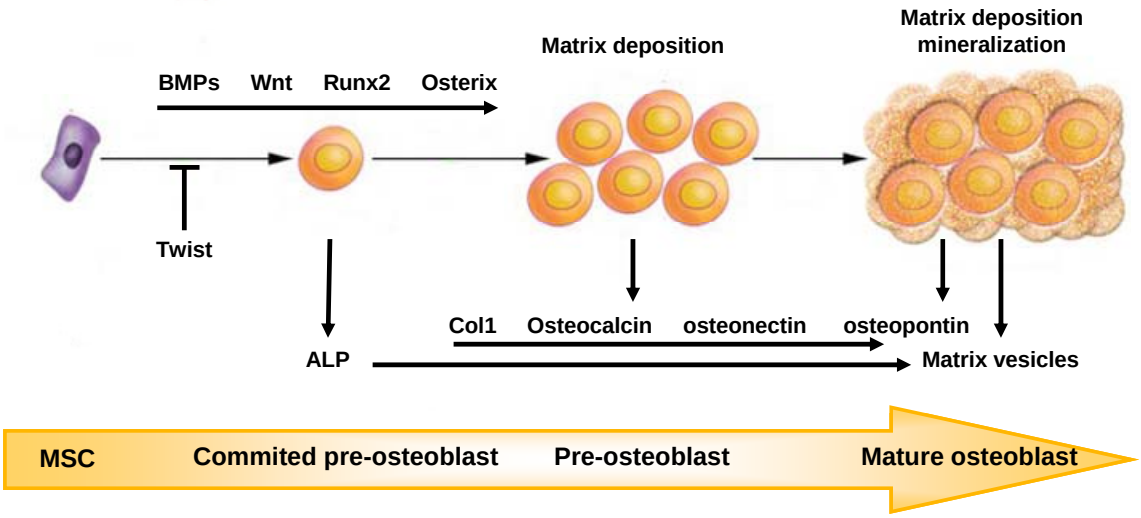

Figure 5. Osteoblast differentiation, maturation and key factors involved. After commitment to the osteoblast lineage, matrix deposition starts. Mature osteoblasts are responsible for both osteoid production and mineralization. See text for details. Redrawn from Krishnan et al. 2006 [122].

Among the downstream targets of Bmp2 are two key regulators for osteoblasts: Runt-related transcription factor 2 (Runx2) and the zinc finger containing transcription factor Osterix [119-121]. Estrogen and 1,25-dihydroxy vitamin D_3 are among the hormones shown to increase osteogenic differentiation via up-regulation of osteogenic growth factors, such as BMP2 [117,118].. The Wnt/ß-catenin pathway may also induce Runx2 expression in MSC [122]. Runx2 is further regulated through phosphorylation or interactions with other transcription factors, such as the basic helix-loop-helix (bHLH) transcription factor Twist [123]. Twist works as a negative regulator of osteoblastogenesis by inhibiting expression of genes downstream of Runx2 [123]. Downstream targets of Runx2 and Osterix include both collagenous (e.g. col1α and col1ß) and non-collagenous (e.g. osteopontin, osteocalcin, osteonectin, bone sialoprotein and alp) proteins, which make osteoblasts capable of producing and mineralizing bone matrix (osteoid). Col1 is the major structural component of bone, whereas the non-collagenous proteins binds inorganic minerals and are involved in the mineralization process [124-127]. Collagen synthesis is dependent on vitamin C, a cofactor participating in the hydroxylation of proline and glycine, turning pro-collagen into mature collagen [128].. Bone matrix is first deposited as un-mineralized osteoid. A time lag where collagen synthesis decreases and mineralization increase appears to be required for allowing modifications of the osteoid so that it is able to support mineralization and hydroxyapatite ($Ca_{10}[PO_4]^6[OH]^2$) formation [82]. Mineralization of bone occurs by deposition of inorganic hydroxyapatite crystals in the ECM. The initiating step of hydroxyapatite formation occurs in ECM vesicles secreted from mature osteoblasts [129,130]. These vesicles create an environment where deposition of minerals (mainly Ca^{2+} and P_i) occurs and hydroxyapatite is produced, a process involving proteins like annexins and alp [131,132]. The attachment of the vesicles to bone is not well understood, but both alp and annexin are reported to anchor to col1 fibrils [133]. Vesicle formation is followed by the linking of hydroxyapatite crystals to ECM components [131]. Osteonectin, Osteopontin, Osteocalcin and Bone sialoprotein all contain Ca^{2+} and hydroxyapatite binding properties [134-136]. As mineralization proceeds, some osteoblasts become entrapped in bone matrix and are called osteocytes. An important role of the osteocytes is to participate in bone mineral homeostasis, assist in regulation of bone resorption and to function as sensors for stress and strain [137-139]. Most teleost have acellular bone, however members of the salmonides and cyprinides (e.g. zebrafish) have osteocytes, characterizing their bone type as cellular [140,141]. Although similar functions may apply for teleost osteocytes, their functions have not been thoroughly studied.

Chondrocyte differentiation and endochondral ossification

The chondrocytes undergo a synchronized process of proliferation, differentiation and maturation. Thus, three zones can be identified in the growing cartilage: resting, proliferating and hypertrophic zones [142]. Chondrocytes in the resting zone are irregularly scattered in cartilage matrix, whereas chondrocytes in the proliferating and hypertrophic zones are arranged in columns [142].The key markers involved in chondrogenesis are shown in Figure 6.

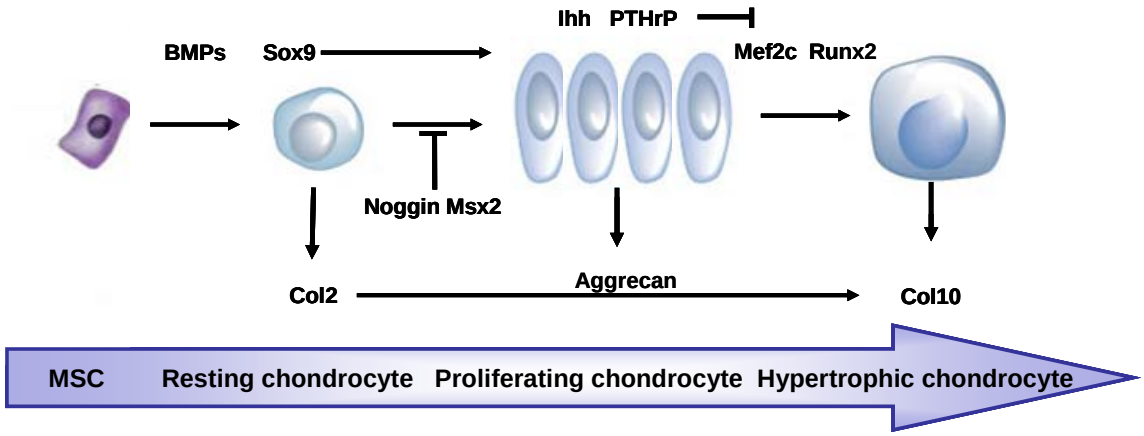

Figure 6. Chondrocytic differentiation, maturation and key factors involved. Resting, proliferating and hypertrophic chondrocytes are clearly visible as zones in the growth plate. See text for details.

The chondrocytes in the resting zone serves as stem-like cells in the growth plate, stimulated by e.g. growth hormone (GH) [143]. Early differentiation of MSC into resting chondrocytes is controlled by Bmps that induce expression of Sex determining region Y box 9 (Sox9) [144]. This transcription factor regulates transcription of col2, the major ECM component of cartilage [145]. Noggin and Muscle segment homeobox-2 (Msx-2) may inhibit expression of Sox9 [146,147]. The proliferating zone is the region for active cell replication and chondrocytes in this zone are mostly devoted to cell cycle processes [148]. When chondrocytes divide, two daughter cells line up in the axis along the bone growth [149]. Parathyroid hormone related protein (PTHrP) and Ihh appear to play important roles in proliferating chondrocytes by maintaining cells in a proliferative condition, hence preventing chondrocyte hypertrophy [150-152]. Chondrocyte hypertrophy is the final step of chondrocyte maturation, regulated by the transcription factors Myocyte enhancer factor 2c (Mef2c) and Runx2 [153,154]. After commitment to the hypertrophic state, chondrocytes start expressing Col10 [153]. Col10 is a unique component of the matrix produced by hypertrophic cells and

extensively used as a marker for chondrocyte hypertrophy [155]. Once hypertrophy is reached, matrix calcification may be initiated [156]. Hypertrophic chondrocytes induce angiogenesis by secreting angiogenetic factors such as the Matrix metalloproteinases (Mmps) [157]. Upon vessel formation, osteoblasts and osteoclasts may enter and initiate endochondral bone formation. Terminally differentiated hypertrophic chondrocytes may die by apoptosis (review [158]). However, hypertrophic chondrocytes are also capable of initiating calcification processes by releasing similar matrix vesicles as osteoblasts and it has been suggested that hypertrophic chondrocytes may participate actively in bone formation [159,160]. Moreover, hypertrophic chondrocytes express genes like osteocalcin, osteonectin and alp [161,162]. Cancedda et al. [163] showed that hypertrophic chondrocytes from chicken can be induced to obtain a strictly osteoblastic phenotype *in vitro.* These findings are supported by Yasui et al. [164] who suggested that hypertrophic chondrocytes are able to trans-differentiate into osteoblasts and produce bone through a process called trans-chondroid ossification. More than 10 different forms of cartilage and several other tissues with histological characteristics between bone and cartilage have so far been identified in fish [140,165]

Osteoclast differentiation and bone resorption

Both mineralized bone and cartilage is broken down through the activity of osteoclasts, cells involved in removing damaged bone, repair mechanisms, mineral homeostasis and replacement of cartilage with bone (review [113]). Osteoclasts provide an acidic environment where mineralized matrix may be dissolved through secretion of cathepsins, mmps and tartrate resistante acid phosphatase (TRAP) [166-169]. As in mammals, osteoclasts in Atlantic salmon are multinucleated and the mechanisms involved in activation and differentiation of osteoclasts are conserved (review [170]). However, mononucleated osteoclasts are also found in both mammals and teleosts and are considered to participate in minor, fine tuning bone resorption [170]. Since teleost lack haemapoietic tissue in bone marrow, the question of the origin of these cells remains unknown. Mononuclear cells respond to macrophage colony stimulating factor (M-CSF) produced by nearby stromal cells and osteoblasts, through activation of c-fms, the receptor for M-CSF [171,172]. The other signaling system essential for osteoclast differentiation is triggered when receptor activator of nuclear factor kappa (κ) B ligand (RANKL), a member of the tumor necrosis factor (TNF) family,

activates its receptor RANK (review [173]). The key markers involved in osteoclastogenesis are shown in Figure 7.

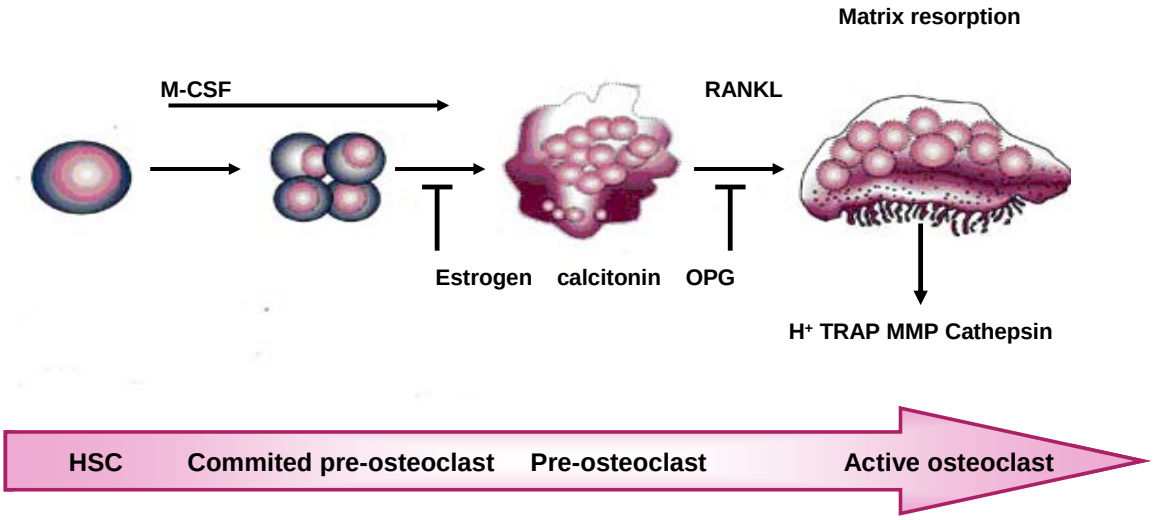

Figure 7. Osteoclast differentiation, maturation and key factors involved. Fully mature osteoclasts are able to dissolve bone. See text for details. Redrawn from Boyle et al. 2003.

Several transcription factors have been found crucial for osteoclast differentiation downstream of M-CSF/c-fms and RANKL/RANK signaling. Amongst these genes is the Microphthalmia-associated transcription factors (MITF) which directly regulates genes important for osteoclast function (e.g. TRAP and Cathepsin K) [174]. Osteoclast differentiation may be inhibited by Osteoprotegerin (OPG), a RANKL decoy receptor secreted by stromal cell and osteoblasts [175]. Furthermore, estrogen and calcitonin are the main osteoclast inhibiting hormones, whereas PTHrP and Vitamin D are the main osteoclast stimulating hormones [176,177]. Once activated, osteoclasts move to areas where bone is to be resorbed and attach to the surface [178]. A H^+-ATPase proton pump linked to Cl^- transporters/channels facilitates the release of H^+ into the sealed lacuna, dissolving the underlying minerals and generates high local levels of Ca^{2+} and P_i [179-181]. TRAP, MMPs and Cathepsin K are also secreted through the ruffled border to digest organic matrix [167-169,182]. In the vertebrae of Atlantic salmon, multinucleated osteoclasts have been identified in the arch centra and trabeculae but not in the compact bone of the amphicoel [170].

General Discussion

Spinal deformities in Atlantic salmon have been intensively studied during the past years due to the importance of this specie to the aquaculture industry. However, previous studies have been largely descriptive and based on x-ray findings, histological staining and field studies to reveal important factors inducing deformities. By combining molecular tools with targeted x-ray based sampling and histology, a more complete description on how spinal deformities in Atlantic salmon develop are presented. To increase the understanding of normal and pathological bone development in Atlantic salmon we exposed fish to two different temperature regimes. During egg and yolk stage, low temperature group was reared at 6±0.3ºC whereas high temperature group was reared at 10±0.3ºC. Temperature was gradually increased at first feeding (750dº) to 10±0.3ºC and 16±0.3ºC for low and high temperature group, respectively. These regimes were kept until 20g, thereafter both groups were held at ambient temperature until termination at 60g. Similar temperature regimes have previously been shown to increase both growth rate and the number of spinal deformities in Atlantic salmon [74,76]. As expected, fish reared at high temperature regime grew significant faster compared to those reared at low temperature, e.g. the former group reached 2g in 6 weeks and 60g in 7 months post first feeding compared to 11 weeks and 10 months for the latter group. Rearing at high temperatures further resulted in higher frequencies of vertebral deformities with the main type being vertebral fusions. At 60g size, more than 28% had developed fused vertebral bodies in the high temperature group compared to 8% in the low temperature group. Most of these fusions were located underneath the dorsal fin. As previously described, the initial steps in the mineralization of the vertebrae starts with the formation of chordacentra underneath the dorsal fin [17]. This process was progressing at the time when temperature was increased in this experiment, which might explain the increased risk of malformations in these regions. However, temperatures ≥8ºC prior to hatch is also considered high, increasing the risk of malformations. The vertebral pathology observed in this study was therefore most likely induced both during the embryonic development and after start-feeding since the incidence of deformities continued to increase throughout the experiment.

Non-deformed fish from the low and high temperature group

In order to understand the susceptibility of developing deformities in the high temperature group, non-deformed fish from the two temperature regimes were compared (paper I). One of the

challenges for salmon deformity studies has been the lack of information on bone formation during development and reliable biochemical markers to characterize specific changes. Further analyses were accomplished through the identification of more than 20 genes involved in the formation of the Atlantic salmon vertebrae. Importantly, results showed similar spatial expression with other vertebrates supporting that most of the factors and pathways that control skeletal formations are conserved in vertebrates [135,183,184]. An overview of major findings in normal vertebrae is shown in Figure 8.

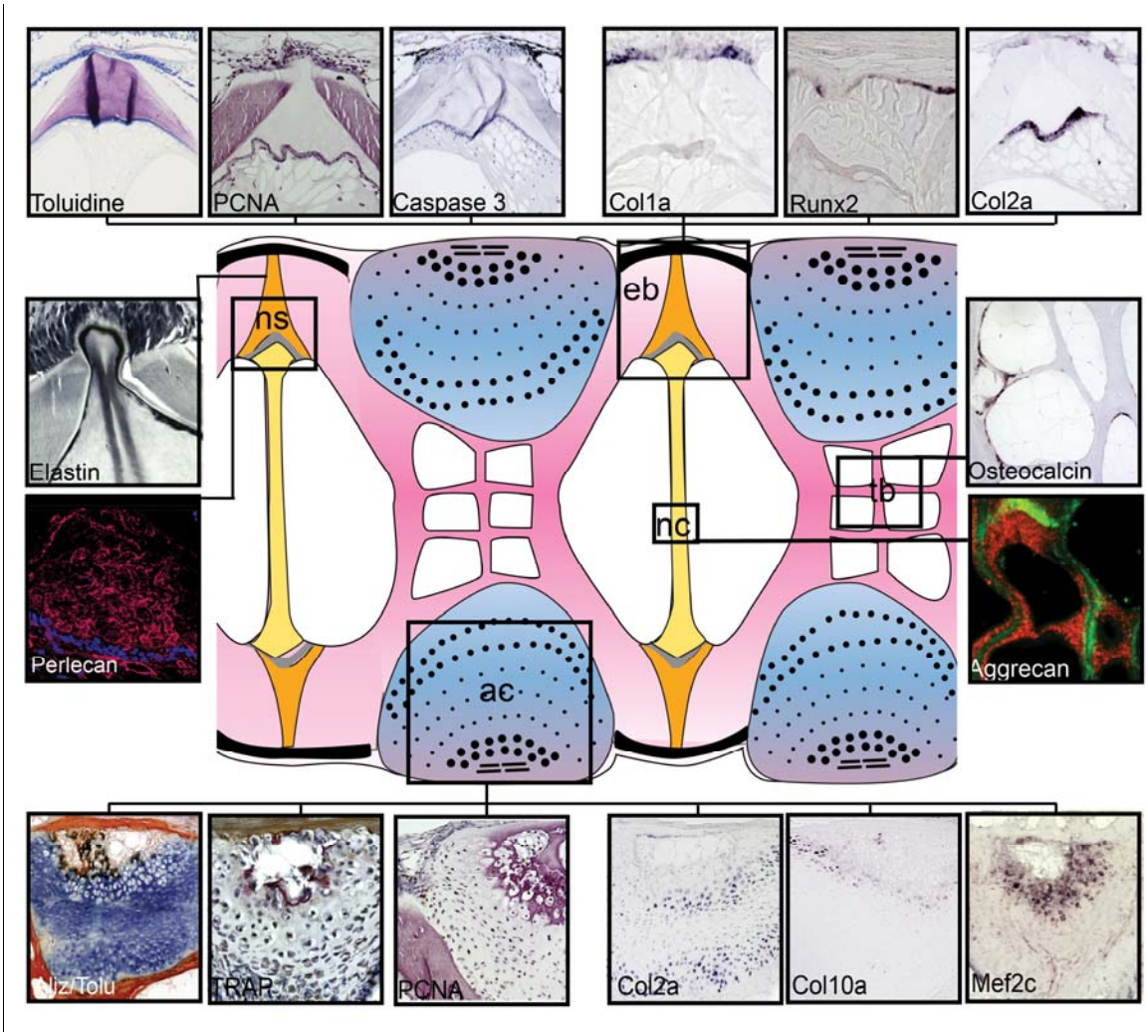

Figure 1. Overview of histological, immunohistochemical and molecular findings in non-deformed vertebrae. Vertebral endbones (top): Toluidine, PCNA, Caspase 3, *col1a, runx2*, *col2a* Elastin in elastic membrane (left), Perlecan in notochordal sheath (left). *Osteocalcin* in trabeculae (right). Aggrecan in chordocytes (right) Arch centra (bottom): Alizarin red/Toluidine blue, TRAP, PCNA, *col2a, col10a, mef2c*. Trabeculae, tb; Notochordal sheath, ns; Notochord, nc. Redrawn from paper I, II and III.

In osteoblast at the vertebral growth zones and in osteoblasts lining the trabeculae, *in situ* hybridization (*ISH*) confirmed the transcription of the osteogenic marker genes *runx2, col1a, osteocalcin* and *osteonectin* (Paper I and II). Weaker signals were detected in fibroblasts lining the intervertebral ligament. Restricted proliferation and apoptosis at the osteoblast growth zones of the vertebral body endplates were shown with proliferating cell nuclear antigen (PCNA) and caspase 3 antibodies, respectively (Paper II). In the arch centra *ISH* identified sub-populations of chondrocytes corresponding to the resting, proliferating and hypertrophic chondrocytes described in mammals [142]. Chondrogenic markers *col2a, col10a, sox9* and *mef2c* identified chondrocytes in the specific maturation zones, where *col10a* and *mef2c* marked hypertrophy (Paper I and II). TRAP activity was present at the ossifying borders and Alizarin red S indicated the ossification front (Paper I). Verhoeff`s hematoxylin stained the elastic membrane surrounding the notochord (Paper III). This membrane had a thickened structure in the intervertebral regions. Immunohistochemistry (IHC) with the PG component Perlecan revealed that this protein is abundantly present in the notochordal sheath (Paper III). This marker further showed that the notochordal sheath have a highly folded structure, which probably contributes to increased flexibility and normal functioning of the mature spinal column. In the notochordal epithelium containing the chordoblasts, *col2* was identified through *ISH,* as previously described [14-16,185]. Substance P (SP) and Aggrecan were expressed in vacuolated chordocytes in the central notochord. Based on findings in non-deformed spinal columns from the low temperature group we suggest that well organized osteoblasts, chondrocytes and chordocytes, strictly controlled proliferation and cell death and a structured notochordal sheath are important for a normal functioning spinal column.

Non-deformed fish from the high temperature group

The non-deformed vertebrae from the high temperature regime appeared shorter in cranio-caudal direction and appeared to have a lower radiodensity than non-deformed vertebrae from the low temperature regime, as observed through radiography (Paper I). Even though malformations were absent, rearing at increased temperatures induced consistent transcriptional changes in several genes that correlated with the observed phenotype and the higher risk of developing vertebral fusions later in ontogeny. Quantification of mRNA revealed a reduced transcription of important genes encoding structural proteins taking part in the bone matrix and mineralization, e.g. *col1a1*, *osteocalcin* and *osteonectin*. Furthermore, generally stronger *ISH* signals in the low temperature

group were detected for probes targeting these ECM transcripts in areas where intramembranous ossification takes place. These findings correlate to an impaired mineralization and support the assumption that disturbances in bone formation constitute an important part of the mechanisms involved in "soft" bone formation [76]. Furthermore, observations were consistent with the osteoblast *in vitro* experiment carried out in paper IV, where long-term 16°C heat exposed cells showed a decreased transcription of *alp*, *col1a1* and *osteocalcin*. Based on *in vitro* and *in vivo* results we suggest that Atlantic salmon osteoblasts may be particularly sensitive to elevated temperatures during the early stages of differentiation. It has further been shown that the matrix structure and composition is important for signal transduction, cell recruitment and organization [186-188]. As for the ECM genes involved in osteoid production and mineralization, high temperature treatment had a significant effect on the transcription of transcription factors (*runx2* and *osterix*) and signaling molecules (*bmp2* and *bmp4*) involved in these processes. Hence, results indicated that osteoblast proliferation and differentiation were restrained in the fast growing group.

Furthermore, results indicated that changes in chondrocytic maturation are important for the susceptibility for developing spinal fusions. Morphological changes in the arch centra identified chondrocytes with a distorted maturation pattern and an increased zone of hypertrophic chondrocytes. The increased zone of hypertrophic chondrocytes correlated with up-regulated transcription of hypertrophic marker genes (*col10a1* and *mef2c*). In addition, TRAP activity, essential for completing endochondral ossification, was absent in the erosive front of the arch centra and *mmp9, mmp13* and *runx2* transcription was severely reduced. Absence of Mmps may cause delays in endochondral ossification and *runx2* deficiency may inhibit *mmp* expression and lead to mild disturbances of chondrocyte differentiation [160,189,190]. Moreover, *ihh*, shown to prevent the final steps in chondrocyte hypertrophy [150,152] had increased transcription. These results suggested an arrest prior to the final maturation of chondrocytes in the high temperature group. A number of studies have linked skeletal malformations to disturbances in chondrocytic maturation [39,84,191]. An interesting comparative pathological condition to our findings has been shown in rat ulnae, where increased loading was associated with an increased hypertrophic zone in the growth plate [192] along with a suppressed mineralization rate [193]. In the high temperature group, delayed endochondral ossification correlated with reduced transcription of genes involved in mineralization. Hence, osteoid production and mineralization through both endochondral and intramembranous ossification seemed delayed in fish from the high temperature regime.

Developing vertebral fusions

Spinal fusions at different developmental stages in Atlantic salmon and other teleosts have previously been described morphologically as shape alterations of vertebral body endplates, reduced intervertebral space, transformation of intervertebral notochord tissue into cartilage, mineralization of the intervertebral cartilage and replacement of intervertebral cartilage by bone [44-46,194]. Paper II and III of this thesis were devoted to further characterize the molecular mechanisms involved in the development of spinal fusions using a combination of specific staining techniques, quantitative and qualitative gene expression analysis and immunohistochemistry. An intermediate and a terminal stage of the fusion process were analyzed to characterize bone and cartilage formation in the centra (paper II) and notochord (paper III) of developing fusions. Both stages were sampled when Atlantic salmon reached 15g. The percentage of vertebral fusions markedly increased from 15g to 60g and pathological changes other than fusions were low in numbers. The development of vertebral fusions is a dynamic process but the underlying cellular and molecular mechanisms may be summarized as four key events. These events included both notochord and centra. Disorganized and proliferating osteoblasts and chordoblasts characterized early stages in the fusion process. A metaplastic shift was further detected through *ISH* in these proliferating cells. As the pathology progressed, the elastic membrane became fragmented and notochordal sheath lost integrity. Finally, ectopic mineralization of intervertebral regions and arch centra was observed. The different events are important in understanding the progression of vertebral fusions and provide basic knowledge on bone formation in Atlantic salmon. The events are more thoroughly described in the following chapters.

1. Disorganized and proliferating cells

Several mammalian studies have suggest that changes in the balance between cell death and cell proliferation is involved in bone and cartilage defects which may lead to malformations [195-200]. Disorganized osteoblasts at the growth zones of the vertebral body endplates were evident in vertebrae with modest alterations, which made us suggest that this is an early event in the fusion process (Paper II). IHC with PCNA further showed that these osteoblasts had a markedly increased cell proliferation rate and that PCNA positive cells in the growth zones extended along the rims of fusing vertebral bodies. The increased proliferation of osteoblasts at the growth zones was partly counteracted by increased cell death as shown by stronger caspase 3 signaling. In

comparison, cultured osteoblasts kept at the same temperature as the high temperature fish (16°C) died after 72 hours (paper IV). In the notochord of developing vertebral fusions, proliferating chordoblasts were detected, however, no caspase 3 staining was observed in these regions (Paper II). As intervertebral space narrowed down, proliferating chordoblasts and denser packet chordocytes were revealed through toluidine blue staining and PCNA antibody binding, respectively. As the pathology progressed, proliferating chordoblasts seemed to occupy most of the intervertebral space and vacuolated chordocytes disappeared. Chordoblasts retain their germinal properties and continue to secrete the notochordal epithelium and notochordal sheath to sustain normal growth [12]. Interestingly, transcription of *laminin*, which has been shown to control cellular differentiation in basement membranes [201-203] was severely reduced throughout the vertebral fusion process (Paper III). In comparison, both zebrafish and mice *laminin1ß* 0-mutants have disrupted formation of basal lamina, which seems to result in disorganization of chordoblasts, notochordal sheath and elastic membrane [201-204]. Overall, developing fusions from the high temperature group had a high number of proliferating cells both at the vertebral growth zone and in the notochord.

2. Metaplastic shift

Witten et al. [44] has previously suggested that a metaplastic shift is involved in the development of spinal fusions, leading to the formation of chondroid bone which at later stages in the fusion process is replaced by bone. We found that a transcriptional shift appeared in proliferating cells at the border between the osteoblast growth zones and the arch centra. This was shown through *ISH*, where co-transcription of osteogenic (*col1a*, *runx2, osteocalcin* and *osteonectin*) and chondrogenic (*col2a, mef2c* and *col10a*) marker genes were detected (paper II). The marked border between the osteoblast growth zones and the chondrocytic areas connected to the arches became less distinct, as proliferating cells and chondrocytes blended through an intermediate zone. As previously described, chondrocytes associated with calcifying cartilage can acquire properties of osteoblasts [163] and are able to change their phenotype from a primarily cartilage synthesizing cell type to a bone synthesizing cell type [162]. As the pathology progressed, most cells in the intermediate zones seemed to co-transcribe these markers. A similar shift was found in the notochord where co-transcription of genes such as *col2a*, *sox9*, *col1a* and *runx2* increased with proliferation of chordoblasts. In the central notochord of developing fusions, hyperdense regions of denser packet

chordocytes lacking vacuoles were visualized through hematoxylin and toluidine blue staining as the number of proliferating cell increased (Paper II and III). Loss of vacuoles correlated with reduced Aggrecan and SP signals in the chordocytes (paper III). Interestingly, both SP and Aggrecan expression reappeared in hyperdense chordocytes where transcription of osteogenic marker genes was prominent. A role for SP in bone mineralization and proliferation has been suggested [205,206] and increased transcription of osteogenic genes (such as *runx2, col1a* and *osteocalcin*) has been reported from SP treated mammalian osteoblast-like cultures [207,208]. Expression of SP and aggrecan in hyperdense notochordal regions and co-transcription of chondrogenic and osteogenic marker genes in the arch centra and notochord supports the suggestion of an adaptation through metaplastic shifts during development of vertebral fusions. Both the metaplastic shift in the notochord and arch centra may be induced to produce more robust cells that are able to withstand increased mechanical load.

3. Loss of notochordal sheath integrity

Verhoeff`s hematoxylin staining visualized a thinner elastic membrane surrounding the notochordal sheath of developing vertebral fusions (Paper III). These findings were supported by a reduced transcription of elastin at both intermediate and fused stage. In the most severe cases, the elastic membrane was fragmented, which is an observation also noted in compressed vertebrae [45]. An interesting comparative pathological condition to our findings in Atlantic salmon is the sparse and disrupted elastic network in scoliotic IVD [209]. The elastic sheath found in the annulus fibrosus (AF) of mammalian IVD is involved in restoration of shape after brief deformation, thus rupture of this sheath may be involved in the progression of spinal deformities. Furthermore, the highly folded structures in the notochordal sheath observed in non-deformed vertebrae were lost during development of spinal fusions, as visualized by Perlecan IHC. At early life stages, the notochordal sheath of Atlantic salmon is compounded by parallel col2 fibrils forming helices around the longitudinal axis of the notochord [12]. In a similar manner to the AF of mammalian discs, the collagen fibrils form overlapping transversing bands crossing the joint in opposite directions, thus stabilizing intervertebral regions [12,18]. As the cross-helical architecture is important for flexural stiffness of the larval body during development, the folded pattern observed in this study may contribute to increased flexibility and normal functioning of the mature spinal column. Hence, stretching of the folded notochordal sheath during the fusion process will most likely reduce

flexibility and affect functionality of the notochord. Moreover, the heparan sulfate chains of perlecan have been shown to play important roles in mammalian glomerular filtration [210] and Parsons et al. [211] have suggested similarities between the structural role of the teleost notochordal sheath and the kidney membrane filtration machinery [212]. Based on our findings we suggest that thinning of the elastic membrane and structural changes in the notochordal sheath decreases flexibility and possibly nutritional transportation across the notochord. Thus, loss of notochordal sheath integrity is likely a key event in the development of spinal fusions.

4. Ectopic bone formation and remodeling

A last major morphological change during the fusion process was ossification of the arch centra (paper II). The described metaplastic shift and the fact that these areas went through mineralization, indicate that the proliferating cells had not only differentiated towards osteoblast-like cells, but also completed the differentiation to cells that were capable of producing mineralized matrix. Our results suggest that a pathway to bone formation through chondrocytes might be possible during development of vertebral fusions and fast growth, which could be similar to trans-chondroid ossification as described by Yasui et al. [164]. A mixed type of inramembranous and endochondral ossification may also occur [213], however the lack of osteoclast activity observed in the high temperature group makes this less likely. Absence of osteoclasts as indicated by the absence of TRAP activity and down-regulated *cathepsin K* transcription were characteristic during the development of vertebral fusions, indicating that normal endochondral ossification was restrained. The completing step in the fusion process is transformation of notochordal tissue into bone [46]. Trans-differentiation and ectopic calcification has also been suggested as pathological pathways in lordotic sea bass where deformations stimulate ectopic bone formation in the intervertebral regions between two affected vertebral bodies and along the rims of the vertebral body endplates [49]. However, this is the first study linking ectopic bone formation to a metaplastic shift in the chondrocytes. As bone replaced chondrocytic areas throughout the pathology, notochordal tissue did not stain for Alizarin red S until the deformity developed into a severe fusion. We therefore suggest that metaplasia leads to cell types more suited to the new environment but that changes are related to a threshold of the stimuli, in this case, grade of fusion. Similarly, a shift in the mammalian IVD nucleus pulposus (NP) cell population coincides with spinal disorders like intervertebral disc degeneration and changes in the synthesis of matrix molecules differ with the

degree of degeneration [214]. The mammalian AF is further strengthened through cartilage formation upon elevated mechanical load [215,216]. Another comparative pathological process to our findings is mammalian “Bamboo spine”, describing a condition where vertebral bodies have fused and reshaped through ectopic bone formation [217,218]. However, whereas the mineralization of the arch centra involves chondrocytes, intervertebral region involves chordoblasts. In paper II and III we suggested that two different pathways for mineralization from metaplastic cells are involved in developing vertebral fusions; trans-chondroid ossification in the arch centra and trans-chordoid ossification in the notochord. Overall, the vertebral fusion process seen in Atlantic salmon might reflect an effort to restore and strengthen a weakened area in the vertebrae.

Developing an *in vitro* osteoblast culture

In paper IV, Atlantic salmon precursor cells were isolated from muscles and stimulated to osteogenic differentiation by an incubation medium containing components previously shown to direct the differentiation of various mammalian precursor cells towards the direction of osteoblasts [219-221]. Molecular techniques together with observations of cell behavior and cell morphology were used to identify the differentiation and maturation stages of the cells. During the incubation period the precursor cells changed morphology from small, spindle-shaped or triangular cells to become confluent cultures of large cells with a polygonal or cuboidal shape. The morphology of the cultured cells was similar to that of gilthead seabream osteoblasts, which were derived from vertebral explants [222]. In addition, they stained for Alp and transcribed several osteogenic marker genes like *alp, col1a1, osteocalcin* and *bmps*. The cells were characterized as osteoblasts at an early differentiation stage, since no ECM mineralization was detected with von Kossa staining. However, von Kossa staining has shown to be unsuitable for detection of small quantities of mineralized matrix [223], and additional methods, e.g. Alizarin staining, TEM or FTIR, might be desirable to use in further studies. Despite the lack of mineralized matrix, gene expressional findings confirmed that these precursor cells were able to differentiate into the osteoblast lineage. The cultured cells were further used to study the effects of two factors that influence bone formation in salmon under commercial farming conditions; temperature and PUFAs.

The molecular mechanisms behind increased levels of vertebral deformities in fish that grow at elevated temperatures are poorly understood. However, it is suggested that changes in transcriptional processes in osteoblasts and chondrocytes are involved [38,64,197]. To further

study temperature effect upon bone formation, osteoblasts differentiated in osteogenic medium for three weeks were exposed to two different temperatures. One group was kept at 12°C (control) throughout the experiment and the other group exposed to 16°C. Interestingly, transcription of *alp, col1a1* and *bmp4* were up-regulated after 1 hour of heat treatment, which may suggest that a short heat shock stimulates differentiation of osteoblasts in Atlantic salmon. However, in line with *in vivo* findings (paper I), cells exposed to elevated temperatures for more than one hour had decreased transcription of osteogenic marker genes such as *alp*, *col1a1* and *osteocalcin*. Similar studies have been carried out for human MSCs, where exposure to mild heat shock stimulated proliferation and osteogenic differentiation and resulting in enhanced mineralization whereas long-term treatment results in inhibited proliferation and apoptosis [68-70]. The cells exposed to elevated temperature died after 72 hours, even though the temperature to which they were exposed was within the normal range of Atlantic salmon. Hence, our *in vitro* results suggest that Atlantic salmon osteoblasts are highly sensitive to long-term exposure to heat during the early stages of differentiation.

Because marine fish oils are a limiting factor for the growing fish farming industry, it is necessary to evaluate alternative lipid sources for feed production. The use of vegetable oils leads to a reduced n−3 PUFA ratio in the fish diet. In both mammals and salmon, n-3 PUFAs have shown beneficial effects on bone mineral density, whereas increased n−6/n−3 fatty acid ratios may reduce bone formation [97,99-102]. Dietary PUFAs is further known to influence vertebral malformations in sea bass [103,224]. In our experiment, cells differentiated in osteogenic medium for three weeks were incubated for 72 hours in a medium containing different n−6/n−3 PUFA ratios and Conjugated linoleic acid (CLA). Adding different fatty acids to the culture affected both Prostaglandin E_2 (PGE_2) production and transcription of osteogenic marker genes. PGE_2 is the predominant bone cell derived prostaglandin and regarded as a potent regulating agent for bone formation in a concentration dependant manner [225-227]. Increasing the level of CLA or decreased the n-6/n-3 PUFA ratios led to suppressed PGE_2 production and increased *osteocalcin* mRNA transcription, suggesting a possible beneficial effect of n-3 PUFAs and CLA on bone formation in fish. In human osteoblast-like cell lines, Watkins et al. [228] described reduced PGE_2 production in the presence of n-3 PUFAs and Cusack et al. [229] showed that increasing the CLA concentration led to down-regulation of PGE_2 production. Results further correlated with recent *in vivo* results, where the level

of PGE_2 in blood plasma was higher and bone less mineralized in Atlantic salmon fed a soybean oil-based compared to a fish oil-based diet [97].

Both temperature and PUFA treatments correlated with previous results obtained from similar *in vivo* and *in vitro* studies. Even though the cells had not reached final maturation stage, the system seems well suited for further work and shows the potential of using an *in vitro* system in addition to *in vivo* studies.

Potential prognostic application

The defined markers for bone and cartilage cell differentiation and matrix formation may be used to investigate how the progression of skeletogenesis is modulated by a variety of factors. Differences in non-deformed fish from the two temperature groups were undetectable externally, but transcriptional changes were detected and correlated with the higher risk of developing deformities. Hence, this work reveals the potential use of gene transcription profiling as a prognostic approach in aquaculture. To test the detection level of marker genes and to further analyze vertebrae formation during growth at elevated temperatures, non-deformed vertebrae from fish at 2g size were analyzed with qPCR (paper I). Diagnosing Atlantic salmon using radiography at 2g size may be useful, however small and low mineralized vertebrae makes targeted sampling difficult. Pathological stages at this size were therefore not further analyzed. However, the percentage of deformities significantly increased in the high temperature group from 2g till 15g. This period therefore seems to involve important steps for the developmental fate of deformities. Between 2g and 15g we observed a change in transcription pattern from a down-regulated to an up-regulated transcription of 9 genes, where 8 of them were involved in chondrogenesis. This suggested that chondrocytes go through changes in this period that could be important for the development of the observed pathologies. In addition, except *alp,* all genes involved in osteoid production and mineralization were down-regulated at both stages. Temperature and light regimes are factors shown to speed up developmental rate, but also to delay production of osteoid (paper I and [75]). It therefore seems that the bone remodeling in Atlantic salmon is generally sensitive to elevated growth rates. However, the usefulness of molecular markers needs to be tested and approved in different settings and upon different treatments. It has already been suggested that *col1a* could be used as an indicator of vertebral toughness and that *shh* and *col1* combined could be used as markers of osteoid incorporation and osteoblast proliferation [75]. The work presented in this thesis

demonstrates the feasibility of developing a molecular toolbox for analyzing development of spinal fusions. Previous studies on vertebral deformities have suggested metaplastic shifts, trans-differentiation and development of intermediate tissues as pathological events [44,46,48,49]. A cell system that allows for cross-studies of adipocytes, myocytes, chondrocytes and osteoblasts may therefore be of high value to Atlantic salmon bone deformity studies, both for functional and response mechanism experiments. Our cell cultures show for the first time in a fish species the great plasticity of the cells in the MSC linage and open up for study of Atlantic salmon osteoblast biology *in vitro*. In addition, and due to their plasticity, they provide a unique opportunity to address important questions in the salmon industry, e.g. how adipose tissue is transformed into cartilage during development of hyperdense vertebrae [48]. Ultimately, these tools may lead to improved definition of the boundaries between healthy and unhealthy spinal columns and refined guidelines for an improved aquaculture.

Concluding Remarks

The application of radiography and imaging techniques together with cell and molecular biology used in this study has proved to be useful tools to characterize bone malformations in Atlantic salmon. Our findings demonstrate an association between temperature, growth rate, bone architecture and bone cell responsiveness in juvenile Atlantic salmon.

- First, our results describe changes in vertebral tissue not yet manifesting pathological deviations. Our results strongly indicates that temperature induced fast growth is severely affecting gene expression in osteoblasts and chondrocytes, hence change in the tissue structure and composition. It is not unlikely that this disequilibrium is involved in the higher rate of deformities developed in the high temperature group. Even though differences in the two experimental groups were undetectable externally, rearing at increased temperatures induced consistent transcriptional changes in several genes that correlated with the higher risk of developing deformities later in ontogeny.
- Second, we identified four hallmarks in developing spinal fusions in Atlantic salmon. These stages included both notochord and centra. First, disorganized and proliferating osteoblasts and chordoblasts characterized the fusion process. Second, a metaplastic shift was detected through *ISH* in proliferating cells. Third, as the pathology progressed, elastic membrane became fragmented and notochordal sheath lost integrity. Finally, mineralization of intervertebral regions and arch centra was observed. The different events are important in understanding the progression of vertebral fusions and provide basic knowledge on bone formation in Atlantic salmon.
- Third, we report for the first time that unspecialized precursor cells isolated from Atlantic salmon muscle tissue are able to differentiate to osteoblasts-like cells *in vitro*. These cells appear to be a suitable model system for the study of osteoblast biology *in vitro.* The cells had osteoblast-like morphology, stained for Alp and expressed several osteoblast-related markers.

Future Perspectives

Understanding the cellular and molecular mechanisms involved in skeletal tissue formation is crucial for explaining the development of both healthy and pathological vertebrae and to further minimize the prevalence of bone disorders in farmed fish. The results presented in this thesis provide targets for further research on skeletal malformations that might have general applications.

- First and foremost, further unraveling of the molecular events in the developing Atlantic salmon vertebrae is needed. Through this study, Atlantic salmon has shown to be suitable for studying general bone development and to be comparable with similar mammalian models for spinal deformities. More basic knowledge would be beneficial for both the aquaculture industry as well as for comparative studies.
- Paper I described changes in non deformed vertebrae from the high temperature group and suggested that these changes could be related to the increased number of deformed fish observed in this group. However, it is important to further focus on this group, since these fish displayed normal vertebrae at the time of radiological examination. Hence, this group might reveal beneficial changes important for maintaining a normal vertebra during fast growth.
- The data presented in this thesis indicate that both production of bone and cartilage is disrupted when promoting fast growth using elevated temperature. The defined markers of bone and cartilage cell differentiation and matrix formation can be used to further investigate how the progression of skeletogenesis is modulated by a variety of factors, e.g. minerals and exercise. Ultimately, these tools may lead to improved definition of the boundaries between healthy and unhealthy spinal columns and refined guidelines for an improved aquaculture.
- The optimization of the osteoblast *in vitro* system to achieve mineralizing osteoblasts and further create a powerful tool to study factors that have potential impact on bone development in fish is needed. MSCs are available at relatively high levels in fish tissue at early life stages. These cells further provide a valuable tool compared to cell culture of differentiated osteoblast since they still possess the ability to differentiate into osteoblasts, myotubes, chondrocytes and adipocytes. The plasticity of these cell culture systems will be of great value when analyzing lineage determination, response-mechanism upon stimuli and nutrients as well as a mean for decreasing the number of experimental animals.

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Paper I

Molecular pathology of vertebral deformities in hyperthermic Atlantic salmon (*Salmo salar*)

Elisabeth Ytteborg[1,2], Grete Baeverfjord[1], Kirsti Hjelde[1], Jacob Torgersen[1] and Harald Takle[1,3]*

[1]Nofima Marin, Norwegian Institute of Food, Fisheries and Aquaculture Research, P.O. Box 5010, NO-1432 Ås, Norway

[2] Norwegian University of Life Sciences, NO-1432 Ås, Norway

[3]AVS Chile SA, Imperial 0655, Of. 3A, Puerto Varas, Chile

*Corresponding author

Email addresses:

EY: elisabeth.ytteborg@nofima.no

GB: grete.baverfjord@nofima.no

KH: kirsti.hjelde@nofima.no

JT: jacob.torgersen@nofima.no

HT*: harald.takle@nofima.no

Abbreviations: ALP, alkaline phosphatase; BCIP/NBT, 5-bromo-4-chloro-3 indolyl phosphate *p*-toluidine salt/nitro blue tetrazolium chloride; bHLH, basic helix loop helix; BMP, bone morphogenetic proteins; dl, decilitre; Gla, y-carboxyglutamic acid; Ihh, indian hedge hog; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PDGFrb, platelet derived growth factor receptor b; PFA, paraformaldehyde; Runx2, runt related transcription factor 2; shh, Sonic hedge hog; Sox9, sex determining region Y-box 9, Tartrate-resistant acid phosphatase, TRAP.

Abstract

Background: Hyperthermia has been shown in a number of organisms to induce developmental defects as a result of changes in cell proliferation, differentiation and gene expression. In spite of this, salmon aquaculture commonly uses high water temperature to speed up developmental rate in intensive production systems, resulting in an increased frequency of skeletal deformities. In order to study the molecular pathology of vertebral deformities, A. salmon was subjected to hyperthermic conditions from fertilization until after the juvenile stage.

Results: Fish exposed to the high temperature regime showed a markedly higher growth rate and a significant higher percentage of deformities in the spinal column than fish reared at low temperatures. By analyzing phenotypically normal spinal columns from the two temperature regimes, we found that the increased risk of developing vertebral deformities was linked to an altered gene transcription. In particular, down-regulation of extracellular matrix (ECM) genes such as *col1a1*, *osteocalcin*, *osteonectin* and *decorin*, indicated that maturation and mineralization of osteoblasts were restrained. Moreover, histological staining and *in situ* hybridization visualized areas with distorted chondrocytes and an increased population of hypertrophic cells. These findings were further confirmed by an up-regulation of *mef2c* and *col10a*, genes involved in chondrocyte hypertrophy.

Conclusion: The presented data strongly indicates that temperature induced fast growth is severely affecting gene transcription in osteoblasts and chondrocytes; hence change in the vertebral tissue structure and composition. A disrupted bone and cartilage production was detected, which most likely is involved in the higher rate of deformities developed in the high intensive group. Our results are of basic interest for bone metabolism and contribute to the understanding of the mechanisms involved in development of temperature induced vertebral pathology. The findings may further conduce to future molecular tools for assessing fish welfare in practical farming.

Background

Industrial fish farming makes use of intensive production regimes in an effort to decrease production time and costs. Elevated water temperatures are commonly applied, often without explicit control of factors like nutrition, water quality, densities and vaccination. The intensive rearing systems are unfortunately correlated with deformities affecting both skeletal and soft tissues [1,2]. In teleosts, hyperthermia can induce vertebral deformities both during the embryonic development and after the vertebral column has been established [3-5]

The teleost vertebral body is built using a minimal bone mass to reduce negative buoyancy [6]. In salmon, the vertebral body comprises four mineralized or ossified layers. Formation of the different layers involves the balanced and highly regulated formation of bone and cartilaginous structures through patterns of mineralization and matrix deposition [7]. The specialized architecture makes it vulnerable to alterations in its tissue composition. Intramembranous ossification occurs by coordinated processes of production, maturation and mineralization of osteoid matrix [8]. Initially osteoblasts produce a thickening osteoid seam by collagen deposition without mineralization. This is followed by an increase in the mineralization rate and the final stage where collagen synthesis decreases and mineralization continues until the osteoid seam is fully mineralized. As part of the process, mineralization time lag appears to be required for allowing modifications of the osteoid so that it is able to support mineralization [9]. Indeed, fast growing A. salmon has been shown to exhibit low vertebral mineral content and mechanical strength, together with an increased risk of developing vertebral deformities [10,11].

Skeletal growth depends upon the dynamic equilibrium between cartilage production and bone apposition rate [12]. Ontogeny and growth of the vertebral column is under control of regulatory mechanisms involving transcription factors, signaling molecules and extracellular matrix proteins. The pathways of chondrocyte and osteoblast differentiation are interconnected during vertebral formation and must be coordinated. In particular, regulatory proteins, like the transcription factors Sox9, Runx2, Osterix, Twist and Mef2c have distinct functions both in the establishment of the vertebral bodies and later in the differentiation and maturation of specific skeletal cell types (review [13]). Similarly, signaling molecules like bone morphogenetic proteins (Bmp2 and Bmp4), and hedgehog proteins (Ihh and Shh) plays different roles both during cell differentiation and skeletal tissue ontogeny [14-16]. Osteoblasts and chondrocytes secrete the collagen fibers and ground substances of bone and cartilage. These cells are also responsible for the mineralization of the matrix through secretion of specialized molecules, such as Alkaline phosphatase (ALP), Osteocalcin and Osteonectin that binds inorganic minerals [17,18]. A widely accepted view is that the spatial restriction of ECM mineralization to bone is explained by osteoblast-specific gene products that initiate the formation of hydroxyapatite crystals ($Ca_{10}[PO_4]^6[OH]^2$) [19]. The requirement for specifically expressed genes in osteoblasts (e.g. *col1, osteocalcin* and

osteonectin) and chondrocytes (e.g. *col2* and *col10*) to initiate the formation of matrix or control the growth of hydroxyapatite crystals is supported by numerous studies [18,20,21]. Furthermore, Matrix metalloproteinases (MMPs) and Tartrate-resistant acid phosphatase (TRAP) are involved in degradation of ECM and in the bone remodeling process performed by the osteoclasts [22] .

In this work, 20 skeletal genes were used to study the effect of long term hyperthermic exposure on vertebral development and growth in A. salmon. Fish exposed to high temperature (high intensive regime) had a significant higher incidence of deformities than fish from the same origin reared under a conservative temperature regime (low intensive regime). The study was aimed at exposing differences in risk level between the groups, rather than elaborating the pathologies of deformed vertebrae, hence, the study concentrated on phenotypically normal fish from both temperatures. Significant changes in gene transcription were found between phenotypically normal vertebrae of both groups, including down-regulation of genes encoding proteins important for mineralization. Further, *in situ* hybridization (*ISH*) and histological staining revealed phenotypical and functional changes in the arch centra. Our results are of basic interest for understanding bone metabolism and deformities, as well as a tool for assessing fish welfare in practical farming.

Results

In the present study we analyzed and compared A. salmon vertebrae from high and low temperature intensity regimes. Rate of development and growth was influenced by temperature regime as observed through SGR and time of sampling. The development from fertilization to first feeding lasted 5 months in the low intensive regime at 6°C, compared to 3 months in the high intensive regime at 10°C. Juveniles of the high intensive group also grew more rapidly after start-feeding than the low intensive group, where the former reached 2g in 6 weeks after first feeding, 15g in 3 months and 60g in 7 months after first feeding, at a rearing temperature of 16°C. In comparison, the low intensive group at rearing temperature of 10°C reached similar sizes in 11 weeks, 5 months and 10 months, respectively. Accordingly, after start-feeding fish from the high intensive temperature regime displayed a higher SGR than the low temperature fish, 2.82 and 1.96 respectively.

Radiography, morphology and mineral analyses

On radiography analysis, the incidence of fish with skeletal abnormalities at 2g size was 4.0 ± 2.8 % and 10.0 ± 1.7 % in the low and high intensive groups, respectively (n.s.; not significant). At 15g size, the difference was more pronounced, 3.4 ± 2.0 % and 17.9 ± 1.3 % ($p<0.001$). At the final sampling at 60g size, 8 ± 1.4 % of the fish in the low intensive group displayed some degree of skeletal pathology compared to 28.1 ± 2.3 % in the high intensive group ($p<0.0001$), results are shown in figure 2.

Morphometric analyses of vertebral shape demonstrated that fish classified as having a normal phenotype in both groups had more or less regularly shaped vertebrae, but that there was a difference in length-height proportion of vertebrae between fish from the two temperature regimes. Measurements on X-ray images showed that vertebral bodies from the high intensive groups were significantly shorter in craniocaudal direction compared to those from the low intensive groups. The ratios for the high and low intensive group were at 2g 0.68 ± 0.02 and 0.76 ± 0.02 , at 15g 0.78 ± 0.03 and 0.89 ± 0.06 and at 60g 0.86 ± 0.01 and 0.94 ± 0.01, respectively ($p<0.001$). Examples of vertebral columns with normal phenotype from the high and low intensive group at 15g are shown in figure 3.

Due to the built-in image contrast enhancement procedures of the semi-digital X-ray system, evaluation of skeletal mineralization as judged by radio density in images was impaired. Nevertheless, a lower contrast in skeletal structures was observed in the high intensity fish, in particular at the 15g sampling, indicative of a lower mineralization rate at this stage.

Whole body mineral content at the end of the experiment (60g size) showed low values for Ca, P and Zn content for both temperature regimes (Table 3), with no significant differences between treatments. There was a small, but significant lower level of whole body Fe and Na in the high intensive group. All Fe and Na values were lower than reference values [23], but in correspondence with Ca, P and Zn values, they were within a range which is commonly seen in commercially reared salmon.

Quantitative vertebral mRNA expression

The skeletal genes were divided into three groups according to function; ECM constituents, transcription factors, and signaling molecules (Fig. 4 A-C).

ECM constituents included genes involved in bone matrix production and mineralization and 7 out of 9 of these genes were found to be down-regulated in high intensive group at 2 and 15g (Fig. 4A). Transcription of *col1a1, osteocalcin, decorin, osteonectin, mmp9* and *mmp13* were reduced in the high intensive group compared to the low intensive group. *Col2a1* transcription was also down-regulated at both developmental stages, however the values were insignificant. *Osteocalcin* was severely down-regulated in 2g high intensive group. Converse transcription profiles could be observed for *col10a1* and *alp* between 2g and 15g fish; *col10a1* was down-regulated at 2g and up-regulated at 15g whereas *alp* was up-regulated at 2g and down-regulated at 15g.

Temporal changes in transcription factor mRNA expression were found between high and low temperature group, and all genes except *sox9* showed opposite expression at 2 and 15g (Fig. 4B). In the high intensive group, *sox9* was down-regulated at 2g (n.s.) and 15g, but more pronounced in the latter. Investigation of the two osteoblast markers *runx2* and *osterix*, revealed opposite mRNA expression levels at 2 and 15g. *Runx2* was up-regulated at 2g (n.s), but down-regulated at 15g. On the

contrary, *osterix* was down-regulated (n.s.) at 2g, but up-regulated at 15g. *Mef2c* and *twist* was also down-regulated at 2g, while up-regulated at 15g (*twist* n.s.).

Signaling molecules included *bmp2, bmp4, shh* and *ihh*. Expression analysis of mRNA for signaling molecules showed statistically significant differences in expression levels between the temperature regimes and all transcripts were found more abundant in the 15g group when compared to 2g vertebrae. *Bmp2* was the only up-regulated signaling molecule at 2g, while all signaling genes were up-regulated at 15g (Fig. 4C).

To further examine changes in chondrocyte recruitment and structure between the temperature regimes, we included *platelet derived growth factor receptor b* (*pdgfrb*) and *vimentin*, because of their importance in proliferation and the cytoskeleton, respectively [24,25]. Both transcripts were significantly down-regulated in 2g, while significantly up-regulated at 15g (Fig. 4A, B).

In summary, we found that out of the 20 genes we analyzed, 8 were down-regulated in both temperature groups (*col1a1, col2a1, osteocalcin*, *sox9, decorin, osteonectin, mmp9* and *mmp13*), 9 genes were up-regulated in the 15g high intensive group, but down-regulated at 2g (*bmp4, col10a1, osterix, ihh, shh, mef2c, twist, vimentin* and *pdgfrb*). And finally, *alp* and *runx2* were up-regulated at 2g but down-regulated at 15g.

Vertebral tissue morphology and spatial mRNA expression

In areas where osteoblasts secrete the osteoid matrix, a generally stronger *ISH* signals was apparent in the low intensive group for all probes. The osteogenic marker gene *col1a* showed distinct staining to osteoblasts at the growth zone of the endbones of the vertebral bodies from fish of both temperature regimes (Fig. 5A-C). Moreover, *col1a* signal was identified in the bone lining osteoblast cells situated at the lateral surfaces of the trabeculae and along the rims of the vertebral bodies. Investigation of *osteocalcin* mRNA revealed an expression pattern similar to *col1a,* with staining of cells in the osteogenous areas and in bone lining osteoblasts and apical surfaces of the trabeculae (Fig. 5D-G). Specifically high *osteocalcin* signal was detected in the proliferative osteoblast growth zones on the endbones of the vertebral bodies. *Osteonectin* mRNA was detected in the osteogenic growth zone of the endbones and lining the exterior part of the vertebral body (Fig. 6A-D). The chondrocytic marker *col2a*, hybridized heavily to chordoblasts in the notochord (Fig. 6E-G), whereas *col10a* was detected in a continuous layer of cells along the rims of the vertebral body (Fig. 6J-L).

Alizarin red S and toluidine blue stained chondrocytes in the arch centra and revealed distinct morphological differences between vertebrae from the two temperature groups. The low intensive group was defined by distinct sub-groups of chondrocytes in the different maturational stages i.e. resting, proliferating and hypertrophic. In contrast, the equivalent chondrocytes were more distorted in the high

intensive group (Fig. 7A, B). *ISH* analysis of *col2a*, *col10a* and *osteonectin* enabled classification of the different chondrocytes into distinct sub-populations of maturational development. *Col2a* hybridized to resting and pre-hypertrophic chondrocytes in two distinct bands of both low and high intensive group, but the mRNA expression was more evenly distributed in all cells of the latter group (Fig. 6H, I). There were also generally less proliferating chondrocytes that tended to be less compact in this group. In proliferating chondrocytes we detected strong *col2a* mRNA expression in the high intensive group, but no expression in the low intensive group. Analysis of *col10a* showed restriction to the pre-hypertrophic and hypertrophic chondrocytes located in the deep cartilage zone (Fig. 6M, N). *Osteonectin* was also expressed in chondrocytes and the signal increased towards the hypertrophic chondrocytes (Fig. 6A-C). The pre-hypertrophic chondrocyte zone was found to be expanded in the high intensive fish and both *col10a1* and *osteonectin* showed an expanded expression domain corresponding to an increased hypertrophic zone. No signal was detected in any of the samples hybridized with sense probes (data not shown).

In normal spinal columns from the low intensive group, positive TRAP staining was detected at the ossifying boarders of the hypertrophic chondrocytes in the arch centra. No positive staining was detected in samples from the high intensive group (Fig. 8A, B).

Discussion

The presented study aims at describing the molecular pathology underlying the development of vertebral deformities in A. salmon reared at a high temperature regime that promotes fast growth during the early life stages. Within the period investigated, vertebral bodies form and develop and the skeletal tissue mineralizes. Rearing at high temperatures resulted in higher frequencies of vertebral deformities, as expected. The vertebral pathology observed in this study was most likely induced both during the embryonic development and after start-feeding, since the incidence of deformities continued to increase throughout the experiment after the first radiographic examination at 2g. Similar temperature regimes before and after start-feeding have independently been shown to induce vertebral defects in juvenile salmon [1,26]. However, whereas high temperatures during embryonic development is commonly related to somitic segmentation failure, deformities later in development may possibly be linked to fast growth induced by elevated temperatures and the impact this might have on the natural maturation and ontogeny of the vertebral bodies [3,10,11]. This causative relation has been shown for fast growing underyearling smolt that has a higher incidence of vertebral deformities than slower growing yearling smolt [27]. Further, morphometric analyses showed that elevated water temperature and faster growth is manifested by a difference in length-height proportion of vertebrae between fish from the two temperature regimes. Similar decrease in length-height proportion was described for the rapid-growing

underyearling smolt [27]. Radiographic observations indicated a lower level of mineralization of osteoid tissues in the high temperature fish. However, we could not find any pronounced altered mineral content between the two temperature regimes. The observed values were low compared to reference values [23], but in a range commonly observed in commercially reared salmon. Apparently, whole body mineral analysis seems insufficient to assess problems related to the development of spinal deformities.

To determine whether the difference in likelihood of developing vertebral deformities between the two groups could be traced back to an altered gene transcription, we examined the expression of selected skeletal mRNAs in phenotypical normal salmon fry at 2 and 15g. Histological examination of 15g fish was included to improve interpretation of the transcriptional data. The selected genes showed both phylogenetic conservation and similar spatial expression with those examined in other vertebrates, supporting that most of the factors and pathways that control skeletal formation are highly conserved in vertebrates.

The lower transcription of ECM genes such as *col1a1*, *osteocalcin*, *osteonectin* and *decorin* suggests a defect in the late maturation of osteoblasts [17,21,28]. The correlation to impaired mineralization is supported by the shorter vertebral bodies in the high intensive groups throughout the study, as well as the impaired mineralization indicated by low contrast observed on X-ray. *Col1a1* is the primary ECM component secreted by osteoblasts in the trabecular bone and growth plate and defects in the synthesis of *col1* or type 1 procollagen have been found in several heritable disorders of connective tissue. Likewise, defects in the assembly of Col1 fibrils have been reported to cause abnormally thin and branched structures [29]. Decreased diameter and crosslink density of the collagen fibers have been suggested to reduce thermal stability of collagen and thereby the tissues ability to support load during elevated temperatures [30]. In chum salmon, *Oncorhynchus keta*, the denaturation temperature of collagen type 1 from skin has been reported to be about 19°C [31]. The collagen fibres are further organized and stabilized by a range of non-collagenous proteins, which functions by linking other proteins and minerals to the ECM scaffold. Decorin, which belongs to the small leucine rich repeat proteoglycan (PG) group (SLRPs) is involved in determining the mature collagen fibril structural phenotype and tissue function by facilitating protein–protein interaction with a range of other matrix components (mainly collagen fibres) and with the mineral phase during the formation of calcified tissues [32]. As a result, decorin has been shown to increase tensile strength of the collagen-decorin fiber [33]. Further, osteonectin is a phosphorylated glycoprotein that binds to collagen fibrils, calcium, and hydroxyapatite, linking the bone mineral and collagen phases and perhaps initiating active mineralization in normal skeletal tissue [17,34]. *Osteonectin*-null mice display decreased trabecular bone volume and have bone of lesser stiffness than control mice [35]. *Osteocalcin* mRNA expression

also serves as a useful molecular marker of mineralization because it is associated with the maturation of bone cells and mineralization [18,36]. *Alp* is another marker gene for bone cell maturation and mineralization. Inhibition of *alp* activation, by for example heat or by gene knockout, inhibits calcification and causes mineralization defects in cultured bone cells and mice [37]. In addition, mutations in the *alp* gene lead to hypophosphatasia, in which bone matrix formation occurs, but mineralization is inhibited [38]. Our results showed that *alp* was down-regulated in the high intensive 15g group, but up-regulated in 2g fish. This may indicate that *alp* is a limiting factor for mineralization after long term exposure to the high temperature regime. Altogether, the simultaneous down-regulation of genes encoding structural proteins taking part in the bone matrix and mineralization strongly supports an assumption that disturbances of these processes constitute an important part of the mechanisms of development of vertebral deformities.

As for the ECM genes involved in osteoblast development and mineralization, high intensive temperature treatment had a significant effect on the transcription of transcription factors and signaling molecules involved in these processes. Intriguingly, Runx2 and Osterix, known as master regulators of osteoblast differentiation [39,40], exhibited opposite mRNA expression levels at 2 and 15g. *Runx2*-null mice have osteoblast differentiation arrested [41], while *osterix*-null mice embryos have a significant reduction of *col1* expression and do not express the late osteoblast specific marker osteocalcin [39]. In addition, we analyzed the bHLH transcription factor *twist*. This gene works as a negative regulator of osteoblastogenesis by inhibiting expression of genes downstream of *runx2* [42]. At 2g when *osterix* and *twist* was down-regulated while *runx2* was up-regulated, osteocalcin was heavily down-regulated as was *col1a1*. The mRNA expression pattern was inverted at 15g. Then *osterix* and *twist* was up-regulated and *runx2* down-regulated, while *osteocalcin* and *col1a1* were weakly down-regulated. Linking these results to the pathways involved in osteoblast development, the required simultaneous activation of *osterix* and *runx2* did not appear at 2g or at 15g. However, Osterix function downstream of Runx2 during osteoblast differentiation, but may be regulated by Bmp2 in a Runx2-independent pathway [43]. Bmp2 can induce ectopic bone and cartilage formation in adult vertebrates [44]. Spinella-Jaegle et al [16] found that cooperation between Bmp2 and Shh was necessary to promote a strong induction of the osteoblast marker *alp* in human mesenchymal cell lines. At both 2 and 15g, *bmp2* was highly up-regulated in the high intensive group, possibly as a response to the low ECM mRNA expression and under-mineralized tissue. In addition, *osterix* and *shh* was up-regulated at 15g, as was *bmp4*. Bmp4 treatment has been shown to stimulate new bone formation and is also expressed in osteoblasts prior to formation of mineralized bone nodules [45,46]. However, in comparison to Spinella-Jaegles *in vitro* findings, we did not detect an increase in *alp* mRNA expression. Further, we detected a weaker signal of *osteocalcin* and *osteonectin* in osteoblasts from the *ISH* of the high intensive group at 15g. Hence, despite the

possible attempt of *bmp2* to restore bone formation and mineralization, there was still lower transcription of ECM components in the high intensive group at 15g. Summarized, our results may indicate that osteoblast proliferation and mineralization were restrained in the fast growing group.

The percentage of deformities significantly increased in the high intensive group from 2g till 15g, while the percentage was stable in the low intensive group. Hence, this period seems to involve important steps for the developmental fate of deformities. Between these two size stages we observed a change in expression pattern, from a downregulated to an upregulated transcription, of 9 genes, where 8 of them are involved in chondrogenesis. This suggested that chondrocytes go through changes in this period that could be important for the development of the observed pathologies.

In vertebrates as mouse and human [47,48], the growth zones of long bones consists of well defined layers of progenitor, proliferative and hypertrophic chondrocytes [49] . These chondrocytes differ in their morphology, proliferation abilities and secretion of ECM components. For example, transcription of *col2a1* is characteristic for the proliferative state whereas *col10a1* is restricted to the hypertrophic state [47,50]. *ISH* of these genes revealed that 15g A. salmon raised at the low intensive regime also had distinct sub-populations of progenitor, proliferative and hypertrophic chondrocytes at the growth zone of the neural and haemal arches. On the contrary, more distorted layers were found in A. salmon raised at the high intensive regime. Moreover, an increased zone of hypertrophic chondrocytes was found in the proximity of the mineralized bone matrix in the high intensive group. Once these hypertrophic chondrocytes are fully differentiated, matrix calcification would normally be initiated [12]. However, we could not identify any variance in mineralization at the ossifying borders of the hypertrophic chondrocytes when examined by histological Alizarin red S staining.

The increased zone of hypertrophic chondrocytes in the high intensive group and the up-regulated transcription of hypertrophic marker genes suggest an arrest prior to the final maturation of chondrocytes. Thus, these chondrocytes seems unable to initiate mineralization. The chondrocyte hypertrophy marker *col10a1* and its activator *mef2c* [51] were both up-regulated at 15g in the high intensive group. Moreover, *ihh,* a repressor of terminal hypertrophic differentiation [52], was found to be highly up-regulated, whereas *sox9*, which is involved in early chondrocyte differentiation, and its downstream structural protein *col2a* [53], were down-regulated. The severely down-regulation of *runx2* at 15g is of interest, since *runx2*-null mice embryos have a narrow zone of proliferating chondrocytes and a wide zone of hypertrophic chondrocytes [54]. In addition, *bmp4*, which was up-regulated at 15g, has been shown to accelerate the hypertrophic maturation process [55]. Interestingly, we also found an up-regulated expression of *pdgfrb* mRNA at 15g. Kieswetter and collaborators [25] have reported that chondrocytes respond to PDGF by enhancing proliferation and cartilage matrix production while

maintaining the cells in a less mature phenotype; corroborating our findings that the chondrocytes are some how arrested in the late hypertrophic stage at 15g with a reduced possibility of completing the endochondral ossification process with calcified bone as end product. Similar findings have also been shown in rat ulnae, where loading was associated with an increased hypertrophic zone in the growth plate [56], but mineralization rate was suppressed [57]. Another interesting comparative pathological condition to our findings in salmon is tibial dyschondroplasia (TD), a metabolic disease of young poultry that affects the growth of bone and cartilage. The lesion is morphologically characterized by an accumulation of chondrocytes that appear to be unable to differentiate past a pre-hypertrophic stage [58]. TD often occurs in broilers and other poultry that have been bred for fast growth rates. The tibial cartilage does not mature enough to ossify, which leaves the growth plate prone to fracture, infection, and deformed bone development.

The observed shorter phenotype of vertebral bodies from the high intensive group might have been a consequence of higher mechanical load in fast growing fish coincidental with a lower transcription of supportive ECM components. Together with the up-regulation of hypertrophic genes in high intensive fish at 15g, we also found increased transcription of *vimentin*. Vimentin filaments have been shown to regulate the swelling pressure of chondrocytes [59] and strengthen resistance to mechanical stress [60]. Hence, the increased activation of *vimentin* and the increased proportion of hypertrophic chondrocytes in the high intensive temperature group at 15g may reflect an adaptation to the fast growth by prioritizing maturation of chondrocytes that are more resistant to mechanical stress. At 2g, however, the reduced level of *vimentin* mRNAs might possibly be linked to the mal-adaptive down-regulation of chondrocytic genes in high intensive group. Indeed, disruption of vimentin filaments has been shown to result in loss of cell contact with the surrounding matrix which may alter the signaling dynamics of the cell and in effect shut down transcriptional events [24].

Mineralizing hypertrophic chondrocytes acquire and express most of the phenotypic characteristics of osteoblasts, including high Alp activity and expression of *osteonectin* and *osteocalcin* [61]. These phenotypic traits shared with osteoblasts may be needed to bring about the final phase of endochondral ossification and replace mineralized cartilage with bone [62]. They may also permit mineralized cartilage to act as bone-like structural tissue and allow for a transition from cartilage to bone. In contrast to the down-regulated transcription of *osteonectin* and *osteocalcin,* as determined by real time qPCR, we observed an increased transcription pattern of these genes in the arch centra in the high intensive group by *ISH*. We also observed a tendency of lower transcription of the same genes in osteoblasts of the high intensive group. However, establishment of a calcifiable matrix requires degradation of some matrix molecules. Endochondral bone formation includes the participation of MMPs, which degrade cartilage matrix and allow vascular invasion [63]. At least two proteases are

involved in this process; MMP13 which regulates remodeling of the hypertrophic cartilage matrix and MMP9 which has a role in vascularisation of the growth plate [64,65]. When analyzing these MMPs in salmon vertebral columns, a significant down-regulation of both *mmp9* and *mmp13* in the high intensive group at 2g were observed. At 15g, *mmp13* mRNA expression decreased even more, while *mmp9* was significantly up-regulated. Indeed, MMP13 is known as the dominant collagenase in cartilage and its absence cause delay in endochondral ossification [65]. Further supporting the hypothesis that endochondral ossification was in some way delayed in the spinal columns from the high intensive group, *runx2* deficiency has been shown to inhibit *mmp* expression [66] and lead to mild disturbances of chondrocyte differentiation, as discussed above. In addition, TRAP activity, essential for completing endochondral ossification [22], was absent in the erosive front of cartilage in neural and heamal arches of spinal columns from the high temperature group.

Conclusion

The presented results contribute to the understanding of the mechanisms involved in development of temperature-induced vertebral pathology by describing changes in vertebral tissue not yet manifesting pathological deviations. Our results strongly indicate that temperature induced fast growth is severely affecting gene transcription in osteoblasts and chondrocytes, leading to a change in the tissue structure and composition. The data presented here indicate that both production of bone and cartilage were disrupted when promoting fast growth using elevated temperature. It is not unlikely that this disequilibrium is involved in the higher rate of deformities observed in the high intensive group. Importantly, management control of deformities and health in general demands precise tools and knowledge to depict any problem as early as possible in the production line. The defined markers of bone and cartilage cell differentiation and matrix formation can be used to investigate how the progression of skeletogenesis is modulated by a variety of factors. Even though differences in the two experimental groups were undetectable externally, rearing at increased temperatures induced consistent transcriptional changes in several genes that correlated with the higher risk of developing deformities later in ontogeny. Hence, this article reveals the potential use of gene transcription profiling as a prognostic approach in aquaculture.

Materials and methods

Experimental design

The fish experiment was done at Nofima Marine at Sunndalsøra, Norway, in 2007 with A. salmon from the Salmobreed strain. Two experimental temperature regimes were set up; a high intensive

temperature group and a low intensive temperature group (Fig. 1). Pooled batches of unfertilized eggs and milt were transported on ice to the hatchery and were fertilized, rinsed and disinfected according to standard procedures. The eggs were incubated in a hatchery designed for incubation of small egg volumes, with approximately 0.2 liters of eggs per unit in six units per temperature regime. During egg rearing water supply was continuous from two temperature controlled tanks stabilized at 10±0.3ºC and 6±0.3ºC, respectively, monitored twice daily. At 850dº (day degrees=sum of daily temperature), a selection of fry were mixed and transferred to 150 liter tanks for start-feeding, four tanks per temperature regime. The number of fry per tank was 400. Water flow in the tanks was adjusted throughout the experimental period to secure oxygen supply in excess. The fish were fed commercial diets and the light was continuous. The temperature for the high intensive tanks was gradually increased at first feeding to 16±0.3ºC and the temperature for the low intensive tanks was gradually increased to 10±0.3ºC (1ºC per day). These temperatures were kept stable until the average size in each group reached 20g. At this size, the differentiated temperature treatment was ended. 100 fish per tank were selected randomly, and were tagged individually with pit-tags in the abdominal cavity. Fish from the four tanks on same temperature regime were mixed in a larger tank, and reared at ambient temperature until termination at 60g. Specific growth rates (SGR) in the period between start-feeding and 60g were measured according to equation SGR=((endweight/startweight)^(1/days)-1)x100).

Tissue sampling, radiography, morphology and mineral analyses

Vertebral columns of phenotypically normal specimens from both temperature groups were sampled for gene expression analysis at 2 and 15g size and histological analysis at 15g size. The term phenotypically normal was defined as vertebral columns without any obvious aberrations or deformities when imaged by radiography at sampling. For this purpose, fish were heavily sedated in MS 222 (Tricaine methane sulphate, Pharmaq, Overhalla, Norway) (150mg/litre) and imaged with an IMS Giotto mammography system (model number 6020/3, IMS Giotto, Bologna, Italy) equipped with a FCR Profect phosphorus film plate (Fuji Medical Inc., Japan). The resulting 20 pixels/mm images were enhanced with digital software (Fuji Computed Radiography Console) and evaluated manually concurrent with sampling. Fish without any specific pathology of the vertebral column were identified for sampling, and killed by an anesthetic overdose. Approximately ~5 vertebral bodies (~1cm) were carefully dissected from the area under the dorsal fin. For gene expression analyses, samples were flash-frozen in liquid nitrogen and transported on dry-ice to a -80°C freezer for storage. For histological analysis, vertebrae were fixated in 4% PFA for 24h at 4°C, dehydrated in ethanol (25, 50 and 70%) and stored at 70% ethanol at -20°C. At 2g size, 350 fish were screened and a total of 40 were sampled for this study. At 15g size, 900 fish were screened, and 70 were sampled. Fish that were not selected for sampling

following radiography were transferred to clean water and returned to the rearing tank. At 60g size, following an on-growing period on ambient temperatures, 800 fish were radiographed, 100 per original first feeding tank.

Incidence of skeletal deformities was recorded on radiographs from all samplings, and the presence or absence of vertebral pathology was recorded. It should be noted that fish with deviant vertebral morphology, mainly those with fusion type changes, were heavily sampled on basis of live X-ray at 2g and 15g (Ytteborg, manuscript in progress). This gives an underestimation of the differences between the two groups. In order to quantify differences observed in proportions of vertebral bodies, length and height of vertebral bodies were measured on X-rays (ImageJ 1.39, NIH, USA), The length (craniocaudal) and height (dorsoventral) of 5 vertebral bodies under the dorsal fin was measured in 12 individuals from each group at 2, 15g and 60g, and the length: height ratio was calculated.

At termination of the experiment, fish were sampled for analysis of whole body mineral content. Four samples per treatment were taken, one per each of the original first feeding tanks. Each sample consisted of 10 fish, which were pooled before analysis. The samples were stored frozen at -20°C, and were homogenized prior to analysis. The dry matter of samples was determined after drying at 104°C for 16h. For mineral analysis, samples were prepared as described [67,68] before analyzed by inductive coupled plasma (ICP) mass-spectroscopy.

Statistical analyses

A one-way analysis of variance model on incidence of deformities were carried out by SAS 9.1 software (SAS Institute Inc., USA), including the fixed effect of temperature regime. Statistics for gene transcription analysis are described in the real time qPCR section.

RNA isolation and cDNA synthesis

Tissue homogenization from 15 replicates from each treatment and developmental stage was achieved in a mortar with liquid nitrogen. Total RNA from the powdered vertebrae was isolated by using TRIzol™ and Micro to Midi Kit® (Invitrogen, MD, USA). Samples were treated with DNase1 (Invitrogen) before cDNA synthesis using oligo(dT) and Taqman Gold RT-PCR kit (Applied Biosystems, CA, USA). The cDNA synthesis was performed with 10min primer incubation at 25°C, 60min RT step at 48°C and 5min RT inactivation at 95°C in accordance to the manufacturer's protocol. All reactions were performed in accordance to the manufacturer's protocol.

Sequence information and primer design

Primers for expression analysis were based on known A. salmon sequences or on conserved regions of known teleost sequences paralogues. Primers were designed using the Vector NTI Advance 10 (Life technologies, MD, USA), and NetPrimer (PREMIER Biosoft, CA, USA) software. All PCR products were cloned using pGEM T-easy (Promega, WI, USA) and sequenced with Big Dye Terminator chemistry and the ABI 3730 automated sequencer, both delivered by Applied Biosystems. The obtained A. salmon sequences were analyzed by BLAST and deposited in the Genbank database (Table 1).

Real time PCR

Triplicate real-time qPCR reactions were performed using the Light cycler 480 and SYBR Green chemistry (Roche, Switzerland) at the following thermal cycling conditions: 95°C for 10min, followed by 45 cycles at 95°C for 15s, 60±1°C for 15s and 72°C for 15s. Further, specificity was assessed by the melting curves, determined post PCR (95°C for 15s, 60°C for 1min and 97°C continuous). PCR efficiencies for each target and the three housekeeping genes; *elongation factor 1a* (*el1a), heat shock protein 90 ß (hsp90ß*) and *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) were tested as endogenous controls. Relative target gene mRNA was normalized to relative *el1a* mRNA levels for all sample, as recommended by Olsvik et al. [69]. The transcription ratios of the 20 genes in all individual vertebrae from the two developmental stages were tested by using the Relative Expression Software Tool, REST, according to Pfaffl et al. [70]. Differences between the transcription ratios were tested for significance by the Pair Wise Fixed Reallocation Randomization Test© [70].

In situ hybridization and histology

Samples of phenotypically normal vertebrae from low and high intensive group at the 15g developmental stage were analyzed by *ISH* and histological analysis. Samples were dehydrated stepwise for 24h and clearing carried out in xylene (Merck, Darmstadt, Germany) for 2x24h before embedding in Technovit 9100 (Heraeus Kulzer, GmbH, Wehrheim, Germany), according to the procedure described by Torgersen et al. [71]. Parasagittal serial sections were cut from vertebral columns by using a Microm HM 355S (Thermo Fisher scientific Inc., PA, USA) and mounted on pre-coated slides (0.01% Poly-L-lysine (Sigma) and 2% polyvinyl acetate glue (Casco, Arnheim, Germany)). *ISH* was carried out with digoxigenine labeled (DIG RNA Labeling Kit, Roche) probes as described [71]. A total of five ECM producing genes were analyzed, including *col1a*, *col2a*, *col10a*, *osteocalcin* and *osteonectin*.

Histological examination of vertebrae with toluidine blue and alizarin red S double staining was carried out on deplastified and rehydrated sections. Briefly, the sections were stained for 2-3min at RT in 0.1% toluidine blue (Sigma-Aldrich) (pH 2.3) and rinsed in distilled H_2O followed by alizarin red

(Sigma-Aldrich) (pH 4.2) staining for 5min. Prior to microscopy, the stained sections were dehydrated in ethanol and mounted with Cytoseal 60 (Electron Microscopy Science, Fort Washington, PA, USA). Bright field microscopic analyses were performed on a Zeiss Axio Observer equipped with an AxioCam MRc5 camera and AxioVision software (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

Specimens for paraffin embedding were stepwise rehydrated in ethanol (50 and 25%) and decalcified for 7 days in 10% EDTA solution buffered with 0.1M Tris base (Merck) at pH 7.0. The decalcified specimens were rinsed in PBS and stepwise dehydrated in ethanol (50, 70 and 100%), before being embedded in paraffin. We used 3 paraffin infiltration steps carried out at 60°C for 2x2h and 1x3h. The specimens were embedded in paraffin, stiffened at room temperature and hardened over night at 4°C. 5µm serial sections were prepared using a Microm HM 355S. Paraffin sections were floated on demineralised water (25°C), mounted on uncoated slides and dried ON at 37°C. Prior to staining the sections were de-waxed with Clear Rite (Richard-Allan, MI, USA), followed by 2x washes in xylene (Merck) for 5min each. Sections were then rehydrated before rinsed in dH_2O. To demonstrate TRAP activity, the Acid phosphatase leukocyte kit No. 387 (Sigma-Aldrich) was used and followed according to the manufacturer's protocol; except that incubation lasted for 2h at 37°C. Subsequently, slides were rinsed in dH_2O. Specimens were counterstained with Mayers hematoxylin (Sigma-Aldrich) for 30s and rinsed in running tap water before dehydrated, cleared and mounted with Cytoseal 60 (Electron Microscopy Science). Controls were incubated without substrate.

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Figure legends

Figure 1 Experimental overview of the two temperature regimes. Developmental stage is shown along the x-axis and temperature along the y-axis. The low temperature intensive rearing conditions are shown as a black line and the high temperature intensive conditions as a stippled line. Sampling was conducted at 2g, 15g and 60g. Experiment was terminated when the fish reached 60g.

Figure 2 Frequency (%) of deformities in the vertebral column based on radiographic examination of A. salmon sampled from the low temperature intensive group (white bars) and the high temperature intensive group (black bars) from fertilization until 60g. Each bar represents the total number of deformities, scored as present or absent. Data are given in percentage ± st.dev, different letters indicate significant differences ($P \leq 0.01$) within the same size group, n=4 tanks per treatment.

Figure 3 Radiographic images of A. salmon at 15g from the low temperature intensive group (A) and the high temperature intensive group (B). Scale bar=1.5cm. Enlarged picture (to the right) corresponds to the dissected area from underneath the dorsal fin. Scale bar=1cm.

Figure 4 Relative gene transcription of A. ECM components, B. Signaling molecules and C. Transcription factors in phenotypical normal spinal columns from 2g (grey bars) and 15g (black bars) high temperature intensive group compared to the low temperature intensive group (white bars). Data are given as mean values + SE, n = 15 and significant differences (P=0.05) are indicated by *. Expression ratios are shown in relative mRNA expression along the y-axis, genes along the x-axis.

Figure 5 Transcription of *col1a* (A-C) and *osteocalcin* (D-G) in parasagittal sections of phenotypically normal vertebrae from 15g salmon reared at low intensive (l.i.) and high intensive (h.i.) temperature intensive regime. A. *Col1a* staining of a large section from l.i. covering 2 vertebrae shows that *col1a* mRNA (arrows) is highly expressed in osteoblasts lining the growth zone on the endbones of the vertebral bodies and the trabeculae. B. Higher magnification of the endbones; C. The corresponding area in h.i., notice weaker staining. D. Osteocalcin expressed in l.i. at the symmetrical growth zones of 2 adjacent vertebrae; E. The corresponding area in h.i.; F. L.i. Transcription in trabeculae; G. The corresponding area in h.i. Arrows indicate positive staining. Ventral side to the left. Scale bar A:200µm, B-G:100µm.

Figure 6 Transcription of *osteonectin* (A-D), *col2a* (E-I) and *col10a* (J-N) in parasagittal sections of phenotypical normal vertebrae from 15g salmon reared at low intensive (l.i.) and high intensive (h.i.) temperature intensive regime. Numbered bars indicate zones of chondrocytes divided into the sub-groups: 1.resting, 2.proliferating and 3.hypertrophic chondrocytes. A. *Osteonectin* specific staining of two vertebrae from l.i. revealed staining in zone 1 and 2. B. Strong transcription of *osteonectin* is present in osteoblasts lining the growth zone at the endbone and in the hypertrophic chondrocytes (arrow) in l.i. C. The arch centra from h.i. Notice increased transcription of *osteonectin*; D. Transcription of *osteonectin* in osteoblasts from h.i. appears decreased compared to l.i. E. *Col2a* staining of 3 vertebrae from l.i. *Col2a* mRNA localized to chondrocytes in zone 1 and 2. F. Higher magnification show distinct staining of *col2a* in the chordoblasts. G. Higher magnification of the arch centra with *col2a mRNA* in zone 1 and 2. H. In the corresponding area of h.i. a similar, but weaker pattern of *col2a* transcription is visible in the chordoblasts. I. Increased temperature lead to a distorted morphology and a more homogenous but weaker staining of *col2a*. J. Staining of a large section from l.i. covering 2 vertebrae show that *col10a* mRNA localizes to zone 3 (arrows). K. and L. Higher magnification reveal *col10a* specific staining in hypertrophic chondrocytes (arrow) and along the rims of the vertebral body (arrowhead). Black bar indicates the narrow zone of transcription restricted to hypertrophic chondrocytes. M and N. Corresponding area from h.i. show a wider zone of *col10a* positive hypertrophic chondrocytes (black bar). Ventral side to the left. Scale bar A,E,J:200µm, B-D,F-I,K-N,:100µm.

Figure 7 Parasagittal sections from 15g salmon vertebrae stained with alizarin red-toluidine blue, showing the arch centra (dorsal side). A. The chondrocytes localize into bands of 1. resting, 2. proliferating and 3. hypertrophic chondrocytes in fish reared at low temperature. B. In comparison, a high temperature regime results in a more uniform cartilage structure without the distinct chondrocytes sub-populations and the hypertrophic chondrocytes appears less calcified. Scale bar: 100µm.

Figure 8 TRAP staining of parasagittal sections from 15g salmon vertebrae, showing the arch centra (dorsal side). A. Positive staining (arrow) in the erosive front of cartilage indicating born resorption in neural arches of spinal columns from the low temperature group. B. No TRAP activity was found in the corresponding areas (indicated with an arrow) in samples from the high temperature group. Scale bar: 100µm.

Table legends

Table 1: Primers used for Real time qPCR (RT) and probes for in situ hybridization (ISH)

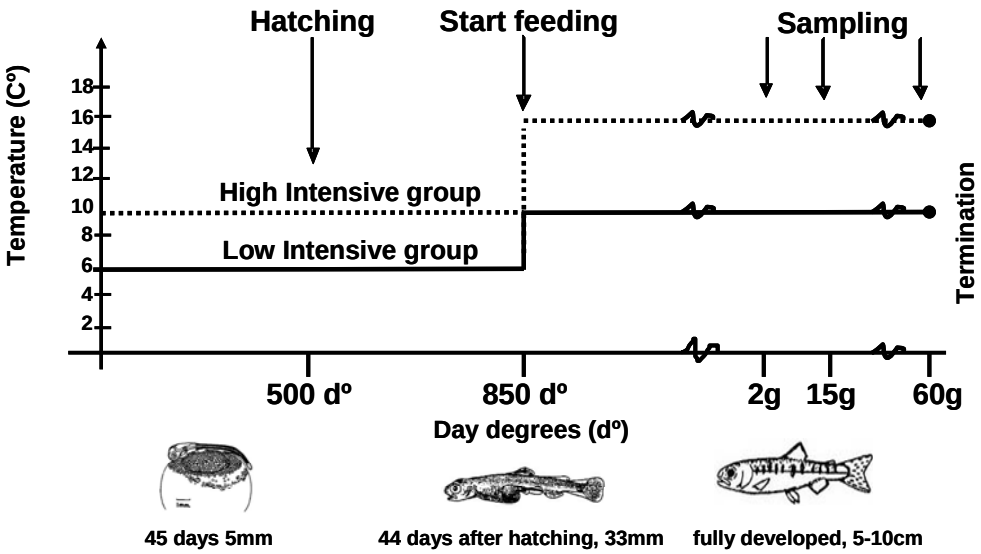
Primers used for Real time PCR (RT) and probes for in situ hybridization (ISH)				
Gene	Orientation	Genbank	Use:	Sequence (5`-3`)
Extracellular Matrix constituents:				
Col1a1	Forward	FJ195608	RT	AGAGAGGAGTCATGGGACCCGT
	Reverse		RT	GGGTCCTGGAAGTCCCTGGAAT
	Forward		ISH	TAGCCGTGGTTTCCCTGGTT
	Reverse		ISH	CCGGGAGGTCCAAATCTACC
Col2a1	Forward	FJ195613	RT	TGGTCGTTCTGGAGAGACT
	Reverse		RT	CCTCATGTACCTCAAGGGAT
	Forward		ISH	GCTGGCGAGACAGGAGAGA
	Reverse		ISH	GCCTCATCAGCCCTCATGTA
Col10a1	Forward	EG837148	RT	TGGTGCTCTTTGACTGCCTGTAA
	Reverse		RT	CATCCTGTGTGTTGCAATATCACA
	Forward		ISH	AACAAGGGCTTCTTGGATCA
	Reverse		ISH	CATAATGCATCCTCAGGCAT
Alp	Forward	FJ195609	RT	CTAGTTTGGGTCGTGGTATGT
	Reverse		RT	TGAGGGCATTCTTCAAAGTA
Osteocalcin	Forward	FJ195616	RT	GTGAACCAACAGCAAAGAGA
	Reverse		RT	CCAGGTCCTTCTTAACAAACA
	Forward		ISH	CTCATACTTGTTGATCGTCCAG
	Reverse		ISH	TCTTTCTCTCTCGCTCTCCC
Osteonectin	Forward	FJ195614	RT	ATTACTGAGGAGGAGCCCATCATT
	Reverse		RT	CCTCATCCACCTCACACACCTT
	Forward		ISH	CTGAACGATGAGGGTGTGGA
	Reverse		ISH	CGAGTGGTGCAGTGCTCCAT
Mmp9	Forward	CA342769	RT	AGTCTACGGTAGCAGCAATGAAGGC
	Reverse		RT	CGTCAAAGGTCTGGTAGGAGCGTAT
Mmp13	Forward	DW539943	RT	TGATGTCCAAGTCAGCCGCTTC
	Reverse		RT	TGGTCTGCCACTTGCGATTGTC
Cathepsin K	Forward	NM_001140399	RT	ATGACCAACGCCTTCGAGTAC
	Reverse		RT	AAGGTGGAGAGGGTGGCATC
Transcription factors:				
Sox9	Forward	EU344852	RT	CCTGCAAACAAGACAAGGT
	Reverse		RT	GGGTCGAGTAGATTCATACGA
	Forward		ISH	GGGGATACTATTTGACTGGATC
	Reverse		ISH	TCTGTCTTGATGTGTGTGGG
Runx2	Forward	FJ195615	RT/ISH	CCACCAGGGACAGACACAGAT
	Reverse		RT/ISH	GAACGGACTGAGATCTGACGAA
Osterix	Forward	FJ195612	RT	TCCCATAGACTTTCCCACA
	Reverse		RT	TGCCTCAGGACATGTACAA
Mef2c	Forward	GU252207	RT	CACCGTAACTCGCCTGGTCT
	Reverse		RT	GCTTGCGGTTGCTGTTCATA
	Forward		ISH	GACAGAGACTGTGTGGTGTTCCCT
	Reverse		ISH	AGGTGGAGGGAGCTACCACTGTTA
Signalling molecules:				
Bmp4	Forward	FJ195610	RT	TCAAGTTGCCCATAGTCAGT
	Reverse		RT	CACCTGAACTCTACCAACCA
Bmp2	Forward		RT	ATGTGGTATTGCACCCATT
	Reverse		RT	ATGGACAGTTTCCCAATGA
Shh	Forward	AY370830	RT	CCGGCTCATGACTCAGAGATG
	Reverse		RT	TATCCCTGGCCACTGGTTCA
Ihh	Forward	FJ195617	RT	CAGATGACCCACTGGACTGAT
	Reverse		RT	GCTTGGTTGGGAGATATGCA
Housekeeping gene:				
Ef1a	Forward	DQ834870	RT	CACCACCGGCCATCTGATCTACAA
	Reverse		RT	TCAGCAGCCTCCTTCTCGAACTTC

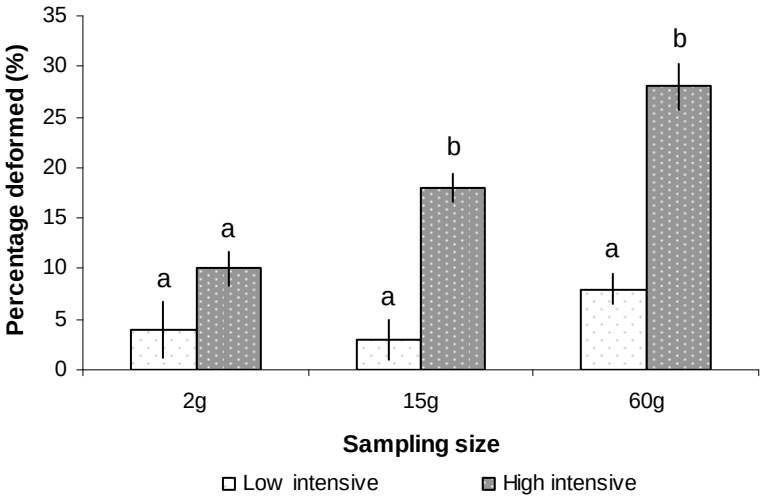
*Wargelius et al. [3] **Olsvik et al. [69] ***Takle et al. [2]

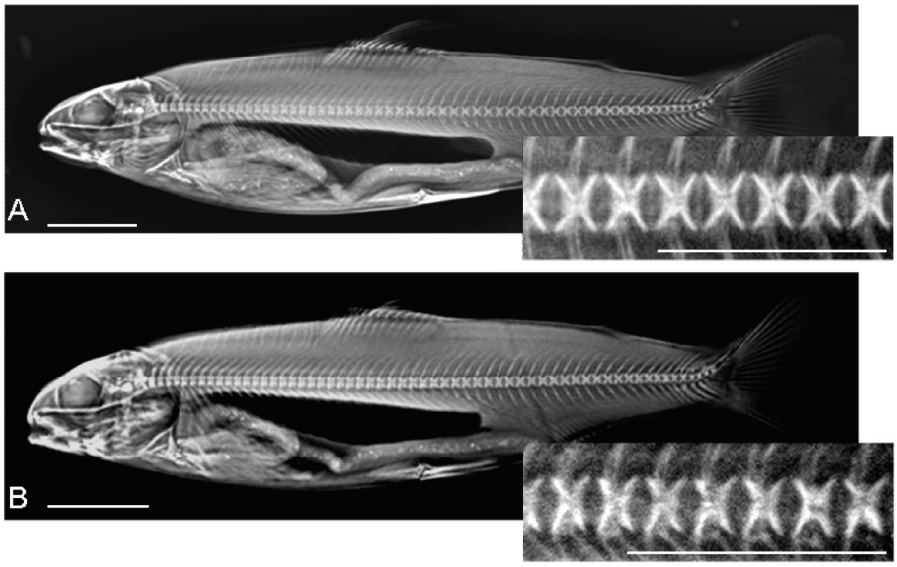
Table 2: Mineral analyzis

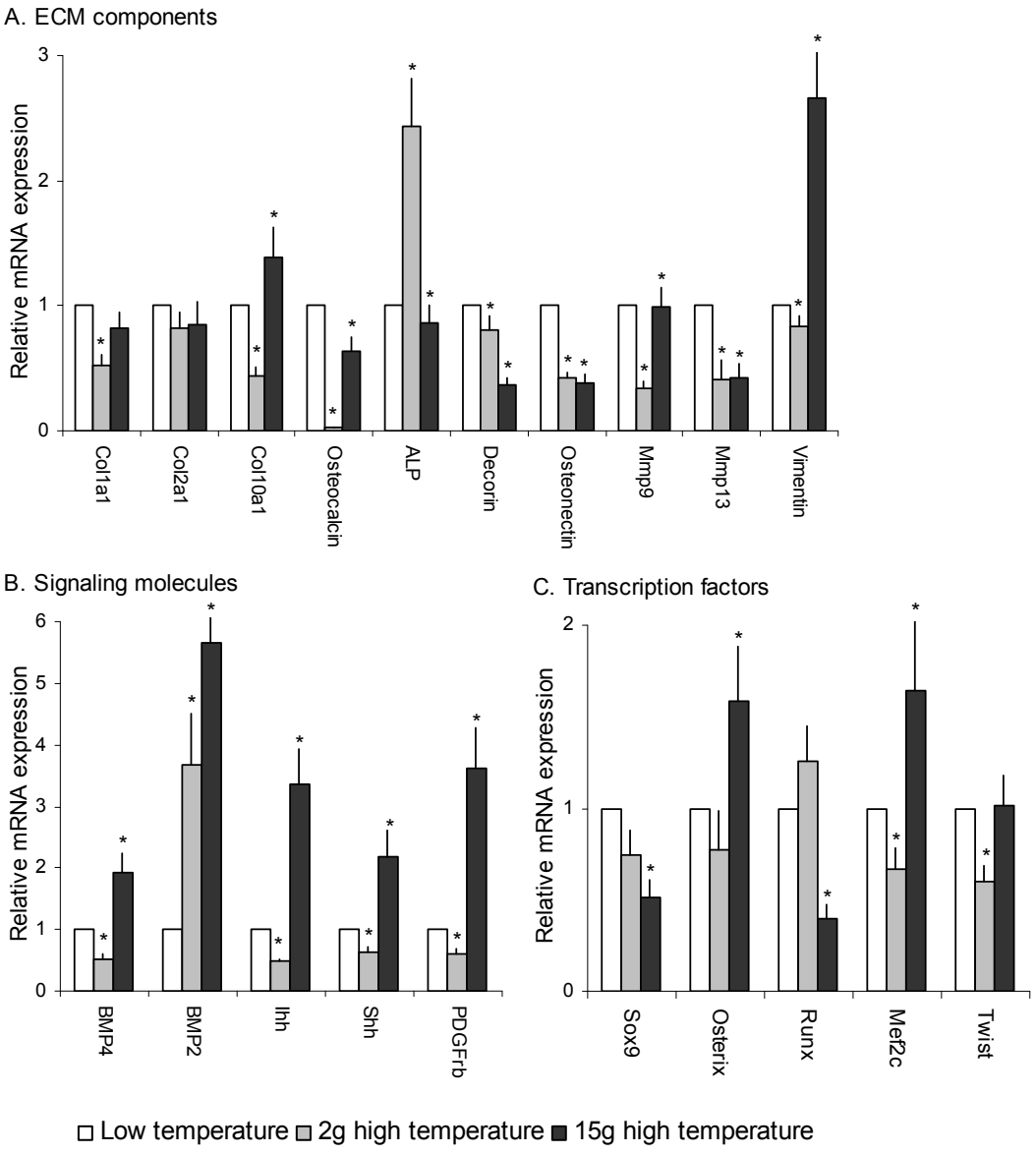
Mineral content (ppm) in whole body of A. salmon at 60g from low and high intensive temperature group. Total mineral content was measured in whole fish. Levels were low, but within the range of what are common in commercially reared salmon. Significant numbers (P=0.05) indicated by *, n=40 (means ± st.dev).

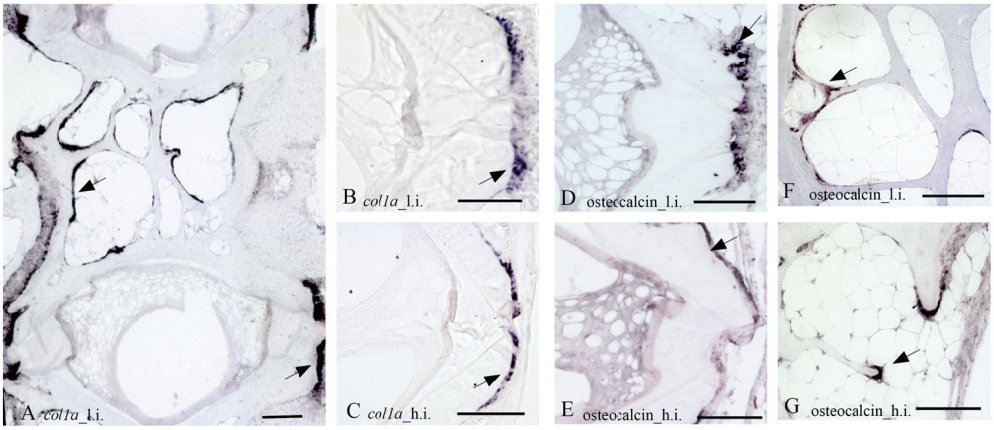
	Low intensive group	**High intensive group**
P	3823 ± 162	3886 ± 285
Ca	3655 ± 341	3700 ± 677
Mg	302.3 ± 8	299 ± 7
Na	1303 ± 70*	1084 ± 87*
Fe	9.7 ± 1.2*	8.1 ± 0.2*
Mn	0.86 ± 0.05	0.8 ± 0.09
Zn	31.8 ± 2.6	33.5 ± 1.7
Cu	0.8 ± 0.12	0.7 ± 0.09

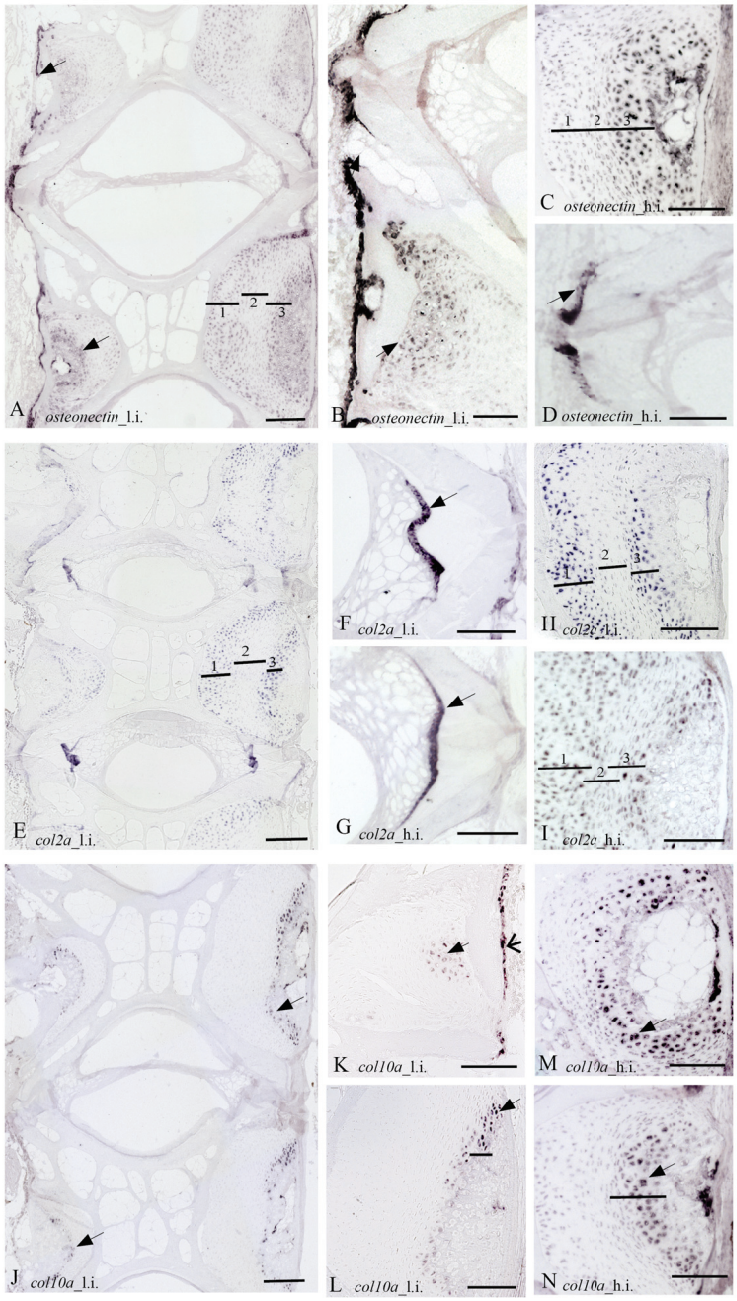

Hatching
Start feeding
Sampling
Temperature (C°)
18
16
14
12
10
8
6
4
2
High Intensive group
Low Intensive group
Termination
500 dº
850 dº
2g
15g
60g
Day degrees (dº)
45 days 5mm
44 days after hatching, 33mm
fully developed, 5-10cm

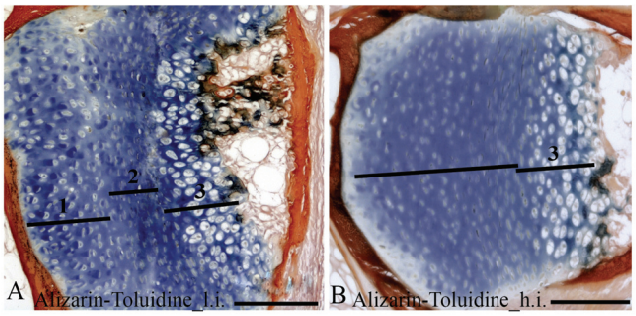

35
30
25
20
15
10
5
0
Percentage deformed (%)
a
a
b
a
b
a
2g
15g
60g
Sampling size
Low intensive
High intensive

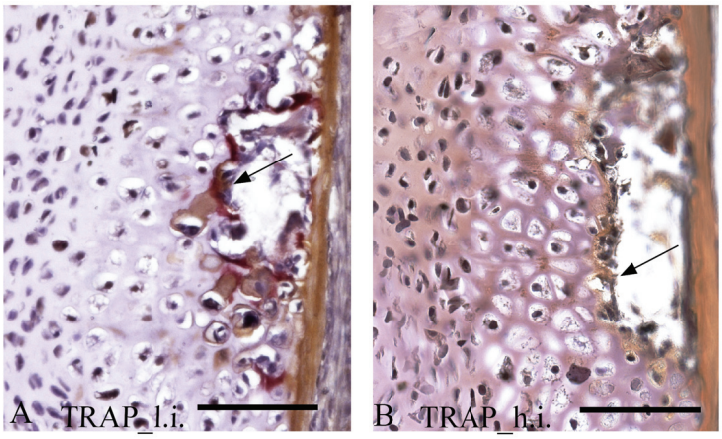

A
B


A. ECM components
Relative mRNA expression
Col1a1
Col2a1
Col10a1
Osteocalcin
ALP
Decorin
Osteonectin
Mmp9
Mmp13
Vimentin
B. Signaling molecules
BMP4
BMP2
Ihh
Shh
PDGFrb
C. Transcription factors
Sox9
Osterix
Runx
Mef2c
Twist
Low temperature
2g high temperature
15g high temperature


A col1a_l.i.
B col1a_l.i.
C col1a_h.i.
D osteccalcin_l.i.
E osteo:alcin_h.i.
F osteocalcin_l.i.
G osteocalcin_h.i.


A osteonectin_l.i.
B osteonectin_l.i.
C osteonectin_h.i.
D osteonectin_h.i.
E col2a_l.i.
F col2a_l.i.
G col2a_h.i.
H col2c_l.i.
I col2c_h.i.
J col10a_l.i.
K col10a_l.i.
L col10a_l.i.
M col10a_h.i.
N col10a_h.i.


1
2
3
A Alizarin-Toluidine_l.i.
3
B Alizarin-Toluidire_h.i.


A TRAP_l.i.
B TRAP_h.i.

Paper 2

Morphological and molecular characterization of developing vertebral fusions using a teleost model

Elisabeth Ytteborg[1,2], Jacob Torgersen[1], Grete Baeverfjord[1] and Harald Takle[1,3]*

[1]Nofima Marin AS, Ås, Norway
[2]Norwegian University of Life Sciences, Ås, Norway
[3]AVS Chile SA, Puerto Varas, Chile

*Corresponding author
Mail address: Norwegian University of Life Sciences, NO-1432 Ås, Norway
Tel: +47 45 40 04 56
Fax: +47 64 94 95 02

Email addresses:

EY: elisabeth.ytteborg@nofima.no

JT: jacob.torgersen@nofima.no

GB: grete.baverfjord@nofima.no

HT*: harald.takle@nofima.no

Abbreviations: Alp, alkaline phosphatase; BCIP/NBT, 5-bromo-4-chloro-3 indolyl phosphate *p*-toluidine salt/nitro blue tetrazolium chloride; Bmp, Bone morphogenetic proteins; Col1a1, collagen type 1a1; Col2a1, collagen type 2a1; Col10a1, collagen type 10a1; Ef1a, Elongation factor 1; Ihh, Indian hedge hog; IVD, intervertebral disk degeneration; IDD, Intervertebral disc disease; ISH, In situ hybridization; Mef2c, myocyte enhancer factor 2c; Mmp, Matrix metalloproteinase; PBS, phosphate-buffered saline; PFA, paraformaldehyde; qPCR, quantitative polymerase chain reaction; Runx2, runt-related transcription factor 2; Shh, Sonic hedge hog; Sox9, (sex determining region Y) box 9; TRAP, Tartrate resistant acid phosphatase.

Abstract

Background: Spinal disorders are a major cause of disability for humans and an important health problem for intensively farmed animals. Experiments have shown that vertebral deformities present a complex but comparable etiology across species. However, the underlying molecular mechanisms involved in bone deformities are still far from understood. To further explicate the mechanisms involved, we have examined the fundamental aspects of bone metabolism and pathogenesis of vertebral fusions in Atlantic salmon (*Salmo salar*).

Results: Experimentally, juvenile salmon were subjected to hyperthermic conditions where more than 28% developed fused vertebral bodies. To characterize the fusion process we analyzed an intermediate and a terminal stage of the pathology by using x-ray, histology, immunohistochemistry, real-time quantitative PCR and *in situ* hybridization. At early stage in the fusion process, disorganized and proliferating osteoblasts were prominent at the growth zones of the vertebral body endplates. PCNA positive cells further extended along the rims of fusing vertebral bodies. During the developing pathology, the marked border between the osteoblast growth zones and the chondrocytic areas connected to the arches became less distinct, as proliferating cells and chondrocytes blended through an intermediate zone. This cell proliferation appeared to be closely linked to fusion of opposing arch centra. During the fusion process a metaplastic shift appeared in the arch centra where cells in the intermediate zone between osteoblasts and chondrocytes co-expressed mixed signals of chondrogenic and osteogenic markers. A similar shift also occurred in the notochord where proliferating chordoblasts changed transcription profile from chondrogenic to also include osteogenic marker genes. In progressed fusions, arch centers and intervertebral space mineralized.

Conclusion: Loss of cell integrity through cell proliferation and metaplastic shifts seem to be key events in the fusion process. The fusion process involves molecular regulation and cellular changes similar to those found in mammalian deformities, indicating that salmon is suitable for studying general bone development and to be a comparative model for spinal deformities.

Background

The vertebral column is the defining character of vertebrates providing the organism with a unique ability of movement, form and function. Obviously, abnormalities to this organ can lead to severe and often painful pathological conditions. Spinal disorders are a major cause of disability for humans and an important health problem for intensively farmed animals. A number of animal models have been used to further explore the pathology and revealed that vertebral deformities present a complex but comparable cross species etiology [1,2]. Morphological changes like altered bone formation and cell density, thinning of osteoblasts along with increased cell proliferation and cell death are changes found in spinal deformities and intervertebral disc degeneration (IDD) in mammals [3,4]. Discs from patients with spinal deformities further have ectopic calcification of the vertebral endplates and sometimes in the disc itself [5]. Cells of the mammalian disc are derived directly from the phylogenetically conserved notochord [6]. Whereas only remnants of the notochord exists in the nucleus pulposus (NP) in humans by the age of 4, the notochord persist throughout all life stages in teleosts. Spinal disorders in teleosts like sea bass, sea bream, rainbow trout, halibut and salmon [7-12] have mostly been descriptive and few molecular studies have been carried out. However, in Atlantic salmon (*Salmo salar*) compression (platyspondyly) and/or vertebral fusion (ankylosis) accounts for 9 out of 20 recently described vertebral deformities [13]. Spinal fusions involves transformation of intervertebral notochord tissue into cartilage, shape alterations of vertebral body endplates, mineralization of the intervertebral cartilage and replacement of intervertebral cartilage by bone [14], pathological processes resembling those of IDD in mammals.

Skeletogenesis in salmon involves activity from the three main bone and cartilage cell types; chondrocytes, osteoblasts and osteoclasts. Bone formation further occurs via two basic mechanisms; compact bone of the amphicoel and trabeculae is formed directly through intramembranous ossification, whereas the cartilaginous template is replaced by bone in the arch centra through endochondral ossification. Bone formation is brought about by a complex set of highly regulated molecular pathways, involving extracellular matrix (ECM) constituents (e.g. collagens and osteocalcin), signaling molecules (e.g. hedgehogs and bmps) and transcription factors (reviewed [15-17]). Some of the key transcription factors in bone metabolism include runx2 and osterix [18], involved in the differentiation of mesenchymal stem cells (MSC) into osteoblasts that express bone matrix (col1a) and matrix mineralizing (osteocalcin and osteonectin) genes. Early chondrocyte differentiation is controlled by sox9, which regulates transcription of col2a [19], the major ECM component of cartilage. Further, before endochondral ossification may occur, mef2c assures that chondrocytes mature into col10a producing hypertrophic cells [20]. Both mineralized bone and cartilage is remodeled through the activity of osteoclasts. These multinucleated cells provide and acidic environment, express cathepsins and matrix metalloproteinases (mmps) and are tartrate acid phosphatase resistant (TRAP). Hence mineralized matrix may be broken

down [21,22]. The skeletal pathways described in mammals are currently being understood in teleosts. In a recent study, we investigated 20 genes for their role in salmon spinal column skeletogenesis [23]. However, the genetic interactions of bone and cartilage development are currently becoming more entangled, as chondrocytes and osteoblasts are shown to intersect through the formation of chondroid bone. This process has been described through normal maturation, differentiation plasticity and trans-chondroid ossification [24-26]. Though, the molecular pathways involved are still far from understood.

During the last decade problems with spinal disorders in salmon have been increasingly in focus due to the importance of this species in the aquaculture industry. To further elucidate the mechanisms involved in the development of vertebral deformities, we analyzed an intermediate and terminal stage of the fusion process at a morphological level by using radiography and histology and gene transcriptional changes using quantitative PCR (qPCR) and *in situ* hybridization (*ISH*). We found that loss of cell integrity and ectopic bone formation characterizes the development of spinal fusions. During the fusion process a metaplastic shift appeared in the arch centra where cells in the intermediate zone between osteoblasts and chondrocytes co-expressed mixed signals of chondrogenic and osteogenic markers. A similar shift also occurred in the notochord where proliferating chordoblasts changed transcription profile from chondrogenic to also include osteogenic marker genes. We suggest that hyperthermic induced development of spinal fusions involve a metaplastic shift in cells from the chondrocytic lineage. With this work, we bring forward salmon to be an interesting organism to study development of spinal fusions.

Materials and methods

Rearing conditions

This trial was performed under the supervision and approval of the veterinarian that has appointed responsibility to approve all fish experiments at the research station in accordance to regulations from the Norwegian authorities regarding the use of animals for research purposes. The experiment was carried out at Nofima Marins research station at Sunndalsøra, Norway, in 2007, as described in Ytteborg et al [23]. During egg rearing, water supply was continuous from temperature controlled tanks stabilized at 10±0.3ºC. The temperature was gradually increased at first feeding to 16±0.3ºC (1ºC per day). Temperatures exceeding 8ºC during egg rearing and 12ºC after start feeding elevate the risk of developing spinal fusions.

Radiography and classification

Sampling was directed from radiographs so that the sampled area corresponded to the deformed or normal area. Fish were sedated (Tricaine methane sulfonate, Pharmaq, Norway) and radiographed

during the experiment at 2g, 15g and 60g. Fish that were not sampled were put back into oxygenated water to ensure rapid wakening. The x-ray system used was an IMS Giotto mammography system (model number 6020/3, IMS Giotto, Bologna, Italy) equipped with a FCR Profect image plate reader and FCR Console (Fuji Medical Inc., Japan). At 15g size, fish were sampled for histological and gene transcriptional analysis. Samples for *ISH* and histology were fixed in 4% PFA (n=24) and samples for RNA isolation were snap frozen in liquid nitrogen and stored at -80C° (n=45).

All fish were divided into three categories where the first group was non-deformed. These spinal columns had no observable morphological changes in the vertebral bodies or in intervertebral space. We further sampled vertebral areas at two different stages in the pathological development of fusions, termed intermediate and fused. Vertebrae diagnosed as intermediate included various degrees of reduced intervertebral space and compressions. Samples characterized as fused ranged from incomplete fusions to complete fusions.

Statistical analyses

Incidence of fusions were observed through radiography and calculated using a one-way analysis of variance model (GLM procedure, SAS 9.1 software, SAS Institute Inc., USA). Results are represented as means ± standard deviation (st.dev).Statistics for mRNA transcription analysis are described in the real-time PCR chapter.

Sample preparation

Histological staining and *ISH* was carried out on five µm Technovit 9100®New sections according to the protocol [27]. Serial sections were prepared in the parasagittal orientation from vertebral columns, starting at the periphery and ending in the middle plane of the vertebrae using a Microm HM 355S (Thermo Fisher Scientific Inc., MA, USA). For immunohistochemistry, tissue was decalcified for seven days in 10% EDTA, dehydrated in ethanol, cleared and embedded in paraffin. Five µm serial sections were prepared as described above, de-waxed with Clear Rite (Richard-Allan, MI, USA), followed by two times washing in xylene (Merck Chemicals Ltd.) for five min each. Sections were then rehydrated before rinsed in dH_2O.

Histology and immunohistochemistry

Bone and cartilage formation in the spinal columns were assayed by Alizarin Red S/Toluidie Blue (Sigma-Aldrich, MO, USA) staining. Sections were stained for 5min in Alizarin red (pH 4.2) and for 2min in 0.1% Toluidine blue (pH 2.3), with a brief rinse in dH_2O in between. Single staining with the two dyes was also performed. All sections were dehydrated in ethanol and mounted with Cytoseal 60 (Electron

Microscopy Science, PA, U.S.A) prior to microscopy. To demonstrate osteoclast activity, TRAP was visualized with the Acid phosphatase leukocyte kit No. 387 (Sigma-Aldrich) was applied according to the manufacturer's protocol, with the exception of a 2h incubation at 37°C. Subsequently, slides were rinsed in dH_2O and counterstained with Mayers hematoxylin (Sigma-Aldrich) for 30s. Cell proliferation and apoptosis were assessed by immunohistochemical detection of proliferating cell nuclear antigen (PCNA) and cleaved Caspase 3, respectively [28-30]. Slides were placed in 0.1M citric acid, 0.05% Tween 20 (pH 6) and heated in microwave, 5min at 900W and 4min at 650W. Endogenous peroxidase activity was blocked 10min in 3% H_2O_2 in methanol. The sections were washed 3x in PBS and incubated with a mouse anti PCNA monoclonal antibody (clone PC10, Zymed Laboratories Inc., CA, USA) or Cleaved Caspase 3 (Asp175 5A1, Cell Signaling Inc. Boston, MA, USA), following the manufacturer's instructions. Slides were washed 3x 5min in PBS-Tween 20 before counterstained with Mayer's hematoxylin for 2min, washed in water, dehydrated in a graded series of ethanol solutions, cleared with xylene, and mounted with Cytoseal60 (Electron Microscopy Science). Controls were incubated without substrate. Microscopic analyses were performed by the stereomicroscope Zeiss Axio Observer Z1 using brightfield illumination and digitized images obtained with an AxioCam MRc5 camera using AxioVision software (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

Primer design

Primers (Table 1) for transcription analysis were based on known salmon sequences or on conserved regions of known teleost sequences paralogues. Primers were designed using the Vector NTI Advance 10 (Invitrogen, CA, USA) and NetPrimer (PREMIER Biosoft, CA, USA) software. All PCR products were cloned using pGEM T-easy (Promega, WI, USA) and sequenced with Big Dye Terminator chemistry and the ABI 3730 automated sequencer, both delivered by (Applied Biosystems, CA, USA). The obtained salmon clones were analyzed by BLAST and deposited in the Genbank database.

RNA isolation and cDNA synthesis

Tissue homogenization from 15 replicates from each group was achieved in a mortar with liquid nitrogen. RNA was extracted using Trizol reagent and Micro to Midi Kit® (Invitrogen). Brief, tissue was homogenized in a mortar with liquid nitrogen and total RNA was extracted using Trizol reagent and Micro to Midi Kit® (Invitrogen) before DNase treatment (DNase1, Invitrogen). The quality of the RNA was assessed spectrophotometrically (NanoDrop Technologies, DE, USA) 1µg RNA was reverse transcribed to cDNA using oligo(dT) primer and the Taqman Gold RT-PCR kit (Applied Biosystems). The cDNA synthesis was performed with 10min primer incubation at 25°C, 1h RT step at 48°C and

5min RT inactivation at 95°C. All reactions were performed in accordance to the manufacturer's protocol.

Real-time quantitative RT-PCR

Real-time qPCR was conducted using the Light cycler 480 and SYBR Green chemistry (Roche, Switzerland) at the following thermal cycling conditions: 95°C for 10min, followed by 45 cycles at 95°C for 15s, 60±1°C for 15s and 72°C for 15s. Further, specificity was assessed by the melting curves, determined post PCR (95°C for 15s, 60°C for 1min and 97°C continuous). To determine the efficiency of target genes and reference gene (*ef1a*), we used the standard curve method. Relative target gene mRNA was normalized to relative *ef1a* mRNA levels for all sample, as recommended by Olsvik et al. [31]. The transcription ratios were analyzed using the Relative Expression Software Tool (REST) and tested for significance by the Pair Wise Fixed Reallocation Randomization Test© [32].

In situ hybridization

Digoxigenin labeled antisense and sense riboprobes were synthesized according to the manufacturer's protocol (Roche), using 250 ng of SP6 and T7 tailed PCR fragments as template. *ISH* was carried out on five µm Tw9100 sections as described [27], and microscopic analyses of the NBT/BCIP stained sections were conducted on a Zeiss Axio Observer Z1 equipped with an AxioCam MRc5 camera and AxioVision software (Carl Zeiss Microimaging GmbH).

Results

The elevated temperature regime used in this study induced mainly vertebral deformities of the fusion type. The incidence of complete fusions was 10.0 (not significant = n.s.), 17.9 ($p \leq 0.001$) and 28.1% ($p \leq 0.0001$) at 2, 15 and 60g, respectively (Fig. 1). The incidence in the two later samplings are underestimated, since these numbers do not take into consideration that fish sampled at 2 and 15g could develop into fusions at the following samplings. Some fish displayed more than one type of pathology, but pathological changes other than fusions were low in numbers and were not investigated. The fusion process is a dynamic process as visualized by x-ray in figure 2.

Histology and immunohistochemistry

Histological examination revealed more detailed morphological characteristics of intermediate and fused vertebral bodies (Fig. 3A, B). The osteoblasts at the growth zones of the vertebral endplate appeared well-organized in non-deformed vertebrae and little aberrancy was found when staining with toluidine

blue (Fig. 3C). The corresponding growth zones in intermediate vertebrae displayed alterations in vertebral endplates and more disorganized osteoblasts (Fig. 3D). These findings became more pronounced at fused stage. The osteogenic zone of the vertebral endplate extended abaxial in-between two vertebral body endplates (Fig. 3E). In addition, arch centra had decreased in fused vertebral bodies and chordocytes appeared denser compared to non-deformed (Fig. 3B). Alizarin red S visualized more calcified tissue in areas with reduced arch centra in intermediate and fused vertebrae (Fig. 3F-H). In fusions, normal vertebral hour-glass shape was replaced by a more compact and squared shape morphology, as the arch centra were more or less replaced by bone (Fig. 3H). Alizarin red S stained calcified tissue and showed calcification of the centra and around hypertrophic chondrocytes (Fig. 3I). No calcification was detected in the intervertebral space of incomplete fusions (Fig. 3I). In fusions, growth zones of opposing vertebral bodies had fused and intervertebral space mineralized (Fig. 3K).

A balance between bone resorption and bone formation is required for maintaining bone integrity during remodeling. Thus, we examined osteoclast activity using TRAP staining. Weak positive TRAP staining was detected at the ossifying border of hypertrophic chondrocytes in the arch centra in one sample from the intermediate group (Fig. 4A, B). No positive staining was found in samples from the fused group (Fig. 4C).

To analyze if the morphological changes observed during development of fusions could be linked to an imbalanced cell cycling, we used immunohistochemistry with antibodies specific to PCNA for detection of proliferation and caspase 3 for detection of apoptosis. A few PCNA positive cells were apparent at the osteoblast growth zone at the endplates in non-deformed vertebral bodies (Fig. 4D). PCNA positive cells were almost entirely restricted to these areas and were rarely found in chordoblasts or chordocytes (Fig. 4E). However, we detected a markedly increase in PCNA positive cells at the growth zone of the endplates, and in cells extending axial at intermediate and fused stages (Fig. 4F and G). Further, high abundance of proliferating chordoblasts were found in the notochord of vertebrae with reduced intervertebral space (Fig. 4H and I). A few positive caspase 3 signals were detected at the rims of the osteoblast growth zone of the endplates in non-deformed vertebral bodies (Fig. 4J). Increased caspase 3 signals were found in these areas of intermediate and fused vertebral bodies. Caspase 3 positive cells were also prominent at the transition between the intervertebral and vertebral regions (Fig. 4K and L). The positive signal was further spreading along the rims of the vertebral bodies in axial direction (Fig. 4M) and in cells harboring the joints of the trabeculae (Fig. 4N). Caspase 3 was not detected in the notochord in any of the groups. The cells that stained positive had characteristic apoptotic morphology with membrane blebbing (Fig. 4O).

Spatial and temporal gene transcription in developing fusions

To examine transcriptional regulations involved in development of fusions, we analyzed non-deformed, intermediate and fused vertebrae with real-time qPCR, while the spatial gene transcription in intermediate and fused vertebrae were characterized by *ISH*. *ISH* of non-deformed vertebral bodies have previously been described in Ytteborg et al [23]. Quantification of mRNA revealed that most genes were transcriptionally down-regulated during the pathogenesis of vertebral fusions and that the suppression was more profound at the intermediate stage than in fused specimens (Fig. 5). We divided the 19 analyzed genes into two groups; structural genes and regulatory genes (transcription factors and signaling molecules).

Structural genes

Nine out of 11 structural genes had a down-regulated transcription in the intermediate group compared to only five in the fused group (Fig. 5A). Four genes were down-regulated in both groups, including genes involved in bone (*col1a1*) and hypertrophic cartilage ECM production (*col10a1*) and mineralization (*osteocalcin* and *alp*). *Col2a1* transcription was down-regulated in intermediate while up-regulated in the fused group (n.s.). *Osteonectin* was up-regulated in both groups. Of genes involved in osteoclast activity, *mmp9* showed opposite transcription, being down-regulated in intermediate while up-regulated in fused. *Mmp13* and *cathepsin K* showed similar transcription pattern in the two groups, *mmp13* up-regulated and c*athepsin K* down-regulated (n.s. in fused).

ISH analyzes of *col1a, col2a, col10a, osteonectin* and *osteocalcin* revealed cells exhibiting characteristics of both osteoblasts and chondrocytes. These findings were more pronounced in fused than intermediate specimens. *Col1a* was expressed in osteogenic cells along the rims of the vertebral body endplates and in osteoblasts at the lateral surfaces of trabeculae at the intermediate stage (Fig. 6A). In incomplete fusions, we could locate osteogenic *col1a* positive cells in the growth zone of the vertebral endplate extending abaxial in-between vertebral bodies (Fig. 6B, C). In addition, *col1a* was expressed in high abundance in the intervertebral space of incomplete fusions (Fig. 6D). The chondrocytic marker *col2a* was observed in chordoblasts in intermediate samples (Fig. 6E). Furthermore, *col2a* was expressed at the growth zone of the vertebral body endplates in both intermediate and fused samples (Fig. 6F, G). Positive staining of *col2a* in the notochord became stronger as intervertebral space narrowed down (Fig. 6F, G). Transcription of *col10a* was observed in hypertrophic chondrocytes and in osteogenic cells lining apical surfaces of trabeculae in intermediate and fused vertebrae (Fig. 6H, I). *Col10a* seemed to be less expressed in both intermediate and fused vertebrae, as also observed from the down-regulated transcription of this gene from the qPCR results. *Osteonectin* showed a similar transcription pattern as *col10,* but transcription seemed increased in the

trabeculae (Fig. 6J). Transcription of *osteonectin* was also associated with chondrocytes in regions where arch centra fused (Fig. 6K, L). Strong *osteonectin* transcription correlated with an up-regulated mRNA transcription observed from qPCR. *Osteocalcin* was transcribed in osteogenic cells lining surfaces of trabeculae of fused vertebrae (Fig. 6M) and in cells located abaxial in-between two opposing vertebral body endplates (Fig. 6N). When the vertebral growth zones blended with the arch centra, chondrocytes expressing *osteocalcin* was observed (Fig. 6O).

Regulatory genes –transcription factors and signaling molecules

All of the regulatory genes (Fig. 5B) were less expressed in the intermediate compared to the fused group. Except of *osterix*, regulatory genes showed similar transcription patterns in the two groups. *Twist* involved in osteoblast inhibition and *mef2c* involved in chondrocyte hypertrophy were down-regulated in both groups. However, the chondrogenic marker s*ox9* was up-regulated in both groups. The osteogenic markers *runx2* and *osterix* had up-regulated transcription in the fused group, *runx2* in intermediate group. Osterix was down-regulated in intermediate group, however n.s. Except of *bmp2* in fused vertebral bodies, signaling molecules (*shh*, *ihh, pdgfrb* and *bmp4)* were down-regulated in both intermediate and fused group.

When analyzing selected genes by *ISH*, *runx2* was never detected in chordocytes, chordoblasts or chondrocytes in non-deformed vertebral bodies. Positive *runx2* staining was however detected at the osteoblast growth zone of the vertebral endplate (Fig. 7A). In intermediate and fused samples we detected transcription at the corresponding growth zone and along the lateral surfaces of the trabeculae (Fig. 7B). We observed an increased transcription of *runx2* in the chordocytes of incomplete fusions (Fig. 7C) and in the chordoblasts and chordocytes in more severe fusions (Fig. 7D). These findings corresponded to the up-regulated transcription found by qPCR. *Sox9* was expressed in chondrocytes in non-deformed vertebral bodies (Fig. 7E) and in chordoblasts. In intermediate and fused samples, strong signals of *sox9* were detected in intervertebral space (Fig. 7F). *Sox9* was also transcribed at the vertebral growth zones of the endplates (Fig. 7G) and the signal was extending axial in severe fusions (Fig. 7H). *Mef2c* was expressed in a wide zone of hypertrophic chondrocytes in non-deformed vertebral bodies (Fig. 7I). Hypertrophic chondrocytes also transcribed *mef2c* in intermediate and fused vertebral bodies (Fig. 7J). Further, *mef2c* was observed at the boundaries between two fused arch centra (Fig. 7J). In fusions were arch centra narrowed down, *mef2c* transcription did not seem restricted to hypertrophic zones (Fig. 7K). Some *mef2c* expressing cells was also detected at the vertebral endplates (Fig. 7L) and abaxial between vertebral growth zones of opposing vertebral bodies in incomplete fusions (Fig. 7M).

Discussion

In this study we present a molecular characterization of mechanisms involved in development of vertebral fusions in salmon. We have previously shown that the non-deformed fish used in this study had indications of soft bone phenotype [23]. They were further characterized by disrupted chondrocytic maturation, increased zones of hypertrophic chondrocytes and delayed endochondral ossification in the arch centra [23]. The number of deformities increased throughout the experiment and an imbalanced bone and cartilage production characterized susceptible fish, predisposed for developing deformities [23]. In this study we wanted to analyze an intermediate and a terminal stage of the fusion process to further characterize developing deformities. Through this experiment, we found that vertebral deformities were developing through a series of events, of which five hallmarks were identified as particularly interesting. First, disorganized and proliferating osteoblasts were prominent in the growth zones of the vertebral body endplates. Second, a metaplastic shift made the borders less distinct between the osteoblastic growth zone and the chondrocytic areas in the arch centra. Third, the arch centra ossified and the endplates became straight, hence giving the vertebral bodies a squared shaped morphology. Fourth, the intervertebral space narrowed down and the notochord was replaced by bone forming cells. Fifth, in a complete fusion all intervertebral tissue was remodeled into bone.

One of the major morphological changes during the fusion process was ossification of the arch centra. Our findings suggest that this ectopic bone formation is a key event in development of vertebral fusions, which involve lack of normal cell differentiation and growth. Immunohistochemistry with PCNA showed that osteoblasts at the growth zone of the vertebral body endplates had a markedly increased cell proliferation during the fusion process. The increased proliferation of osteoblasts was apparently partly counteracted by increased cell death as shown by stronger caspase 3 signaling. Nevertheless, the osteoblasts at the vertebral endplates appeared less organized in intermediate and fused vertebral bodies by toluidine blue staining. In addition, in fused vertebral bodies we observed moderate changes of abaxial translocation of cells from the osteoblast growth zone. Abaxial direction of growth from the borders of vertebral body end-plates and formation of chondroid bone in these areas are also described in previous experiments [14,33]. The findings of increased proliferation and disorganized osteoblast growth were evident in vertebrae with modest alterations, which may suggest that this is an early event in the fusion process.

During the developing pathology, the marked border between the osteoblast growth zones and the chondrocytic areas connected to the arches became less distinct, as proliferating cells and chondrocytes blended through an intermediate zone. PCNA positive cells further extended along the rims of fusing vertebral bodies. This cell proliferation appeared to be closely linked to fusion of opposing

arch centra. During the fusion process a metaplastic shift appeared in the arch centra where cells in the intermediate zone between osteoblasts and chondrocytes co-transcribed *col1a*, *col2a, runx2, osteocalcin* and *osteonectin,* as visualized by *ISH.* Based on histology, Witten et al. [25] have previously suggested the involvement of a metaplastic shift in developing fusions. In more progressed fusions, most cells in the arch centra seemed to co-transcribe osteogenic and chondrogenic markers. Our suggestion is therefore that trans-differentiated cells produce the ectopic bone.

Several *in vitro* studies have demonstrated that chondrocytes associated with calcifying cartilage can acquire properties of osteoblasts [34] and are able to change their phenotype from a primarily cartilage synthesizing cell type to a bone synthesizing cell type [35]. However, hypertrophic chondrocytes able to trans-differentiate into osteoblasts through a process called trans-chondroid ossification has also been described [24]. Interestingly, this type of growth has been identified during distraction osteogenesis in rats [24,36], a process where bone is formed rapidly upon stretching. During trans-chondroid ossification, chondrocytes are found to express both *col1* and *col2* [24]. In a review by Amir et al. [37] it was speculated if tension stress during distraction inhibited final differentiation of chondrocytes and rather trans-differentiated these cells into osteoblastic cells. At fused stage, early markers for osteoblasts and chondrocytes (*runx2, osterix, sox9* and *bmp2)* were upregulated whereas the osteoblast inhibitor (*twist)* and genes involved in chondrocyte hypertrophy (*bmp4, mef2c, col10a, shh* and *ihh)* were downregulated, results also supported by *ISH*. Deletion of *Ihh* has been shown to disrupt the normal pattern of various zones of chondrocyte differentiation in the growth plate [38], whereas Sox9 accelerate chondrocyte differentiation in proliferating chondrocytes but inhibit hypertrophy [39]. Sustained *runx2* expression, as found in our studies, is further associated with trans-differentiation of chondrocytes into bone cells [40]. On the contrary, analyzing the ECM components of both osteoblasts and chondrocytes (*col1a, col2a, col10a, osteocalcin* and *alp*) revealed that these transcripts had reduced activity in both intermediate and fused vertebrae. These findings might reflect the reduced radiodensity described in fish reared at elevated temperatures [23].

To further characterize the pathological bone formation in the chondrocytic areas in the arch centra, we analyzed osteoclast activity. Absence of osteoclasts visualized through TRAP staining was characteristic during the development of vertebral fusions, indicating that normal endochondral ossification was restrained. In addition, *cathepsin k* had a down-regulated transcription level. In normal developing salmon vertebrae, these areas are modeled through endochondral bone formation, a process requiring invasion of osteoclasts and activity of TRAP, Mmps and Cathepsin K [21,41]. Transcription of *mmp*s are up-regulated during IDD [42] and compression-induced IVD [43,44] in mammals. Intriguingly, *mmp9* and *mmp13* were also up-regulated during fusion of vertebral bodies in

salmon. Excessive co-activity of *mmp9* and *mmp13* is linked to development and healing of chronic wounds in rainbow trout [45] and salmon [46]. Lack of osteoclast activity and reduced activity of genes involved in chondrocyte hypertrophy during development of vertebral fusions may therefore suggest that *mmp*`s were up-regulated in fused vertebral bodies as a response to chronic injury rather than bone resorption.

Our results suggest that the ossification type during development of spinal fusions and fast growth could be trans-chondroid ossification. A mixed type of intramembraneous and endochondral ossification, as suggested by Yasui et al [24] and demonstrated by Okafuji et al [36] may also occur, however the lack of osteoclast activity makes this less likely. Our findings indicate that chondrocytes had not only differentiated towards osteoblast-like cells, but also completed the differentiation to cells that were capable of producing mineralized bone matrix. Whether the suggested trans-chondroid ossification is trans-differentiation as a sudden switch from the chondrogenic to the osteogenic phenotype or a continuous differentiation was not assessed in this experiment. However, based on our results, a pathway to bone formation through chondrocytes might be possible during development of vertebral fusions.

The completing step in the fusion process is transformation of notochordal tissue into bone [14]. As intervertebral space narrowed down, proliferating chordoblasts and denser packet chordocytes were revealed through toluidine blue staining and PCNA antibody binding, respectively. The structured chordoblast layer increased and more of these cells stained for *col2a*. As the pathology progressed, proliferating chordoblasts seemed to occupy most of the intervertebral space and vacuolated chordocytes disappeared. Moreover, cells in the notochord had a transcription profile resembling the trans-differentiating cell at the borders between the osteoblast growth zones and the chondrocytic areas connected to the arches. Transcription of marker genes changed from chondrogenic to also include osteogenic, as mRNA of *osteocalcin, runx2, osteonectin* and *col1a* were detected. QPCR further showed up-regulated transcription of both *runx2* and *sox9* throughout the developing deformity. Comparative to our findings, disc cell proliferation and a switch in the synthesis of ECM components are associated with disc degeneration [47,48]. However, *ISH* revealed that whereas *sox9* and *col2a* was present in chordoblasts from the non-deformed stage, *runx2* and *col1a* was only detected in fused samples, when intervertebral space was severely narrowed. This co-transcription of chondrocytic and osteogenic markers in the notochord supports the hypothesis of a metaplastic shift during vertebral fusions in salmon [49].

The metaplastic shift in the notochord and arch centra may be induced to produce more robust cells, able to withstand increased mechanical load. However, as bone replaced chondrocytic areas throughout the pathology, notochordal tissue did not calcify until the deformity developed into severe fusion. We therefore suggest that metaplasia leads to cell types more suited to the new environment but that changes are related to a threshold of the stimuli, in this case, grade of fusion. A shift in NP cell population coincides with spinal disorders like IDD and changes in the synthesis of matrix molecules differ with the degree of degeneration [50]. A comparative pathological process to our findings is mammalian “Bamboo spine”, describing a condition where vertebral bodies have fused and reshaped through ectopic bone formation [51,52]. Similar rescue processes have also been found in the mammalian AF, where it is strengthened through cartilage formation upon elevated mechanical load [53,54]. Overall, the vertebral fusion process seen in salmon might reflect an effort to restore and strengthen a vertebral area of a weakened vertebral column.

Conclusion

Vertebral fusions develop through a series of events. Disorganized and proliferating osteoblasts at the growth zones and along the rims of affected vertebral bodies characterized the fusion process. Moreover, loss of cell integrity through cell proliferation was prominent at the border between the osteoblastic growth zone and the chondrocytic areas in the arch centra and in intervertebral space. During the fusion process a metaplastic shift appeared in the arch centra where cells in the intermediate zone between osteoblasts and chondrocytes co-expressed mixed signals of chondrogenic and osteogenic markers. A similar shift also occurred in the notochord where proliferating chordoblasts changed transcription profile from chondrogenic to also include osteogenic marker genes. As the pathology progressed, ectopic bone formation was detected in these areas. Since transcription turned from chondrogenic to osteogenic, our suggestion is that trans-differentiated cells produce the ectopic bone. In complete fusions, all intervertebral tissue was remodeled into bone. The molecular regulation and cellular changes found in salmon vertebral fusions are similar to those found in mammalian deformities, showing that salmon is suitable for studying general bone development and to be a comparative model for spinal deformities. With this work, we bring forward salmon to be an interesting organism to study general pathology of spinal deformities.

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Tables

Table 1 Primers used for cloning, sequencing, transcriptional analysis and probe synthesis of Atlantic salmon (*Salmo salar*) genes.

Primers used for Real time PCR (RT) and probes for in situ hybridization (ISH)				
Gene	Orientation	Genbank	Use:	Sequence (5`-3`)
Extracellular Matrix constituents:				
Col1a1	Forward	FJ195608	RT	AGAGAGGAGTCATGGGACCCGT
	Reverse		RT	GGGTCCTGGAAGTCCCTGGAAT
	Forward		ISH	TAGCCGTGGTTTCCCTGGTT
	Reverse		ISH	CCGGGAGGTCCAAATCTACC
Col2a1	Forward	FJ195613	RT	TGGTCGTTCTGGAGAGACT
	Reverse		RT	CCTCATGTACCTCAAGGGAT
	Forward		ISH	GCTGGCGAGACAGGAGAGA
	Reverse		ISH	GCCTCATCAGCCCTCATGTA
Col10a1	Forward	EG837148	RT	TGGTGCTCTTTGACTGCCTGTAA
	Reverse		RT	CATCCTGTGTGTTGCAATATCACA
	Forward		ISH	AACAAGGGCTTCTTGGATCA
	Reverse		ISH	CATAATGCATCCTCAGGCAT
Alp	Forward	FJ195609	RT	CTAGTTTGGGTCGTGGTATGT
	Reverse		RT	TGAGGGCATTCTTCAAAGTA
Osteocalcin	Forward	FJ195616	RT	GTGAACCAACAGCAAAGAGA
	Reverse		RT	CCAGGTCCTTCTTAACAAACA
	Forward		ISH	CTCATACTTGTTGATCGTCCAG
	Reverse		ISH	TCTTTCTCTCTCGCTCTCCC
Osteonectin	Forward	FJ195614	RT	ATTACTGAGGAGGAGCCCATCATT
	Reverse		RT	CCTCATCCACCTCACACACCTT
	Forward		ISH	CTGAACGATGAGGGTGTGGA
	Reverse		ISH	CGAGTGGTGCAGTGCTCCAT
Mmp9	Forward	CA342769	RT	AGTCTACGGTAGCAGCAATGAAGGC
	Reverse		RT	CGTCAAAGGTCTGGTAGGAGCGTAT
Mmp13	Forward	DW539943	RT	TGATGTCCAAGTCAGCCGCTTC
	Reverse		RT	TGGTCTGCCACTTGCGATTGTC
Cathepsin K	Forward	NM_001140399	RT	ATGACCAACGCCTTCGAGTAC
	Reverse		RT	AAGGTGGAGAGGGTGGCATC
Transcription factors:				
Sox9	Forward	EU344852	RT	CCTGCAAACAAGACAAGGT
	Reverse		RT	GGGTCGAGTAGATTCATACGA
	Forward		ISH	GGGGATACTATTTGACTGGATC
	Reverse		ISH	TCTGTCTTGATGTGTGTGGG
Runx2	Forward	FJ195615	RT/ISH	CCACCAGGGACAGACACAGAT
	Reverse		RT/ISH	GAACGGACTGAGATCTGACGAA
Osterix	Forward	FJ195612	RT	TCCCATAGACTTTCCCACA
	Reverse		RT	TGCCTCAGGACATGTACAA
Mef2c	Forward	GU252207	RT	CACCGTAACTCGCCTGGTCT
	Reverse		RT	GCTTGCGGTTGCTGTTCATA
	Forward		ISH	GACAGAGACTGTGTGGTGTTCCCT
	Reverse		ISH	AGGTGGAGGGAGCTACCACTGTTA
Signalling molecules:				
Bmp4	Forward	FJ195610	RT	TCAAGTTGCCCATAGTCAGT
	Reverse		RT	CACCTGAACTCTACCAACCA
Bmp2	Forward		RT	ATGTGGTATTGCACCCATT
	Reverse		RT	ATGGACAGTTTCCCAATGA
Shh	Forward	AY370830	RT	CCGGCTCATGACTCAGAGATG
	Reverse		RT	TATCCCTGGCCACTGGTTCA
Ihh	Forward	FJ195617	RT	CAGATGACCCACTGGACTGAT
	Reverse		RT	GCTTGGTTGGGAGATATGCA
Housekeeping gene:				
Ef1a	Forward	DQ834870	RT	CACCACCGGCCATCTGATCTACAA
	Reverse		RT	TCAGCAGCCTCCTTCTCGAACTTC

*Ytteborg et al. [23] **Wargelius et al. [55] ***Olsvik et al [31]

Figure legends

Figure 1 Frequency of deformities

Spinal fusions increased at each sampling point; 2g, 15g and 60g. Each bar represents the means of analysis of variance at each sampling point as registered through radiographic findings in n=4 tanks. Data are given in percentage ± st.dev, asterix indicate significant differences (P=0.01).

Figure 2 X-ray and diagnostics

The development of vertebral fusions is a dynamic process where the final result is a complete fusion of two or more vertebral bodies. Vertebrae were divided into non-deformed (ND), intermediate (IM) and fused (FS). White arrow points to malformed vertebral bodies. Scale bar=0.1cm.

Figure 3 Histological findings

Toluidine blue staining of A. Intermediate vertebrae with irregular shaped vertebral bodies and B. Fused vertebrae with ectopic bone formation and denser notochord. Remnants of the arch center are present at ventral side of the notochord (arrow). Growth zones of vertebral endplates in C. Non-deformed D. Intermediate and E. Fused vertebrae. Osteoblasts appeared more disorganized throughout the pathology (arrow). At fused stage, osteoblasts located abaxial between vertebral bodies were observed. Double staining with Alizarin red S and toluidine blue of F. Non-deformed, G. Intermediate and H. Deformed vertebrae. Ectopic bone formation and reduced (arrow) or fused (white double arrow) arch centra was prominent throughout the developing pathology. Alizarin red S staining of I. Non-deformed J. Intermediate and K. Fused vertebrae. Notochordal tissue did not stain with alizarin red S until fusion was complete (arrow). ND, non-deformed; IM, intermediated; FS, fused, nc, notochord; ns, notochordal sheath, eb, endbone; ec, ectopic bone. Scale bar = 100µm. .

Figure 4 Immunohistochemistry with TRAP, PCNA and Caspase 3

A: One sample from intermediate group showed weak positive TRAP staining (arrow) at the ossifying border of the hypertrophic chondrocytes. B. Higher magnification of black box in A, positive TRAP staining (arrow). C. No TRAP activity was detected in any of the samples from the fused group. PCNA positive cells (brown) in D. Non-deformed. Some proliferating cells can be seen at the growth zones of the vertebral body endplate (arrow). E. Higher magnification of chordoblasts in non-deformed. Positive cells were rarely found in chordoblasts. F. Intermediate vertebrae. PCNA was detected in higher amount at growth zones of the vertebral body endplate and extending abaxial and axial direction (arrow). G. Fused vertebrae. PCNA labeled cells were detected in the corresponding areas, but in higher

abundance (arrow). H. PCNA positive cells were observed in the notochord and also in arch centra (arrow). I. Higher magnification of the black box in H, PCNA positive notochordal cells (arrow). Caspase 3 positive cells (black) in J. Non-deformed. Caspase 3 positive cells can be seen at the fringe of the growth zones of the vertebral body endplate (arrow). No positive cells could be detected in the chordoblasts (arrowhead) in any of the groups. K. Intermediate vertebrae; positive cells were detected in exceedingly higher amount at the corresponding areas (arrows). L. Fused vertebrae; positive cells were detected in the corresponding areas, but in higher amounts (arrow). M. Positive caspase 3 signal increased along the rims of the vertebral body and in trabeculae in fused vertebral bodies where we observed ectopic bone formation (arrow). N. Higher magnification of black box in E showing caspase 3 positive cells in the joints of ectopic bone (arrow). O. Higher magnification of caspase 3 labeled cells showing typical apoptotic phenotype with membrane blebbing (arrow). ND, non-deformed; IM, intermediated; FS, fused, nc, notochord; ns, notochordal sheath, eb, endbone; ec, ectopic bone. Scale bar = 100µm.

Figure 5 Quantitative gene transcription profiles in intermediate and fused vertebral bodies

Relative gene transcription of A. Extracellular matrix constituents and B. Regulatory genes in non-deformed (white bars) intermediate (light grey bars) and fused (dark grey bars) vertebrae, normalized with *ef1a*. Significant values (P=0.05) indicated by a-b-c, n=15, means ± SE. Transcription ratios are shown in relative mRNA expression along the y-axis, genes along the x-axis.

Figure 6 *In situ* hybridization of genes involved in the extracellular matrix

A. *Col1a* transcription observed in trabeculae and at the growth zones of vertebral body endplates of intermediate vertebrae. B. *Col1a* in osteoblasts extending from the growth zones of the vertebral body endplate in intermediate and C. fused vertebrae. D. *Col1a* transcription in the notochord of a fusion. E. *Col2a* transcription in chordoblasts of intermediate vertebrae (arrow). F. *Col2a* transcription increased as intervertebral space narrowed down. In addition, *col2a* was observed at the osteoblast growth zone at the vertebral body endplates (arrow). G. In fused vertebrae, most cells in intervertebral space expressed *col2a* and expression in the osteoblasts increased (arrow). H. *Col10a* transcription along the rims of intermediate vertebrae and in hypertrophic chondrocytes (arrow). I. *Col10a* was more expressed in areas with ectopic bone formation (arrow). J. *Osteonectin* in fused vertebral bodies. Notice transcription along the rims of the vertebral bodies as well as in chondrocytes (arrow). Stronger staining was observed in areas with ectopic bone formation. K. *Osteonectin* transcription in areas with ectopic bone formation. L. *Osteonectin* transcribing chondrocytes. M. *Osteocalcin* was expressed in chondrocytes and along the rims of fused vertebrae. N. *Osteocalcin* transcription at the growth zone of

two vertebral body endplates in a fusion. Notice cells expressing *osteocalcin* abaxial in-between the vertebral bodies (arrow). O. *Osteocalcin* expressing cells blending with chondrocytes. Both osteoblasts and chondrocytes expressed *osteocalcin* in these areas. ND, non-deformed; IM, intermediated; FS, fused, nc, notochord; ns, notochordal sheath, eb, endbone; ec, ectopic bone. Scale bar = 100μm.

Figure 7 *In situ* hybridization of genes involved in regulatory processes

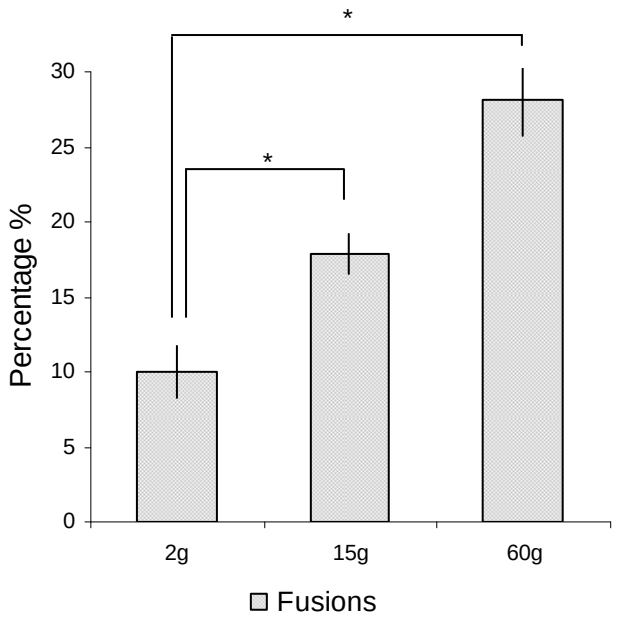
A. *Runx2* at the osteoblast growth zone of the vertebral body endplates in non-deformed vertebral bodies (arrow). Notice no transcription in chordocytes. B. *Runx2* transcription in intermediate vertebrae showed transcription in chordoblasts. C. *Runx2* transcription in fused vertebrae, notice positive staining in the chordocytes and in areas with ectopic bone formation (arrow). D. Strong staining of *runx2* in cells in the notochord of a fusion. E. *Sox9* transcription chondrocytes of non-deformed vertebrae. F. Fused vertebrae had high *sox9* transcription in intervertebral space. G. Higher magnification of cells expressing *sox9* at the vertebral growth zone of a fusion. H. *Sox9* was also expressed along the rims of ectopic bone formation. I. *Mef2c* transcription in hypertrophic zone in non-deformed vertebral bodies. J. *Mef2c* in fusing arch centra. Notice positive cells outside the hypertrophic zone (arrow). K. *Mef2c* in arch center narrowing down. L. *Mef2c* transcription at the growth zones blending with the arch centra. M. *Mef2c* positive cells in intervertebral space of an incomplete fusion (arrow). ND, non-deformed; IM, intermediated; FS, fused, nc, notochord; ns, notochordal sheath, eb, endbone; ec, ectopic bone. Scale bar = 100μm.

Additional files

Sense probes

No staining was detected in *ISH* with sense probes.

nc, notochord; ns, notochordal sheath, eb, endbone; tb, trabecular bone. Scale bar = 100μm.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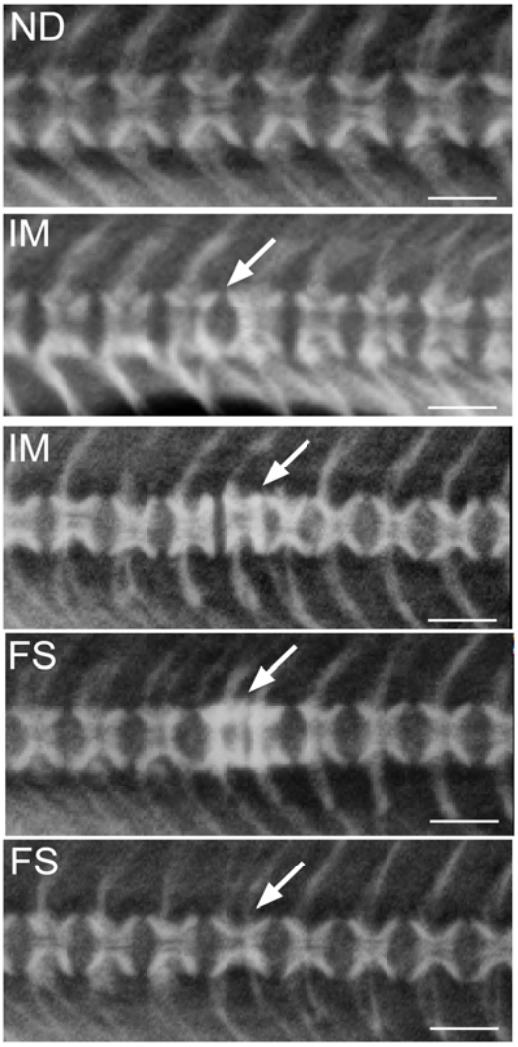
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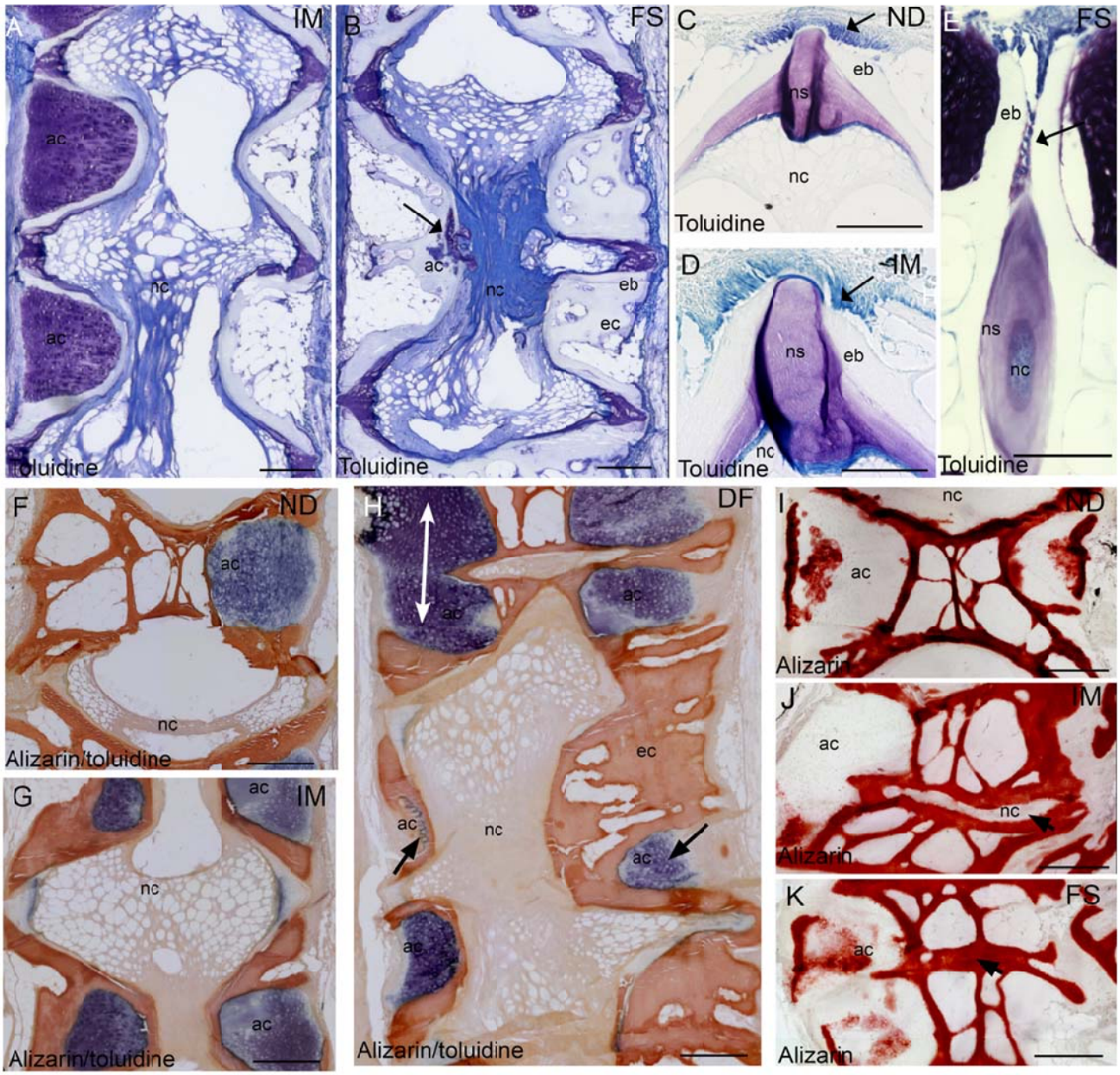
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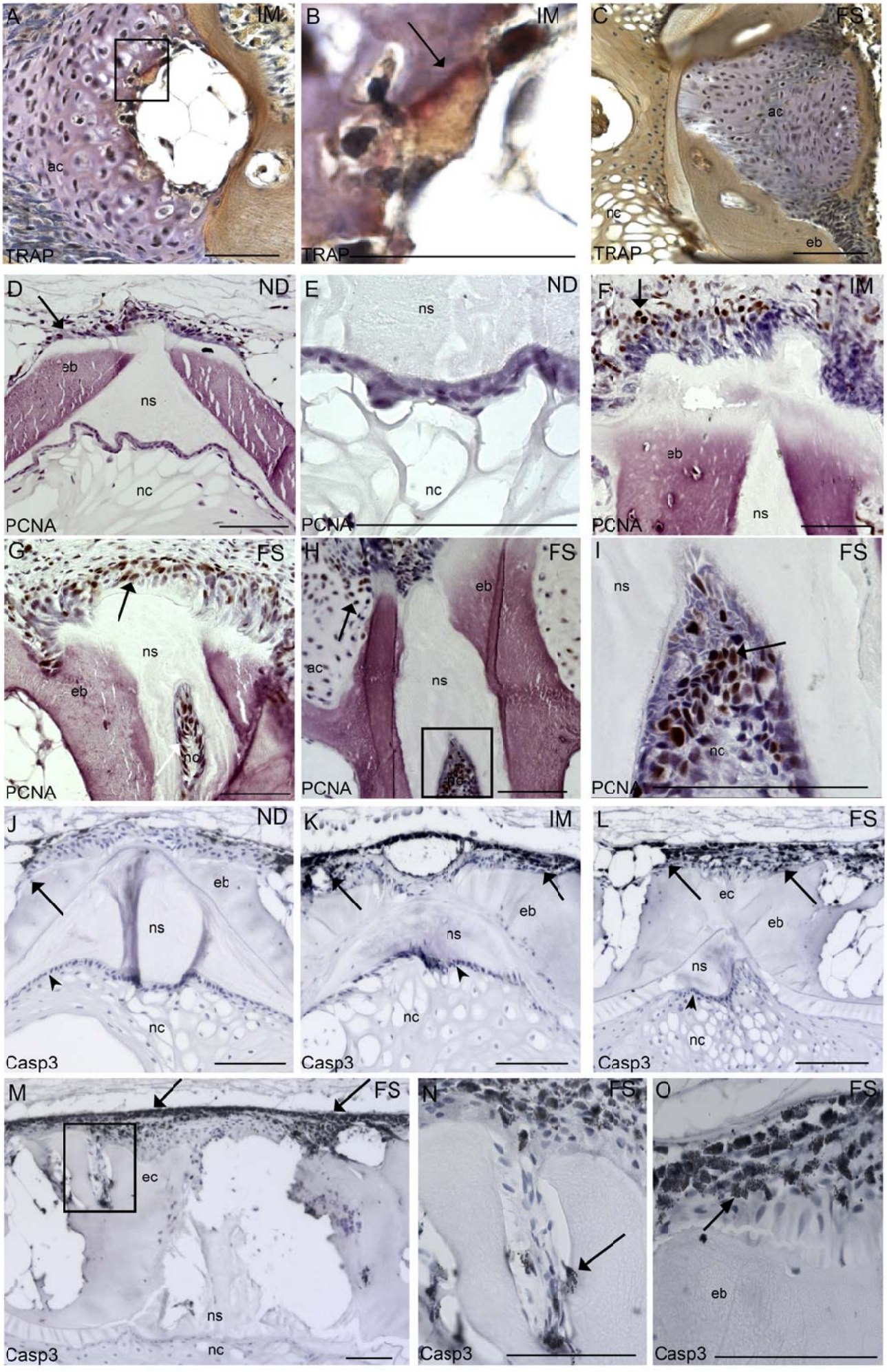
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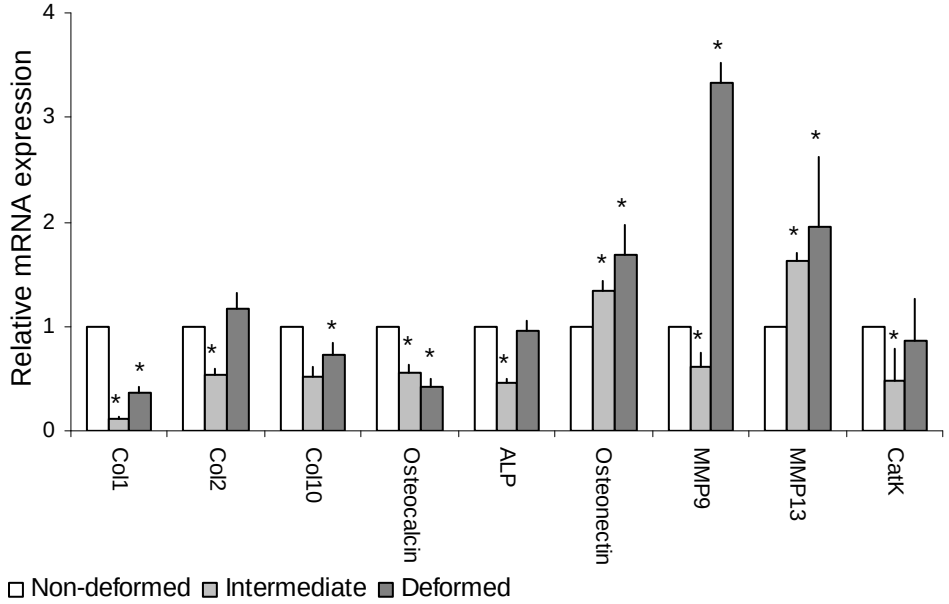
A. ECM components



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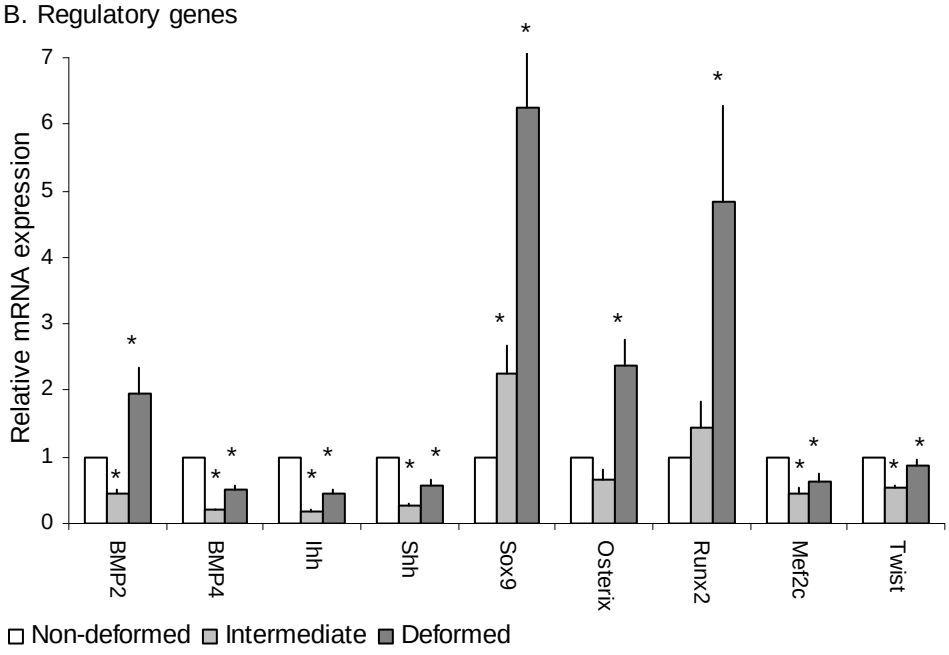

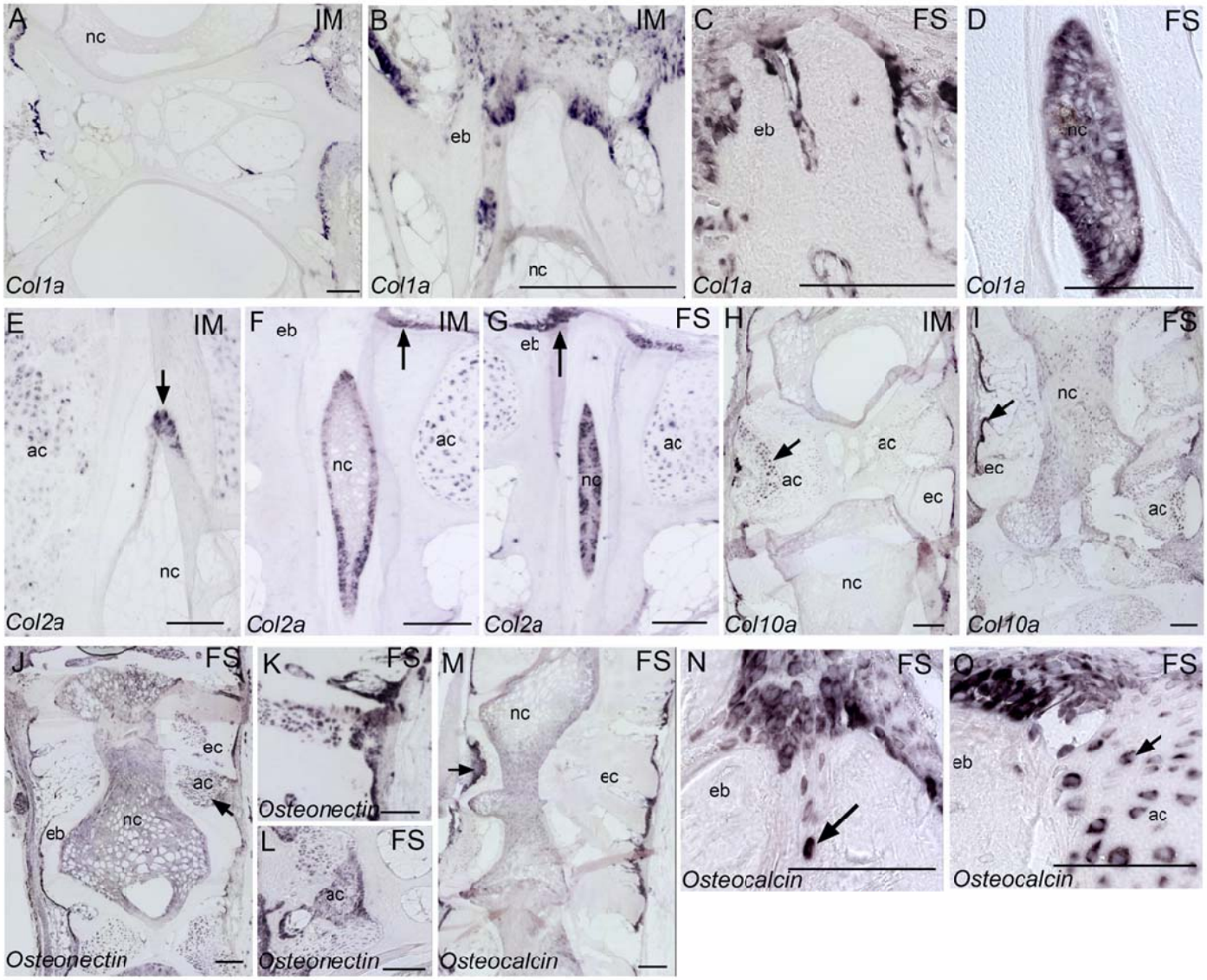
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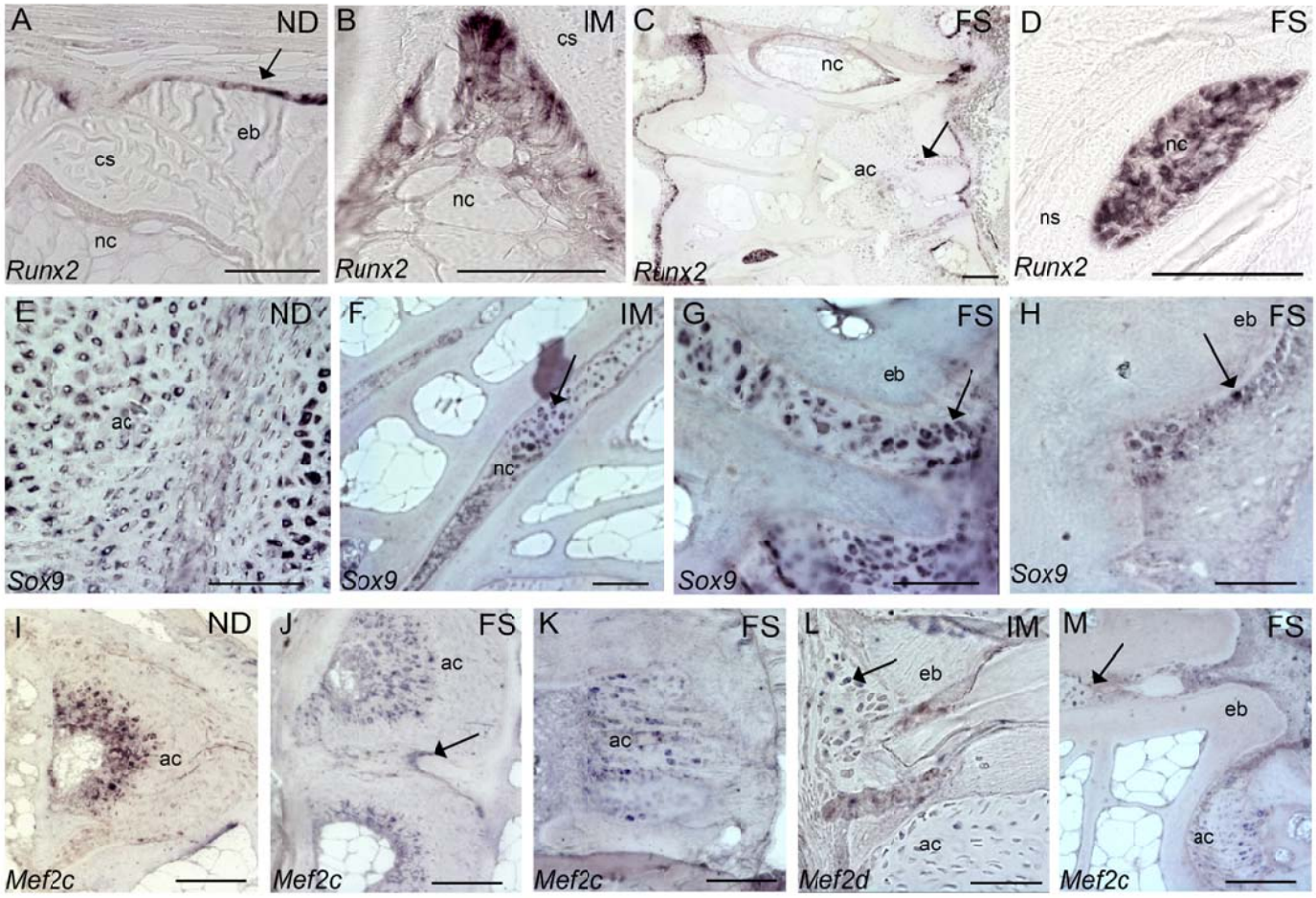
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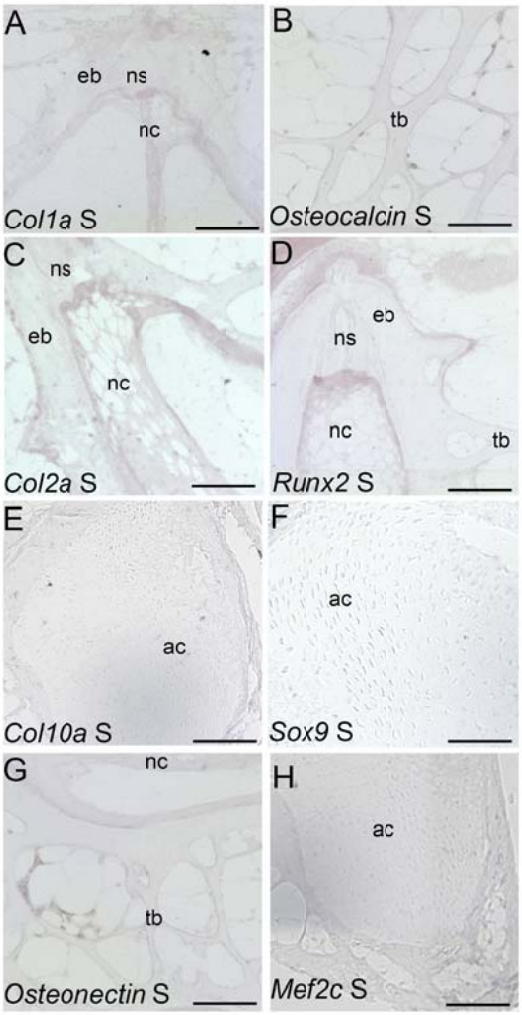
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Paper 3

Remodeling of the notochord during development of vertebral fusions in Atlantic salmon (*Salmo salar*)

Elisabeth Ytteborg[1,5], Jacob Seilø Torgersen[1], Mona E. Pedersen[2], Grete Baeverfjord[3], Kirsten O. Hannesson[2], Harald Takle[1,4*]

[1]Nofima Marine AS, Norwegian Institute of Food, Fisheries and Aquaculture Research, P.O. Box 5010, Aas NO-1432, Norway *
[2]Nofima Food AS, N-1430, Aas, Norway
[3]Nofima Marine AS, N-6600, Sunndalsøra, Norway
[4]AVS Chile, Imperial 0655, Of. 3A, Puerto Varas, Chile
[5]Norwegian University of Life Sciences, Department of Animal and Aquaculture Sciences, P.O. Box 5003, N-1432, Aas, Norway

*Corresponding author
Tel: +47 45 40 04 56
Fax: +47 64 94 95 02
E-mail: harald.takle@nofima.no

Key words:Atlantic salmon, notochord, Perlecan, spinal fusions, Substance P

Abbreviations:

Alp	alkaline phosphatase
Col2a1	collagen type 2a1
Ef1a	Elongation factor 1
GBM	Glomerular kidney membrane
IVD	intervertebral disk
PBS	phosphate-buffered saline
PFA	paraformaldehyde
qPCR	quantitative polymerase chain reaction
Runx2	runt-related transcription factor 2
Sox9	(sex determining region Y) box 9
SP	Substance P
Zn12	zebrafish neuron marker 12

Abstract

Histological characterization of spinal fusions in Atlantic salmon (*Salmo salar*) have described shape alterations of vertebral body endplates, reduced intervertebral space and replacement of intervertebral cells by ectopic bone. However, the significance of the notochord during the fusion process has not been addressed. Thus, in this study we investigated structural and cellular events in the notochord during development of vertebral fusions. In order to induce vertebral fusions, Atlantic salmon were exposed to elevated temperatures from fertilization until 15g size. Based on radiography, an intermediate and a terminal stage of the fusion process were investigated using immunohistochemistry and real time quantitative PCR. Examination of structural extracellular matrix proteins like Perlecan, Aggrecan, Elastin and Laminin revealed reduced activity and reorganization at early stages in the pathology. Staining for elastic fibers visualized a thinner elastic membrane surrounding the notochord of developing fusions and immunohistochemistry with Perlecan showed that the notochordal sheath is stretched upon fusion formation. These findings in the outer notochord correlated with loss of Aggrecan and Substance P positive signals and further loss of vacuoles from the chordocytes in the central notochord. At more progressed stages of the fusion process, chordocytes condensed and expression of Aggrecan and Substance P reappeared. The hyperdense regions are of importance for the formation of notochordal tissue into bone. Based on our results we suggest that remodeling of notochord integrity by reduced elasticity, structural alterations and cellular changes is involved in the development of vertebral fusions.

Introduction

The vertebral column is the defining feature of all vertebrates, composed of an alternating pattern of vertebral bodies (centra) and intervertebral regions. While centra give support and strength to the organism, intervertebral regions provide flexibility (review (Urban and Roberts 2003)). Spinal deformities have for long been established as a major concern for human health and an important welfare problem for farmed animals. A number of animal models have played important roles in understanding the complex pathology of spinal disorders (review (Lotz 2004)), but molecular mechanisms involved have not been described in detail.

The notochord is found in embryos of all chordates, being well conserved between species as the forerunner of the spinal column. However, whereas only remnants of the notochord exist between adjacent vertebrae in the mammalian intervertebral disc (IVD), the notochord persists throughout all developmental stages and throughout the entire length of the fully developed vertebrae in many teleosts, including salmon. The mature notochord of Atlantic salmon (*Salmo salar*) consists of a core of chordocytes surrounded by a layer of chordoblasts, an acellular fibrous sheath and an outer elastic membrane (Grotmol et al. 2006). The chordoblasts continue to divide throughout life in accordance with sustained notochordal growth (Grotmol et al. 2006). Chordoblasts further differentiate to chordocytes, containing large fluid filled vacuoles which functional role is to maintain internal hydrostatic pressure (Nordvik et al. 2005;Glickman et al. 2003;Adams et al. 1990;Kimmel et al. 1995;). Moreover, the chordoblasts produce the basal membrane and ECM components of the notochordal sheath, which in both mammals and teleosts like Atlantic salmon has been shown to consist of mainly collagen type 2 (Col2) (Hayes et al. 2001;Domowicz et al. 1995;Sandell 1994;Linsenma et al. 1973). The acellular notochordal sheath and the elastic membrane surrounding the teleost notochord restricts expansion of the vacuolated chordocytes (Grotmol et al. 2005), thus generating the hydroskeletal properties essential for extension of the larvae (Koehl et al. 2000). The notochord secrete factors to surrounding tissues and contribute to vertebral patterning during embryogenesis (Fleming et al. 2004;Cleaver and Krieg 2001). In vertebrates with limited growth, such as humans, the notochord ceases its regulating role for vertebral development as part of the normal ontogeny, followed by the transformation of notochordal tissue into cartilage (Hunter et al. 2003;Oegema 2002). In Atlantic salmon, however, the notochord fulfils its regulatory role for vertebral development throughout life since salmon and other fish species do not stop growing (Witten et al. 2002).

We have previously presented Atlantic salmon to be an interesting organism for cross-study comparisons of spinal deformities (Ytteborg et al. submitted). High frequency of spinal fusions can be induced by elevated water temperatures during early life-stages (Ytteborg et al. 2010). Furthermore, the overall structural and molecular features of bone and cartilage development in vertebral fusions have

shown resemblance with similar pathological spinal conditions in mammals (Gorman and Breden 2007;Witten et al. 2006;Ytteborg et al. submitted). Spinal fusions in Atlantic salmon are characterized by changes in extracellular matrix (ECM) components and mineralization of the intervertebral regions (Witten et al. 2006;Ytteborg et al. submitted). Similarly, intervertebral disc degeneration (IDD) in mammals involves breakdown of ECM components in the annulus fibrosus (AF) and calcification of the nucleolus pulposus (NP) (Takaishi et al. 1997;Kanemoto et al. 1996;Antoniou et al. 1996). Moreover, breakdown of proteoglycans (PGs), like Aggrecan and Perlecan, may lead to reduced hydrostatic pressure, invasion of nerves and blood vessels and loss of transportation of nutrients and waste products in degenerating IVD (Melrose et al. 2002;Kauppila 1995;Yasuma et al. 1993;Urban and Mcmullin 1985). Loss of Aggrecan resulting in tissue dehydration, reduces the ability of mammalian IVD to transmit and absorb compressive load (Kanemoto et al. 1996;Urban and Mcmullin 1985). Perlecan has structural roles in mammalian cartilage and IVD (Sivan et al. 2006) and is important for proper establishment of basement membranes in different vertebrates including teleosts (Parsons et al. 2002;Aviezer et al. 1994). Furthermore, Substance P (SP), established as a marker for neuronal invasion in both mammals and teleosts (Holmqvist and Ekstrom 1991;Hokfelt et al. 1975), has also shown important roles in mineralization processes of both osteoblasts (Sun HB et al. 2010;Goto et al. 2007) and chondrocytes (Millward-Sadler et al. 2003). Hence, immunohistochemical markers for ECM components, mineralization and neuronal development, such as Collagen, Aggrecan, Perlecan and SP, have been used to characterize pathological vertebral conditions (Kuraishi et al. 1985;Hokfelt et al. 1975).

In the presented study we investigated the remodeling of the notochord during the vertebral fusion process in Atlantic salmon. Aggrecan, Perlecan and SP in combination with elastic staining techniques were used to characterize the intervertebral region of developing spinal fusions. During the pathology, the elastic membrane surrounding the notochordal sheath appeared fragmented. Immunohistochemistry with Perlecan antibodies visualized that the highly folded notochordal sheath were stretched upon development of vertebral fusions. Loss of Aggrecan from chordocytes in the central notochord correlated with loss of vacuoles in these cells at more progressed stages of the fusion process, while increased SP signals were found prior to mineralization in hyperdense notochordal cells. Based on our results we suggest that remodeling of the intervertebral regions are important for the development of spinal fusions.

Materials and methods

This trial was performed and approved in accordance to regulations from the Norwegian authorities regarding the use of animals for research purposes. The fish experiment was done at Nofima Marine at

Sunndalsøra, Norway, in 2007 with Atlantic salmon from the Salmobreed strain, as described in Ytteborg et al. (Ytteborg et al. 2010). Briefly, two experimental temperature regimes were used. During egg rearing, the low temperature group was stabilized at 6±0.3ºC whereas the high temperature group was stabilized at 10±0.3ºC. Temperature was gradually increased (1ºC per day) at first feeding to 10±0.3ºC and 16±0.3ºC for low and high temperature group, respectively. At 15g size, fish were sedated (Tricaine methane sulfonate, Pharmaq, Overhalla, Norway) and radiographed. As described in Ytteborg et al. (submitted), rearing at high temperature resulted in more than 28% of the fish developing vertebral fusions. During sampling, vertebrae were divided into four categories; non-deformed vertebrae from both low and high temperature regimes and two stages in the fusion process; an intermediate and an evolved stage (fused). Non-deformed vertebrae had no observable morphological changes in the vertebral bodies or in intervertebral space. Vertebrae diagnosed as intermediate included various degrees of reduced intervertebral space and compressions. Samples characterized as fused ranged from incomplete fusions to complete fusions. Based on findings in Ytteborg et al. (Ytteborg et al. 2010), where no changes were observed in the intervertebral regions of non-deformed fish reared at high temperature regime, non-deformed vertebrae from the low temperature group were used as control in histological and immunohistological analysis. All four groups were used for quantitative real time PCR (qPCR) analysis.

Sample preparation

Specimens for histology and immunohistochemistry (IHC) were embedded in paraffin as described in Ytteborg et al. (submitted). Five µm serial sections were prepared in the parasagittal orientation from vertebral columns, starting at the periphery and ending in the middle plane of the vertebra using a Microm HM 355S (Thermo Fisher Scientific Inc., MA, USA). The effect of distance from the sagittal plane was taken into account and effort made to ensure that sections were analyzed in corresponding areas. Sections were mounted on coated slides and dried ON at 37°C.

Histology

Histological examination of vertebrae (n=12) was carried out on de-waxed sections; 3x 3 min in Clear Rite (Richard-Allan, MI, USA) followed by 2x washing in xylene (Merck, Darmstadt, Germany) for 5 min each. Sections were then rehydrated in graded series of ethanol before rinsed in dH_2O. To visualize elastic fibers, deparaffinised sections were stained for 30 min with Verhoeff`s hematoxylin, rinsed in dH_2O and differentiated in 2% ferric chloride for 2 min. Sections were subsequently rinsed in dH_2O, dehydrated, cleared and mounted in Cytoseal 60 (Electron Microscopy Science, PA, USA). Microscopic

bright field images were captured using a Zeiss Axio Observer Z1 and post processed for light and contrast in AxioVision software (Carl Zeiss Microimaging GmbH).

RNA isolation and cDNA synthesis

RNA for qPCR analysis was extracted from vertebrae of 15 individuals per categorized group. Tissues were thoroughly homogenized in a mortar with liquid nitrogen, as described (Ytteborg et al. 2010). Total RNA was extracted using Trizol reagent and Micro to Midi Kit® (Invitrogen, CA, USA) and DNase treated with DNase1 (Invitrogen). Total RNA (1ug) was reversed transcribed to cDNA using oligo(dT) primer and reagents from the Taqman Gold RT-PCR kit (Applied Biosystems CA, USA) at the following thermal cycling conditions: 10 min primer incubation at 25°C, 60 min RT step at 48°C and 5 min RT inactivation at 95°C. All reactions were performed in accordance to the manufacturer's protocol.

Immunohistochemistry

The selected antibodies Perlecan (Chemicon, MA, USA), Aggrecan (Santa Cruz Biotechnology Inc., CA, USA) and SP (Sigma) were used to characterize the notochord during the fusion process. Proteins were isolated from the residual fractions after RNA isolation by Trizol® (Invitrogen) and concentrations measured with RC DC Protein Assay (Bio-Rad, USA). Protein (20µg) was solubilised, separated by SDS-gel electrophoresis and transferred to a nitrocellulose membrane using reagents from NuPAGE and iBlot™ (Invitrogen). Non-specific binding sites were blocked using 5% teleostan gelatine in 0.1M Tris-saline pH 7,4 (Sigma, MO, USA). The nitrocellulose membrane was probed with the zebrafish neuron marker Zn12 diluted 1:5000 and Perlecan and Aggrecan diluted 1x1000 in TBS added 0.5% teleostan gelatine. Alkaline phosphatase conjugated anti-mouse IgG(H+L) secondary antibodies (Promega, WI, USA) was applied for Zn12 and Perlecan, Alkaline phosphatase conjugated anti-rabbit IgG (H+L) (Promega) for Aggrecan, both diluted 1:7500. Immunoreactive bands were revealed with Novex® AP NBT/BCIP (Invitrogen). All reactions were performed in accordance to the manufacturer's protocol. Perlecan visualized one band of approximately 200-260 kDa, which is in agreement with previously published sizes for this protein (Sundarraj et al. 1995). We were not able to detect Aggrecan, probably due to the size of this protein. Aggrecan is more than 250 kDa and is likely to contain large number of GAG chains and expected to form a broad band migrating at the top of the gel (Dodge and Jimenez 2003). No smaller bands were detected. Specificity for SP has previously been shown in salmonides (Tarakçý and Köprücü 2010). Zn12 (Developmental Studies Hybridoma Bank, IA, USA), a membrane bound protein that binds cell adhesion glycoproteins in a variety of different tissues, such as in notochordal tissue (Metcalfe et al. 1990;Vincent et al. 1983), was used in this as a marker solely to

visualize structures in the notochord. Western blot visualized two bands with molecular weight between 80-100 kDa, corresponding to previously shown values for Zn12 (Metcalfe et al. 1990).

Fluorescence IHC was initiated on sections by successive rehydration in decreasing ethanol concentrations followed by microwave facilitated antigen retrieval for 10 min in 10mM citric acid pH 6.0. Permeabilization was carried out using 1% Triton in 1x PBST for 10 min, before blocking in 5% non fat dry milk powder dissolved in 1x PBST. The primary antibodies (Aggrecan and Perlecan diluted 50x, SP 100x and Zn12 200x) was incubated over night at 4˚C before washing. Alexa conjugated secondary antibodies (diluted 200x) specific to the primaries were applied and incubated 2 h. Negative controls were incubated with secondary antibodies only. After washing in 1x PBST, the slides were coverslipped using Prolong Gold antifade (Life Technologies). Microscopy was carried out on a Zeiss Axio Observer Z1 equipped with the Apotome system for structured illumination (Carl Zeiss Microimaging). AxioVision software (Carl Zeiss Microimaging) was applied for extended focus and generation of 3D transparency of the captured z-stacks.

Real-time quantitative RT-PCR

Primers for transcription analysis were based on known Atlantic salmon sequences or on conserved regions of known teleost sequence paralogues. Amplicons were cloned using pGEM T-easy vector (Promega) and sequenced with Big Dye Terminator chemistry and the ABI 3730 automated sequencer (Applied Biosystems). The obtained Atlantic salmon sequences were analyzed and confirmed with BLAST and deposited in the Genbank database.

Triplicate real-time qPCR reactions for *elastin* and *laminінß1* were performed using the Light cycler 480 and SYBR Green chemistry (Roche, Switzerland) at the following thermal cycling conditions: 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec, 60°C for 15 sec and 72°C for 15 sec. Specificity was assessed by melting curves, determined post PCR (95°C for 15sec, 60°C for 1 min and 97°C continuous). Primers for *elastin* (GenBank accession no.BF228555) and *laminінß1* (GenBank accession no.FJ195616) were designed using the Vector NTI Advance 10 (Invitrogen, CA, USA) and NetPrimer (PREMIER Biosoft, CA, USA) software; *elastin* forward 5'-GAGGCTACAGACCAGGAGGAGTT and reverse 5'-TCTGGGTCGGTGGGTTTGTA*, laminінß1* forward 5'-CATGTGACATGGACACAGGAA and reverse 5'-CGTCCTCAGCCTCATAGGTGTA. Relative target gene was normalized relative to *elongation factor* (*ef1a*, GenBank accession no. AF321836) for all samples as recommended by Olsvik et al. (Olsvik et al. 2005). Forward primer *ef1a:* 5'-CACCACCGGCCATCTGATCTACAA and reverse primer: 5'-TCAGCAGCCTCCTTCTCGAACTTC.

Triplicate real-time qPCR reactions for *aggrecan* and *perlecan* were performed using a ABI prism 7900HT Sequence detection system with TaqMan PCR core master kit (Applied Biosystem) at the

following thermal cycling conditions 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The primers and TaqMan probe (5'labeled-6-FAM and 3'Quencher TAMRA) were designed by using Primer Express Program (Applied Biosystem); *aggrecan* (GenBank accession no.FJ 179677); forward 5'-TGGCGGCCGAACCA, reverse 5'-CCGTTCTCGTGCCAGATCAT, probe 5'-CCGGATAACTACTTTAACTCTGGAGAAGACTGTGTGG and *perlecan* (GenBank accession no. FJ 825137); forward 5'-GCATACCCTCCTCTGACCAACA, reverse 5'-GCCATGGGTTTGAACTCCAT and probe 5'-CCACAATGACCTGCACATCGA. Gene expression was normalized to *ef1α* (forward 5'-GCCAGATCTCCCAGGGCTAT, reverse 5'-TGAACTTGCAGGCGATGTGA and probe 5'-CCCCTGTGCTGGATTGCCATAC).

PCR efficiency for all target genes and the reference gene were tested and approved. The expression ratios were tested by using the Relative Expression Software Tool (REST) according to Pfaffl et al. (Pfaffl et al. 2002). Differences between the mRNA expression ratios were tested for significance by the Pair Wise Fixed Reallocation Randomization Test© (Pfaffl et al. 2002).

Results

The elastic membrane

Investigation of the elastic layer by Verhoeff`s hematoxylin staining showed the elastic membrane surrounding the notochord of non-deformed vertebrae with a thickened structure in the intervertebral regions (Fig. 1a and b). However, at the intermediate stage of the fusion process, the elastic membrane was found to have a decreased thickness (Fig. 1c and d) which seemed to be connected with expansion of the notochordal sheath in developing fusions. During the fusion process the notochordal sheath increased in area and width in the intervertebral regions and an increased distance between the chordoblasts layer and the vertebral endplates was observed. In severe fusions the elastic membrane appeared fragmented or absent (Fig. 1e and f). Furthermore, quantification of mRNA transcription revealed that *elastin* was down-regulated during the pathogenesis of vertebral fusions (Fig. 2), with most reduction at intermediate stage.

The notochordal sheath

In order to characterize the notochordal sheath structure, IHC with antibodies against Perlecan, Aggrecan and SP were applied (Fig. 3 and 4). Of these markers, Perlecan was abundantly detected in the notochordal sheath at non-deformed, intermediate and fused stages (Fig. 3). Perlecan visualized highly folded structures in dorso-ventral zigzag pattern in the notochordal sheath of non-deformed vertebrae, as being compressed in cranio-caudal direction (Fig. 3a). Transparency 3D models showed

that the notochordal sheath of non-deformed vertebra has a finely organized morphology with evenly distributed tubular structures characterizing the sheath (Fig. 3b). At intermediate stage, the number of folded structures were reduced and more straightened in dorso-ventral as well as stretched in cranio-caudal direction, giving the structures in the sheath a more linear appearance (Fig. 3c). Transparency 3D models revealed a reduced number of structures and visualized better the dorso-ventral and cranio-caudal elongation of the notochordal sheath (Fig. 3d). At fused stage, dorso-ventral patterning was evident and the number of tubular structures reduced (Fig. 3e), as also visualized with transparency 3D models (Fig. 3f). Quantification of *perlecan* mRNA revealed up-regulated transcription at the intermediate and fused stage, with highest transcription at intermediate stage (Fig. 2).

Aggrecan and SP showed only few and scattered signals in the notochordal sheath of non-deformed vertebrae (Fig. 4). However, signals for both Aggrecan and SP were found in the growth zones of the vertebral body endplates, but only SP was found directly associated with the osteoblasts (Fig. 4a and b). In addition, strong signals for SP were detected in the compact bone of the endbones (Fig. 4b). No changes in signal specificity were observed in pathological vertebrae (results not shown).

Chordoblasts and chordocytes

Using IHC, Aggrecan and SP were found in notochordal cells in non-deformed vertebrae were they associated with the cell membranes of these cells (Fig. 5 and 6). Perlecan was also detected in chordocytes and chordoblasts (Fig. 3a and c), but no changes in signal specificity were observed in pathological vertebrae. A well organized layer of chordoblasts in the notochordal epithelium was observed in non-deformed vertebrae (Fig. 5a and b). Aggrecan appeared more abundant in chordocytes than in the chordoblasts layer (Fig. 5a), whereas the opposite was seen for SP (Fig. 5b). An increase in the number of chordoblasts could be seen at both intermediate and fused stage (Fig. 5c-f). At intermediate stage, Aggrecan and SP signals were severely decreased in both chordoblasts and chordocytes (Fig. 5c and d). At fused stage, Aggrecan expression reappeared in the notochordal epithelium and around chordocytes close to this layer (Fig. 5e). Correspondingly, *aggrecan* mRNA transcription was reduced at intermediate stage (n.s.) and increased at fused stage (Fig. 2). Furthermore, *aggrecan* transcription was down-regulated in non-deformed from the high temperature group. SP signal disappeared from the chordocytes at fused stage, but some expression was detected around the chordoblasts (Fig. 5f).

To further analyze the notochordal epithelium, *laminin*ß*1,* which is an important constituent for proper establishment of basal membrane around the chordoblasts, was analyzed with qPCR (Fig. 2). Quantification of *laminin*ß*1* mRNA revealed reduced transcription at both intermediate and fused stage, with most reduction at intermediate stage.

In non-deformed vertebrae, chordocytes containing large vacuoles were present in the center of the notochord. These chordocytes stained for both Aggrecan and SP, with similar pattern as what was seen in chordocytes close to the notochordal epithelium (Fig. 6a and b). At intermediate stage, no aggrecan and only weak signals for SP could be detected (Fig. 6c and d). Weak Aggrecan signals were present at fused stage (Fig. 6e), but most chordocytes appeared to completely lack SP (Fig. 6f). However, in hyperdense areas where chordocytes appeared compact and vacuoles were lost (Fig. 1e), Aggrecan and SP signals reappeared (Fig. 6g and h).

Discussion

In the presented study we investigated structural and cellular events in the notochord during development of vertebral fusions in Atlantic salmon. One of our major findings during this work was the visualization of a thinner elastic membrane surrounding the notochordal sheath of developing vertebral fusions. These findings were supported by a reduced transcription of *elastin* at both intermediate and fused stage. In the most severe cases, the elastic membrane was fragmented, an observation also noted in compressed vertebrae (Kvellestad et al. 2000). An interesting comparative pathological condition to our findings in salmon is the sparse and disrupted elastic network in scoliotic discs (Yu et al. 2005). The elastic sheath found in the AF of mammalian IVD is involved in restoration of shape after brief deformation, thus rupture of this sheath may be involved in the progression of spinal deformities also in Atlantic salmon. The notochord may be stretched to a certain point before elastic membrane becomes fragmented. Structural changes of the notochordal sheath most likely co-evolve with a disrupted elastic membrane. As seen in this study, the highly folded structures in the notochordal sheath were lost during development of spinal fusions, as visualized by Perlecan IHC. Grotmol et al. (2006) have previously described the notochordal sheath of Atlantic salmon. At early life stages, this sheath is compounded by parallel col2 fibrils forming helices around the longitudinal axis of the notochord. The helical geometry shift between adjacent collagen lamella, forming left- and right-handed coils, is important for the hydraulic properties of the notochord (Grotmol et al. 2006;Koehl et al. 2000). Thus, in a similar manner to the AF of mammalian discs, the collagen fibrils form overlapping transversing bands crossing the joint in opposite directions, hence, stabilizing intervertebral regions. At more mature stages in normal developing Atlantic salmon, this sheath is smooth and continuous with a thickened structure in the intervertebral regions (Nordvik et al. 2005). The observed folded pattern in our study appeared in sections as dorso-ventral zigzag bands. Hence, the folded structures seen in non-deformed spinal columns may be the consequence of compressions of the notochordal sheath upon formation of centra. As the cross-helical architecture probably is important for flexural stiffness of the larval body during development (Grotmol et al. 2006), the folded pattern observed in this study may contribute to increased

flexibility and normal functioning of the mature spinal column. Thus, stretching of the folded notochordal sheath during the fusion process will most likely reduce flexibility and affect functionality of the notochord.

Another interesting aspect of Perlecan expression is its link to nutritional transportation over the notochordal sheath. Parsons et al. (2002) have previously suggested similarities between the structural role of the teleost notochordal sheath and the mammalian glomerular kidney membrane (GBM). GBM is an important part of the filtration machinery in the kidneys and involved in hydrostatic pressure maintenance (Timpl 1996). The heparan sulfate chains of perlecan have further been shown to play important roles in glomerular filtration (Morita et al. 2005) and to be involved in diffusion of nutrients during tooth development in mice (Ida-Yonemochi et al. 2005). Impairment of the notochordal sheath and structural distribution of Perlecan may disturb nutritional demands or transport of waste products across the sheath. In comparison, the mammalian IVD is mostly dependent on diffusion for nutrient supply (Urban et al. 2004). However, in degenerated discs, matrix turnover and calcification of the endplates have been shown to disturb this transport (Roberts et al. 1989) and identified as one of the primary causes of IDD (Nachemso et al. 1970;Anderson 1993). If a similar transport system across the notochordal sheath exists in Atlantic salmon, loss of structure may play a central role in developing spinal fusions. Based on our findings we suggest that thinning of the elastic membrane and structural changes in the notochordal sheath decreases flexibility and possibly nutritional transportation across the notochord, and are thereby involved in the development of spinal fusions.

An increased number of chordoblasts in the notochordal epithelium of pathological spinal columns is in line with the increased proliferation found in Ytteborg et al. (submited). Chordoblasts retain their germinal properties and continue to secrete the notochordal sheath to sustain normal growth (Grotmol et al. 2006). Kvellestad and colleagues (2000) have previously suggested that chordoblasts and the notochordal sheath are of paramount importance for proper growth of the notochordal tissue. The increased proliferation of chordoblasts could suggest a response upon stretching of the sheath. However, integrity and organization of chordoblasts were found to be disrupted with increased proliferation (Ytteborg et al. submitted). The way in which the chordoblasts are linked both to each other and to the notochordal epithelium most likely play crucial roles in maintaining the structure of the notochord. The types of junctions that are present in the notochord of lampreys have been thoroughly studied by Bartels and Potter (1998). In lampreys, hemidesmosomes are anchoring the chordoblasts to the notochordal sheath. Junctions have not been studied in Atlantic salmon notochord, but it seems likely that similar mechanisms must apply (Grotmol et al. 2006). Hemidesmosomes contain α and ß integrins, which are receptors for Laminin and Perlecan (Brown et al. 1997;Lee et al. 1992;Hayashi et al.

1992;Sonnenberg et al. 1991). Normal sheath organization and intact basal lamina is dependent on Laminin and Perlecan (Grotmol et al. 2006;Hassell et al. 1980), which also control cellular differentiation (Deng and Ruohola-Baker 2000;Streuli et al. 1995). Interestingly, transcription of *laminin* was severely reduced throughout the vertebral fusion process and any possible anchoring may have been weakened. In zebrafish and mice, *laminin1ß* 0-mutants have disrupted formation of basal lamina, which seems to result in disorganization of both notochordal sheath and elastic membrane (Miner et al. 2004;Smyth et al. 1999;Amenta et al. 1983;Hogan et al. 1980). Furthermore, if perlecan is not expressed in zebrafish, basement membranes around the notochord fail to form and notochordal cells fail to differentiate (Parsons et al. 2002). Hence, the increased *perlecan* transcription found in progressing fusions may reflect an attempt of strengthening weakened notochordal structures.

In non-deformed vertebrae, IHC with SP antibody revealed high abundance of this protein in the osteoblasts at growth zones and in the compact bone of the endbones. SP neurokinin receptors are distributed in osteoblasts (Goto et al. 1998) and its role in bone mineralization and osteoblast proliferation has been suggested (Adamus and Dabrowski 2001;Bjurholm 1991), particularly in regions with high osteogenic activity (Hukkanen et al. 1992). Importantly, a novel observation of SP expression in chordoblasts and chordocytes was identified in non-deformed vertebrae. However, signals were lost in these cells during development of vertebral fusions. Whether SP is receptor associated or transcribed in Atlantic salmon chordoblasts and chordocytes have not been further characterized in this study due to the lack of sequence information to perform *in situ* hybridization. The functional role of SP has been shown to involve vascular permeability, vasodilatation and plasma protein extravasations in different tissues (Gjerde et al. 2003;Barnes 2001;Baluk et al. 1997;McDonald 1988;Lundberg et al. 1984). It is therefore tempting to suggest a role for SP in maintenance of hydrostatic pressure in the chordocytes, since these cells tend to loose their vacuoles in progressed vertebral fusions. Notochordal cells have recently been shown to possess chondrogenic phenotypes, expressing Aggrecan, Sox9 and Col2 (Kim et al. 2009;Ytteborg et al. submitted). Aggrecan was present in chordocytes at non-deformed stage, but were lost from chordocytes in the central notochord during progression of the pathology. These findings correlated with loss of vacuoles and formation of hyperdense regions at more progressed stages of the fusion process. Similarly, loss of aggregating PG components from the mammalian IVD is related to loss of hydrostatic pressure and development of IDD (Melrose et al. 2002;Kauppila 1995;Yasuma et al. 1993;Urban and Mcmullin 1985).

Interestingly, both SP and Aggrecan expression reappeared in hyperdense regions of the notochord. Supporting these findings, *aggrecan* showed increased mRNA transcription at fused stage. Aggrecan has been detected in the cartilaginous matrix surrounding hypertrophic chondrocytes in

ossification centers in mammalian vertebrae (Smith et al. 2009) and increased transcription of osteogenic markers have been reported from mammalian SP treated osteoblast-like cultures (Sun et al. 2010;Goto et al. 2007). Expression of osteogenic markers, such as *runx2, col1a* and *osteocalcin,* have been detected in hyperdense notochordal cells together with chondrogenic marker genes like *sox9* and *col2* (Ytteborg et al. submitted). Thus, our results correlate with previous studies showing increased proliferation and co-transcription of both chondrogenic and osteogenic marker genes in the notochord of Atlantic salmon during the pathogenesis of spinal fusions. Witten et al. (2006) has previously suggested chondroid bone formation in the notochord of developing fusions, which at later stages is transformed into bone. Expression of SP and aggrecan in hyperdense notochordal regions of developing fusions supports the suggestion of an adaptation of these cells through metaplastic shifts (Witten et al. 2006;Ytteborg et al. submitted). Based on findings in this study we suggest that mineralization from these cells at later stages in the fusion process could be termed trans-chordoid ossification.

Conclusion

Remodeling of the notochord through a fragmented elastic membrane, altered structures of the notochordal sheath and loss of vacuoles from the central notochord characterized the notochord of developing spinal fusions. Hyperdense regions in the notochord consist of cells in a metaplastic state. At later stages in the fusion process, these cells may contribute to mineralization of the notochord through a process we suggest could be termed trans-chordoid ossification. With this study we suggest that alterations in the notochord contribute to the progression of vertebral fusions in Atlantic salmon.

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Figure legends

Fig.1 Morphological analysis of elastic membrane

Verhoeff`s hematoxylin staining of the elastic membrane. The elastic membrane completely surrounds the notochord (arrowheads). **a.** Overview of non-deformed vertebrae. **b.** Higher magnification of black box in a, intervertebral region showing a thick elastic membrane (arrow). **c.** Overview of intermediate vertebrae. Notice aberrantly shaped vertebral endbones and notochordal sheath. **d.** Higher magnification of black box in c. A decreasing thickness of the elastic membrane (arrow) was observed in intervertebral regions at intermediate stage. **e.** Overview of fused vertebrae. Notice hyperdense notochord in the centre of the fusion. Chordocytes in hyperdense areas have lost their vacuoles. **f.** Higher magnification of black box in e. The elastic membrane was highly fragmented or absent in severe fusions. Throughout the pathology, the notochordal sheath increased in area and width, indicated by asterix. ND, Non-deformed; IM, intermediated; FS, fused; nc, notochord; ns, notochordal sheath; eb, endbone; ac, arch centra; hd: hyperdense. Nuclei are stained black by hematoxylin. Scale bar a, c, e = 100µm; b, d, f = 50 µm.

Fig. 2 Quantitative gene transcription

Relative gene transcription of *laminin1ß, elastin, aggrecan* and *perlecan* in non-deformed low temperature group (white bars, control), non-deformed high temperature group (light grey bars), intermediate (dark grey bars) and fused (black bars) vertebrae normalized with *ef1a*. Significant values (P=0.05) indicated by *, n=15, means ± SE. Expression ratios are shown in relative mRNA expression along y-axis, genes along the x-axis.

Fig. 3 Perlecan expression in the notochordal sheath

IHC with Perlecan (red) in the notochordal sheath (left row) and structural changes of the same area visualized using transparency 3D models (right row) where different colors indicate different confocal layers (red-yellow: structures most elevated; blue-green: structures that appear deeper in the section). **a.** Perlecan was abundantly expressed in the notochordal sheath of non-deformed vertebrae and formed a dorso-ventral zigzag pattern. **b.** 3D image of a visualized an amorphous structure with evenly distributed peaks. **c.** In intermediate vertebrae, Perlecan signals appeared in more linear structures, straightened in dorso-ventral as well as stretched in cranio-caudal direction. **d.** 3D image of b visualized the stretched structure. **e.** In fused vertebra the stretched structure appeared more pronounced and the linear shape was evident. **f**. 3D image of c visualized dorso-ventral stretches. ND, Non-deformed; IM, intermediated; FS, fused; nc, notochord; ns, notochordal sheath; eb, endbone. Scale bar = 50µm.

Fig. 4 Aggrecan and SP expression in the growth zones of non-deformed vertebrae

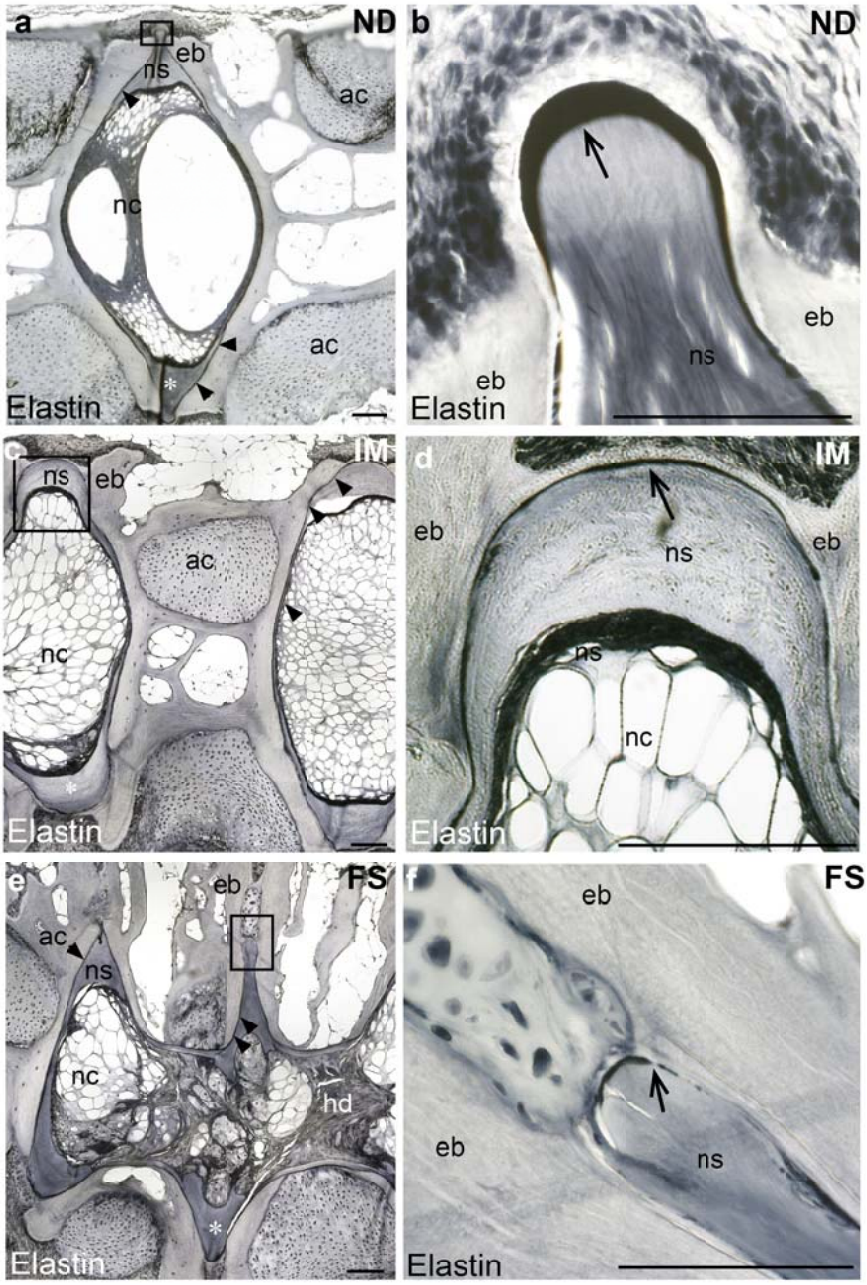
IHC with Aggrecan and SP (red) showed only few scattered signals in the notochordal sheath, but signals were present at the osteoblast growth zones at the vertebral body endplates. **a.** Weak signals of Aggrecan at the growth zones. **b.** SP in endbones and osteoblasts. ND, non-deformed; nc, notochord; ns, notochordal sheath; cb, chordoblast; cc, chordocytes; eb, endbone. Scale bar = 50μm.

Fig. 5 Aggrecan and SP expression in the notochordal epithelium

IHC with Aggrecan and SP (red) in chordoblasts in the notochordal epithelium. Zn12 (green) is used to visualize general structures. **a.** In non-deformed vertebra Aggrecan was present in the chordocytes and **b.** SP in both chordoblasts and chordocytes **c.** At intermediate stage Aggrecan and **d**. SP were severely reduced in both chordoblasts and chordocytes. **e.** At fused stage, Aggrecan was not detected in chordocytes, but expression was detected in the notochordal epithelium. Notice the increased number of chordoblasts. **f.** SP expression was detected at the dorsal side of the notochordal epithelium. Yellow color indicates co-localization with Zn12. ND, Non-deformed; IM, intermediated; FS, fused; ns, notochordal sheath; cb, chordoblast; cc, chordocytes. Scale bar = 50μm.

Fig. 6 Aggrecan and SP expression in the central notochord

IHC with Aggrecan and SP (red) in the central notochord. Zn12 (green) is used to visualize general structures. **a.** In chordocytes in the central notochord of non-deformed vertebrae Aggrecan and **b.** SP had similar expression pattern as in chordocytes close to the notochordal epithelium. **c.** In the central notochord of intermediate vertebrae, Aggrecan was absent (asterix) and **d.** SP severely reduced (asterix). **e.** In the central notochord of fused vertebrae, Aggrecan and **f.** SP were reduced (asterix). **h.** In hyperdense regions of the notochord in fused vertebrae, both Aggrecan and **g.** SP expression reappeared (asterix). Yellow color indicates co-localization with Zn12. ND, Non-deformed; IM, intermediated; FS, fused; FS*, fused with hyperdense notochord; cc, chordocytes. Scale bar = 50μm.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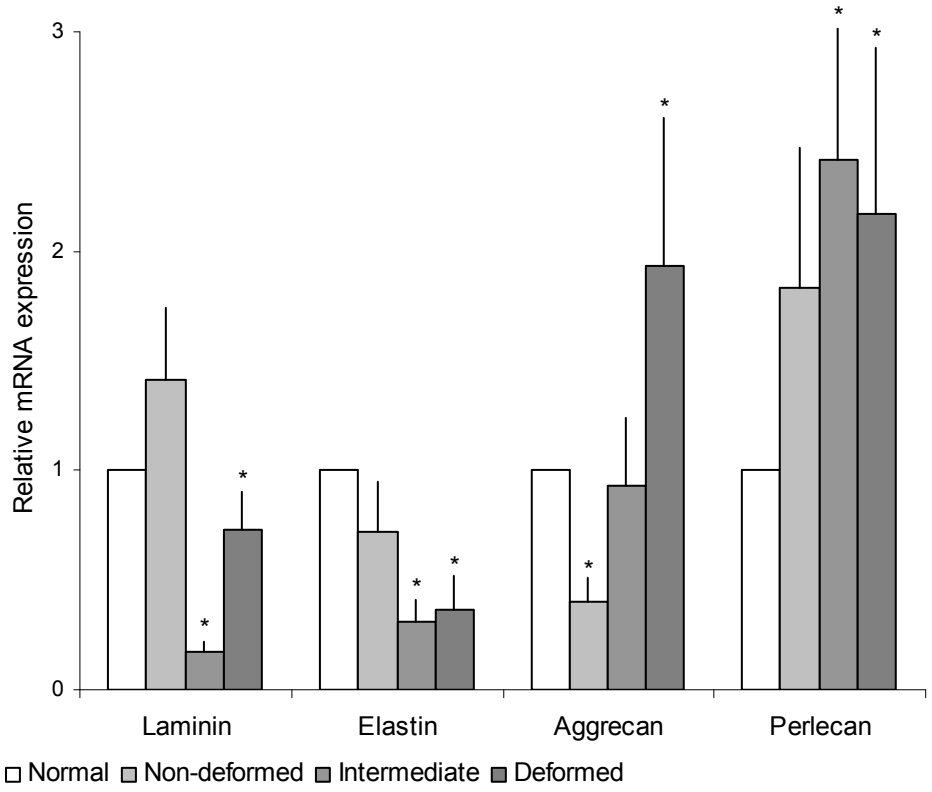
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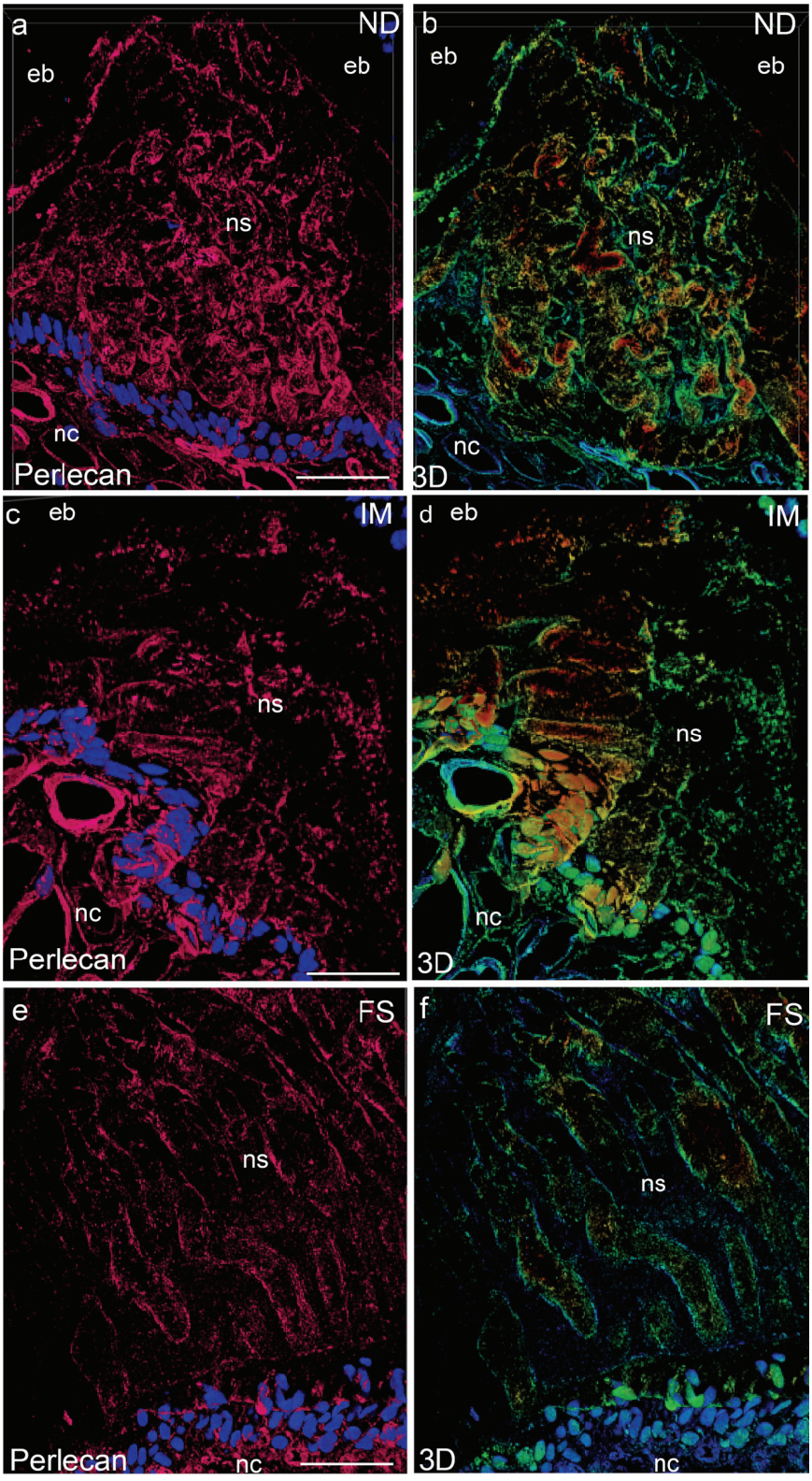
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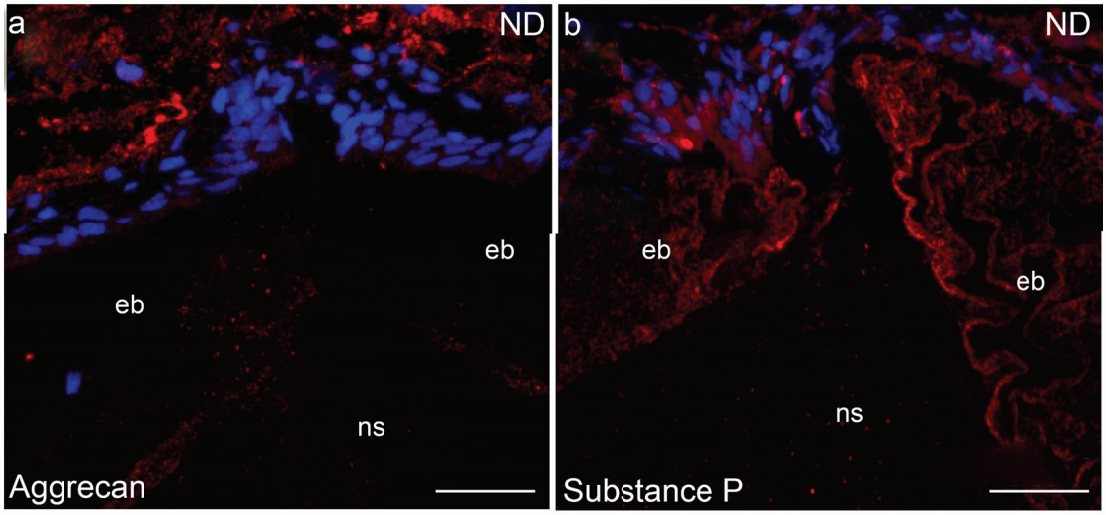
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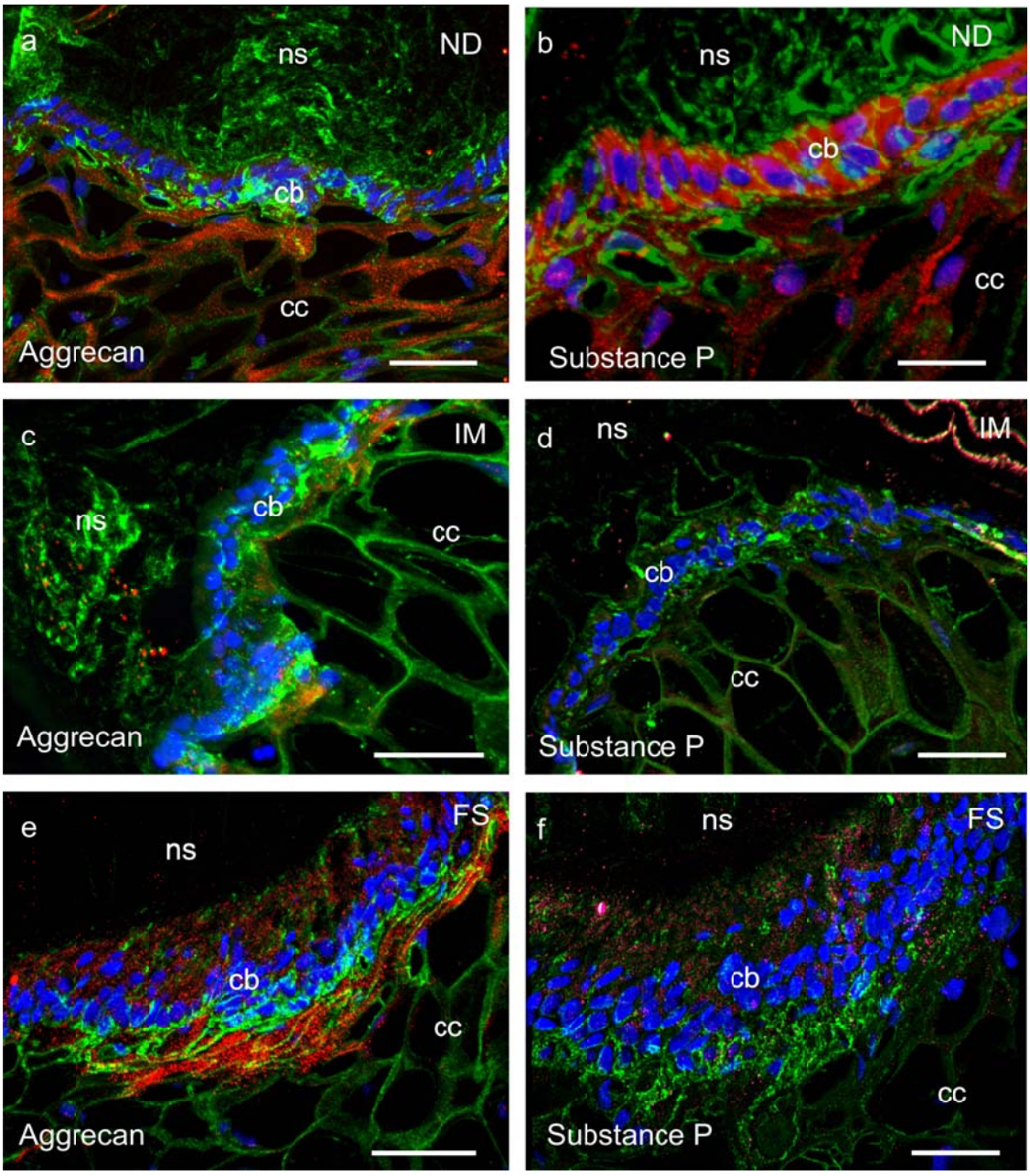
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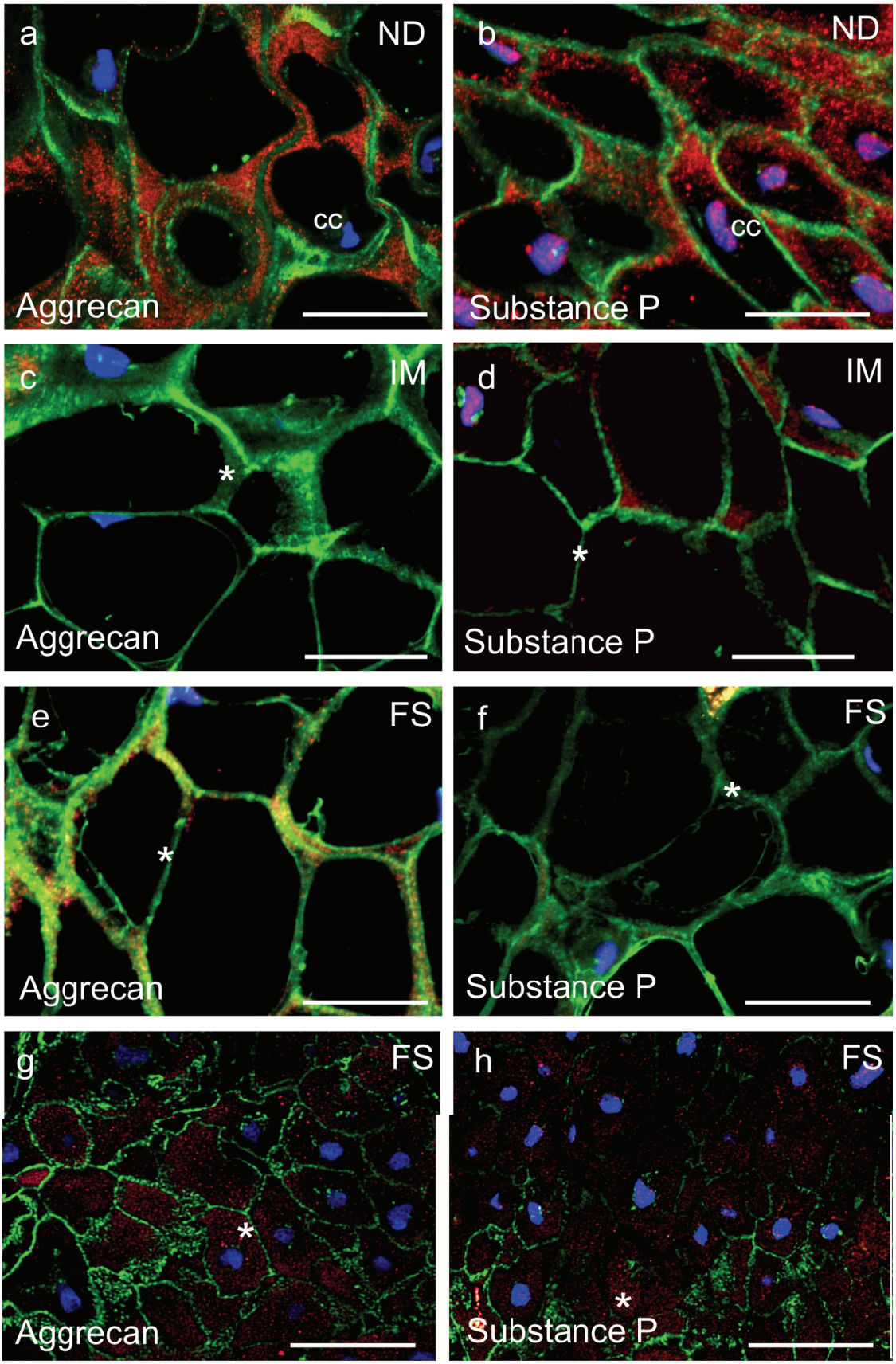
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Paper 4

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Atlantic salmon (*Salmo salar*) muscle precursor cells differentiate into osteoblasts *in vitro:* Polyunsaturated fatty acids and hyperthermia influence gene expression and differentiation

Elisabeth Ytteborg [a], Anne Vegusdal [a], P. Eckhard Witten [b], Gerd Marit Berge [c], Harald Takle [a,d], Tone-Kari Østbye [a], Bente Ruyter [a,*]

[a] *Nofima Marin, Norwegian Institute of Food, Fisheries and Aquaculture Research, P.O. Box 5010, NO-1432 Ås, Norway*
[b] *Ghent University, Department of Biology, Ledeganckstraat 35, B-9000 Ghent, Belgium*
[c] *Nofima Marin, NO-6600 Sunndalsøra, Norway*
[d] *AVS Chile S.A., Of. 3A, Puerto Varas, Chile*

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ABSTRACT

The formation and mineralisation of bone are two critical processes in fast-growing Atlantic salmon (*Salmo salar*). The mechanisms of these processes, however, have not been described in detail. Thus, *in vitro* systems that allow the study of factors that influence bone formation in farmed Atlantic salmon are highly warranted. We describe here a method by which unspecialised primary cells from salmon white muscle can differentiate to osteoblasts *in vitro.* We have subsequently used the differentiated cells as a model system to study the effects of two factors that influence bone formation in Atlantic salmon under commercial farming conditions, namely polyunsaturated fatty acids, PUFAs, and temperature. Muscle precursor cells changed their morphology from triangular or spindle-shaped cells to polygonal or cubical cells after 3 weeks in osteogenic medium. In addition, gene expression studies showed that marker genes for osteoblastogenesis; *alp, col1a1, osteocalcin*, *bmp2* and *bmp4* increased after 3 weeks of incubation in osteogenic media showing that these cells have differentiated to osteoblasts at this stage. Adding CLA or DHA to the osteoblast media resulted in a reduced PGE_2 production and increased expression of *osteocalcin*. Further, temperature studies showed that differentiating osteoblasts are highly sensitive to increased incubation temperature at early stages of differentiation. Our studies show that unspecialised precursor cells isolated from salmon muscle tissue can be caused to differentiate to osteoblasts *in vitro*. Furthermore, this model system appears to be suitable for the study of osteoblast biology *in vitro*.

1. Introduction

Osteoblasts are mononucleated bone-forming cells that originate locally from mesenchymal progenitor cells. Mesenchymal progenitor cells not only form cells of the osteoblast lineage (osteoblasts, osteocytes and bone-lining cells), they also give rise to adipocytes, myocytes and chondrocytes [1,2]. Several studies have shown that these cells have a certain degree of developmental plasticity (modulation) and that differentiated cells also have a certain degree of plasticity (trans-differentiation) [3,4]. *In vitro* culture systems that allow the examination of the cells' capacity for modulation and trans-differentiation are well established for several mammalian species [5] and for the advanced marine teleost species Sea bream (*Sparus aurata*) [6]. Undifferentiated mammalian muscle satellite cells are able to differentiate *in vitro* into one of several cell types, among them osteoblasts [7]. It is not known whether mesenchymal skeletal progenitor cells from Atlantic salmon (*Salmo salar*) have a capacity for modulation or trans-differentiation. However, we have shown that primary precursor cells located in the adipose and muscle tissue of Atlantic salmon can proliferate and differentiate into mature adipocytes and muscle cells, respectively, *in vitro* [8,9].

Specific markers that can confirm the identity of a certain cell type *in vitro* are required in order to determine whether unspecialised precursor cells can successfully mature into specialised cells. Osteoblasts express several phenotypic markers, both collagenous (e.g. *collagen1a*) and non-collagenous (e.g. alp and *osteocalcin*) bone

Abbreviations: ALP, alkaline phosphatase; BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt/nitro blue tetrazolium chloride; BMP, bone morphogenetic protein; CLA, Conjugated linoleic acid; col1a, collagen type 1; col2a, collagen type 2; DHA, Docosahexaenoic acid; EF1a, Elongation factor 1a; ETIF, eukaryotic translation initiation factor; FA, fatty acid; FBS, fetal bovine serum; FS, fish serum; L-15, Leibowitz 15; mlc, myosin light chain; Mrf4, muscle-specific bHLH factor 4; Myf5, Myogenic factor 5; MyoD1b, myoblast determination protein 1b; PBS, phosphate-buffered saline; PFA, Paraformaldehyde; PGE_2, prostaglandin E2; PPARγ, peroxisome proliferator-activated receptor γ; Rpol2, ribosomal protein gene 2

* Corresponding author. Tel.: +47 930 97 531; fax: +47 64 94 95 02.
E-mail address: bente.ruyter@nofima.no (B. Ruyter).

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matrix proteins [10,11]. Osteoblast differentiation is specialized and strictly regulated by a number of transcription factors and signalling molecules. Two members of the bone morphogenetic protein (BMP) family, *bmp2* and *bmp4,* are involved in bone and cartilage development [12,13]. Among the downstream targets of BMPs are *runx2* and other osteoblast-related transcription factors such as *osterix* [14,15]. These osteoblast-related factors directly activate a number of osteoblast markers such as *col1a, osteopontin* and *osteocalcin* [3,10]. In comparison, sox9 *is* an essential transcription factor of chondrocyte differentiation and cartilage formation in vertebrates [16]. Sox9 directly regulates the expression of *col2a*, the gene that encodes the major cartilage matrix protein expressed in chondroprogenitor cells and also expressed at high levels in chondrocytes [17,18]. Further, four muscle-specific bHLH factors (MRFs); mrf4, myogenin, Myf-5, and MyoD are expressed early in embryogenesis, at a time when muscle lineage decisions are established. MyoD and myf5 are required for myogenic determination, whereas myogenin is a downstream transcription factor involved in differentiation [19,20]. Mrf4 has a more complex role and is considered as both a determination and differentiation factor [21]. In addition, developing and mature muscle cells express the structural protein mlc, which may be used as a marker for cells turning into myoblasts [22,23]. PPARγ plays an important role in controlling differentiation programs of multipotent mesenchymal progenitor cells that favor adipogenesis over osteogenesis [24,25]. Teleosts have a single *pparγ* gene homologous to the mammalian *pparγ* [26]. Recent results from our group have shown that two alternatively transcription variants of *pparγ*, named *pparγ-long* and *pparγ-short*, are present in Atlantic salmon and that *pparγ-short* is significantly up-regulated during adipocyte differentiation [27].

Due to the growing need of replacing fish oils in commercial fish feeds, the use of vegetable oils has increased during the latest years. These vegetable oils typically have a high content of n-6 PUFAs that lead to decreased n-3/n-6 PUFA ratios in both the fish diet and the fish tissue [28–33]. Dietary PUFAs are important regulators of many cellular functions in mammals, including those related to bone cell formation. Low n-3/n-6 ratios of PUFAs reduce bone formation and cause greater bone resorption activity in mammals [34–36]. Bioactive fatty acids such as CLA affect bone biology and may have similar effects as those of n-3 PUFAs [37,38]. CLA, like n-3 PUFAs, reduces the production of PGE_2 in rats, but different mechanisms may operate from those in fish [37]. PGE_2 is a potent agent regulating bone formation [39,40] and has been shown to inhibit bone formation at high concentrations *in vitro* [41]. Recent studies on European sea bass larvae have shown that the concentrations of dietary PUFAs, particularly dietary EPA and DHA, are related to vertebral malformations [42,43], but it remains to be determined whether the n-3/n-6 PUFA ratios in fish diets affect bone mineral density.

Use of elevated water temperature during early development to reduce production time is a major factor affecting the prevalence of skeletal abnormalities in farmed fish, [44–46]. In a recent study, we found that by increasing the rearing temperature of salmon from 12 °C to 16 °C the expression of most skeletal genes examined were affected ([47], submitted). Genes that code for extracellular matrix constituents, such as *osteocalcin* and *osteonectin,* were in general down-regulated and results indicated that osteoblast activity was reduced. Nevertheless, there is limited knowledge of how temperature and other factors affect osteoblast differentiation and further bone development, and how these mechanisms are linked to the development of spinal deformities in salmonids. Thus, the development of an Atlantic salmon osteoblast culture is therefore an important step in order to reveal the specific mechanisms underlying the pathogenesis of skeletal deformities. Moreover, such a salmon osteoblast culture will provide improved opportunities for studying the evolutionary development of bone differentiation and remodelling among vertebrates, since experiments with mammalian cell cultures have, to a great extent, already described the major pathways that are involved in skeletal development and remodelling (review [3,48]).

The aim of the present study was to establish a method that would cause unspecialised primary cells from salmon muscle to differentiate to osteoblasts *in vitro.* We used real-time quantitative RT-PCR assays for genes that encode typical osteoblast-related membrane and extracellular matrix molecules (*alp, col1a1, osteocalcin, osteonectin*); for two of the most potent growth factors involved in the recruitment and differentiation of mesenchymal precursors (*bmp2* and *bmp4*); and for the main transcription factor related to osteogenesis (*runx2*). We then analyzed how these genes were expressed in differentiating osteoblasts under different culture conditions: (i) in a medium that induced osteogenic differentiation, (ii) in a medium that had been supplemented with fatty acids having different n-3/n-6 PUFA ratios and different concentrations of CLA, and (iii) under different temperature regimens.

2. Materials and methods

2.1. Materials

Atlantic salmon (*Salmo salar*) fry were obtained from Aqua Gen (Sunndalsøra, Norway) and raised on a commercial diet in an experimental unit at the Agricultural University of Norway.

L-15, fetal bovine serum (FBS), antibiotics, antimycotics, HEPES, L-glutamine, collagenase, trypsin, $CaCl_2$, Ca-ionophore (calimycin), glycerol-2-phosphate disodium salt hydrate, L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, (1,25)-dihydroxyvitamin D_3, dexamethasone, laminin and thermanox cover slips were obtained from Sigma-Aldrich (St. Louis, MO). Tissue culture plasticware was obtained from Nalge Nunc International (Naperville, IL, USA). Paraformaldehyde and formalin were obtained from Electron Microscopy Sciences (Fort Washington, PA, USA). Cells in culture were observed using a Nikon Diaphot inverted light microscope (Japan). A Leica DM6000 light microscope (Germany) was used to view all stained cells. A Leica DC100 camera integrated with the inverted microscope and a Leica DC340 camera integrated with the DM6000 microscope were used to capture cell images. All image acquisitions were controlled by Image-Pro Plus 5.1 software from Media Cybernetics (Silver Spring, MD, USA). Absorbance was measured using a Titertek Multiscan Plus (Ontario, Canada).

2.2. Cell isolation

Myosatellite cells were isolated from Atlantic salmon (average fork length of 50 mm) as described by Koumans et al. [49], using the modifications for Atlantic salmon as described by Vegusdal et al. [9]. Muscle tissue from 80 individual fish was pooled prior to isolation of myosatellites. It was difficult to count myosatellite cells due to the presence of tissue debris and of other cell types in the final suspension; the muscle tissue was therefore weighed after excision. Myosattelites isolated from approximately 5 g muscle tissue were plated to each cell flask of 25 cm^2.

2.3. Osteoblast differentiation

Myosatellite cells were allowed to adhere overnight in an L-15 medium containing 10% FBS, 2 mM L-glutamine, 0.01 M HEPES and 10 ml/l antibiotic-antimycotic, and then extensively washed with pure L-15. The cells were subsequently trypsinated (using 1 ml of stock solution, concentration 2.5 g/l) and re-seeded (day 0) in order to get rid of cell debris and to obtain cleaner cell cultures. After re-seeding, the cultured cells were incubated in an osteogenic differentiation medium composed of growth medium supplemented

with 4 mM $CaCl_2$, 10 mM β-glycerophosphate, 150 μM L-ascorbic acid, 1 μM 1,25-dihydroxyvitamin D_3 and 10 nM dexametasone. Cells were incubated at 12 °C without CO_2 and the medium was changed every second day. Cells grown in the osteogenic medium for 1, 2, 3 or 4 weeks, were further prepared for analysis of gene expression and morphological studies as described below. Cells for *in situ* hybridisation were fixed after 3 weeks in osteogenic medium. The experiment was repeated four times.

2.4. Cell proliferation

Proliferation capacity of the myosatellite cells was evaluated using proliferating cell nuclear antigen (PCNA) staining kit (Invitrogen, MD, USA). After 8 days in culture the cells were washed twice in PBS and stored in 4% PFA at 4 °C (Electron Microscopy Sciences) prior to immunostaining with PCNA. The cells were washed three times in PBS before permeabilisation in 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 min. The manufacturer's protocol for PCNA staining was followed, except for a prolonged incubation period of 10 min in the DAB chromogen. The amount of proliferating cells is expressed as PCNA positive cells in percentage of total cell number.

2.5. Incubation of differentiating osteoblasts with media containing different n-3/n-6 PUFA ratios and CLA (FA experiment)

Both the FA experiment and the temperature experiment were conducted on differentiating cells. After 3 weeks in culture the cells were about to show full osteoblast morphology (see results). Confluent monolayers of differentiating osteoblasts were washed in a FA-free L-15 medium, and osteogenic differentiation medium supplemented with either 10% FBS (n-3/n-6 ratio = 0.7) (control), 10% FBS + 0.05 mM CLA (n-3/n-6 ratio = 0.7), 2.5% fish serum (FS) (n-3/n-6 ratio = 7.0) or 2.5% FS + 0.1 mM DHA (n-3/n-6 ratio = 9.5). The fatty acids (DHA and CLA) were added to the media in the form of their potassium salts bound to BSA (the molar ratio of FA to BSA was 2.5 to 1). The same concentration of BSA was added to the incubations without CLA and DHA (only FBS and FS). Cells were incubated for 72 h at 12 °C. After incubation of cells with different n-3/n-6 PUFA ratios and CLA for 72 h, PGE_2 production was measured and the cells were harvested for the determination of fatty acid composition and levels of gene expression. The experiment was repeated three times.

2.6. Incubation of differentiating osteoblasts at two different temperatures

Cultivated cells intended for the temperature study were grown in an osteogenic medium for 3 weeks at 12 °C before they were divided in two groups. One group was kept at 12 °C (control), the other group was transferred to 16 °C. Cells at the two temperatures were thereafter harvested and prepared for gene expression analysis and for morphological studies at the following time-points: 1 h, 3 h, 6 h, 1 day, and 2 days. Cells were harvested at each time point from both temperature treatments. The morphology of cells was observed by light microscopy and used to follow the differentiation process, as were the levels of gene expression of osteogenic factors. The experiment was repeated four times.

2.7. Alkaline phosphatase staining

Cells cultivated on coverslips for 1, 2 or 3 weeks in osteogenic medium were rinsed with PBS and distilled water, and then dehydrated through a graded series of EtOH. The cells were stained with BCIP/NBT, R&D Systems, Inc. (Minneapolis, USA), for 24 h at 4 °C. The BCIP/NBT stains cells that have ALP activity blue.

2.8. Measurement of PGE2 production

Wells were washed with L-15 and incubated with L-15 supplemented with 5 μM calimycin for 30 min at room temperature. The medium from each well was removed for analysis, and indometacin (10 μg/ml) was added to prevent the breakdown of PGE_2. The medium samples were diluted 1:2 with a calibrator solution and were then immediately analyzed for PGE_2 using the protocol developed by R&D Systems Inc. Protein concentration was determined by the method of Lowry et al. [50].

2.9. FA composition of incubation media and cells

The cells were washed twice in PBS and harvested in PBS prior to analysis of total FA composition. The total FA profiles in the media and in the cells were determined. Lipids were extracted using the Folch method [51], and then transmethylated overnight with 2,2-dimethoxypropane, methanolic HCl and benzene at room temperature, as described by Mason and Waller [52] and by Hoshi et al. [53]. The methyl esters of FAs thus formed were separated in a GC (Hewlett Packard 6890) with a split injector, SGE BPX70 capillary column (length 60 m, internal diameter 0.25 mm and thickness of the film 0.25 μm), flame ionization detector and HP ChemStation software. The carrier gas was helium. The injector and detector temperatures were 280 °C. The oven temperature was raised from 50 °C to 180 °C at a rate of 10 °C/min, and then raised to 240 °C at a rate of 0.7 °C/min. The relative quantity of each FA present was determined by measuring the area under the peak in the GC spectrum corresponding to that FA.

2.10. RNA isolation and cDNA synthesis

Total RNA was isolated using an RNeasy® Mini Kit and QIAshredder columns with on-column RNase-Free DNase set (Qiagen, Hilden, Germany), all in accordance with the manufacturer's protocols. The total RNA concentration and quality were determined by spectrophotometry (NanoDrop® ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA). 1 μg of total RNA was reversed transcribed to cDNA in a total volume of 50 μl using an oligo(dT) primer and reagents from the TaqMan Gold RT-PCR kit (Applied Biosystems, CA, USA).

2.11. Sequence information and primer design

Gene-specific primers for real-time PCR were based on Atlantic salmon cDNA sequence-information available in GenBank® or based on sequences found by BLAST searches with genes from related species in the GRASP EST database. All primers (Table 1) used in mRNA expression studies were designed using the Vector NTI Advance 10 (Invitrogen) and NetPrimer (PREMIER Biosoft, CA, USA) software. PCR products to be sequenced were inserted into pGEM T-easy vectors (Promega, WI, USA), sequenced in both directions and their identity verified using BLAST. All sequencing reactions were carried out using BigDye Terminator chemistry (Applied Biosystems) and analyzed on an ABI 3730 automated sequencer (Applied Biosystems). The obtained coding sequences of Atlantic salmon have been deposited in the GenBank database under the accession numbers shown in Table 1.

2.12. Real-time PCR

Fluorescence-based real-time qPCR was performed using the ABI Prism 7700 Sequence Detection System 2.3 (Applied Biosystems) (*ef1a, etif, col1a1, col2a1, bmp2, bmp4, alp, sox9, runx2, osteocalcin, osteonectin*) and Lightcycler LC480 (Roche, Switzerland) (*ef1a, rpol2, mrf4, mlc, pparγ-short and pparγ-long, myoD1b, myf5, myogenin*). The reactions were run through the following thermal cycles: 95 °C for

Table 1
Primers used for real time PCR expressional analysis of Atlantic salmon (*Salmo salar*) genes.

Gene	Accession number	Orientation	Sequence (5′-3′)
Col1a1	FJ195608	Forward	AGAGAGGAGTCATGGGACCCGTT
Col1a1	FJ195608	Reverse	GGGTCCTGGAAGTCCCTGGAAT
Col2a1	FJ195613	Forward	TGGTCGTTCTGGAGAGACT
Col2a1	FJ195613	Reverse	CCTCATGTACCTCAAGGGAT
BMP4	FJ195610	Forward	TCAAGTTGCCCATAGTCAGT
BMP4	FJ195610	Reverse	CACCTGAACTCTACCAACCA
BMP2	BT059611	Forward	ATGTGGTATTGCACCCATT
BMP2	BT059611	Reverse	ATGGACAGTTTCCCAATGA
ALP	FJ195609	Forward	CTAGTTTGGGTCGTGGTATGT
ALP	FJ195609	Reverse	TGAGGGCATTCTTCAAAGTA
Sox9	EU344852	Forward	CCTGCAAACAAGACAAGGT
Sox9	EU344852	Reverse	GGGTCGAGTAGATTCATACGA
Runx2	FJ195615	Forward	CCACCAGGGACAGACACAGAT
Runx2	FJ195615	Reverse	GAACGGACTGAGATCTGACGAA
Osteocalcin	FJ195616	Forward	GTGAACCAACAGCAAAGAGA
Osteocalcin	FJ195616	Reverse	CCAGGTCCTTCTTAACAAACA
Osteonectin	FJ195614	Forward	ATTACTGAGGAGGAGCCCATCATT
Osteonectin	FJ195614	Reverse	CCTCATCCACCTCACACACCTT
PPARγlong	AJ292963	Forward	CATTGTCAGCCTGTCCAGAC
PPARγ-short	EU655708	Forward	ATACAGCGTGTATCAAGACG
PPARγ-long/ short	AJ292963	Reverse	TTGCAGCCCTCACAGACATG
MLC	AJ557151	Forward	GGCCCCATCAACTTCAC
MLC	AJ557151	Rewerse	CTCCTCCTTCTCCTCTCCGTG
Mrf4	DQ479952	rorward	CCTTTGTACCACGGGAATGACAGC
Mrf4	DQ479952	ʀewerse	TGTCGGTCGGTGCAGACTTTCTT
Myf5	DQ452070	Forward	GGGAACTGGATGGCTCAGA
Myf5	DQ452070	Reverse	TGCTGGACTTACGCTTGCA
MyoD1b	AJ557150	rorward	CCGCAACACGAAGCAACTATTACAGC
MyoD1b	AJ557150	Reverse	GGAACCCTCCTGGCCTGATAACAC
Myogenin	DQ294029	rorward	ATTGAGAGGCTGCAGGCACTTG
Myogenin	DQ294029	Reverse	GTGCGGTAGTGTAAGCCCTGTGTT
Rpol2	CA049789	Forward	TAACGCCTGCCTCTTCACGTTGA
Rpol2	CA049789	Reverse	ATGAGGGACCTTGTAGCCAGCAA
EF1A	AF321836	Forward	CACCACCGGCCATCTGATCTACAA
EF1A	AF321836	Reverse	TCAGCAGCCTCCTTCTCGAACTTC

10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Further, specificity was assessed by the melting curves, which were determined after the PCR (95 °C for 15 s, 60 °C for 1 min, and 97 °C continuous). All reactions were performed as described in the SYBR Green PCR Mastermix protocol (Invitrogen) or LightCycler® DNA Master SYBR Green I protocol (LC480, Roche). PCR efficiencies for each target and the three housekeeping genes *ef1a*, *etif* and *rpol2* were used as endogenous controls. Relative target gene mRNA was normalized to relative *ef1a* mRNA levels for all samples. The expression ratios between the different genes were tested by using the Relative Expression Software Tool, REST, according to Pfaffl et al. [54]. Differences between the expression ratios were tested for significance by the Pair Wise Fixed Reallocation Randomisation Test© [54].

2.13. In situ hybridisation (ISH)

Digoxygenin (DIG)-labeled riboprobe was transcribed *in vitro* following the manufacturer's protocol (Roche, Switzerland), using PCR products as templates. The *col1a* sequence was obtained using the forward primer 5′-TAGCCGTGGTTTCCCTGGTT-3′ and the reverse primer 5′-CCGGGAGGTCCAAATCTACC-3′) [55], and inserted into pGEM T-easy vectors (Promega). Digoxigenin-labeled *in situ* hybridization probes were synthesized from Sp6-tailed and T7-tailed PCR products. Hybridization on sections was performed overnight with a probe concentration of 500 ng/μl at 65 °C. Excess probe was washed away, and heat-inactivated calf serum was used to prevent non-specific binding of the antibody. Sections were incubated with ALP linked to an anti-DIG antibody at 4 °C overnight. Excess antibody was then washed away and staining performed with NBT/BCIP (R&D Systems Inc.). The sections were post-fixed after staining using 4% PFA in PBS and the slides were mounted with Aquamount (Gurr, BDH, UK) for observation in a light microscope. Sections were hybridised with sense probes as a control in all cases.

3. Results

3.1. Cell morphology during differentiation

Changes in cell morphology were studied during a differentiation period of 3 weeks. Newly isolated cells from salmon muscle tissue consisted of a heterogeneous population of spindle-shaped myosatellite cells and small, round or triangular cells (Fig. 1A). The myosatellite cells at this stage had relatively high proliferative capacity, with approximately 50% of the cells staining positive for PCNA.

Few cells had the characteristic elongated appearance of muscle cells after a few days in the osteogenic medium. The majority of cells retained a spindle or polygonal shape, and the cells did not fuse with each other. During the subsequent days, the cells became more elongated and fibroblast-like. The morphologies of the cells were heterogeneous after 1 week in culture. Some extremely thin and elongated cells were present, but the majority of cells had a fibroblast-like, elongated morphology. Most cells exhibited several cytoplasmic extensions and a polygonal shape (Fig. 1B). Some of the fibroblast-like cells after 1 week in osteogenic differentiation medium, stained positive for endogenous ALP activity (Fig. 2A).

During the second week of incubation, most cells gradually changed their morphology from an elongated shape to a more compact shape (Fig. 1C). ALP activity clearly increased between one and 2 weeks, and clusters of cells that stained strongly for ALP were present after 2 weeks (Fig. 2B). The majority of cells that stained positive for ALP were compact, whereas the more elongated cells stained weakly or not at all for ALP activity. Myotubes were observed, but they did not dominate the cultures. A cell count showed that less than 20% of the cells had the elongated morphology typical of myotubes (Fig. 2B).

The cell density increased until the culture reached confluence after approximately 3 weeks. The cultures at this stage consisted mainly of cells with a round ("cobblestone") or ellipsoid appearance, with less than 5% of the cells showing the typical morphology of myotubes (Fig. 1D). ALP staining showed the same pattern as after 2 weeks, with groups of cells exhibiting a high ALP activity (Fig. 2C). The round or ellipsoid shapes of the cells at this stage resembled the shapes of osteoblasts.

3.2. In situ hybridisation

In situ hybridisation of 3-week cultures using a *col1a* RNA probe revealed positive staining in the cytoplasm of a large portion of the cells with a compact shape (Fig. 2D). Some groups of cells were especially strongly stained. Cells with an elongated shape were not stained.

3.3. Gene expression during differentiation

Analysis of gene expression showed that the markers *alp*, *col1a1* and *osteocalcin* increased significantly (p-value ≤ 0.05) from week 2 to week 4, as did the osteogenic signalling molecules *bmp2* and *bmp4* (Fig. 3A–E). The highest expression of *bmp2* occurred at week 2. Expressions of *bmp4, alp, col1a1* and *osteocalcin* increased significantly during the third week of differentiation. *Runx2* was detected at week 3, although the increase was not statistically significant (results not shown). Expression of *col2a1* was down-regulated and *sox9* low during the fourth week of differentiation

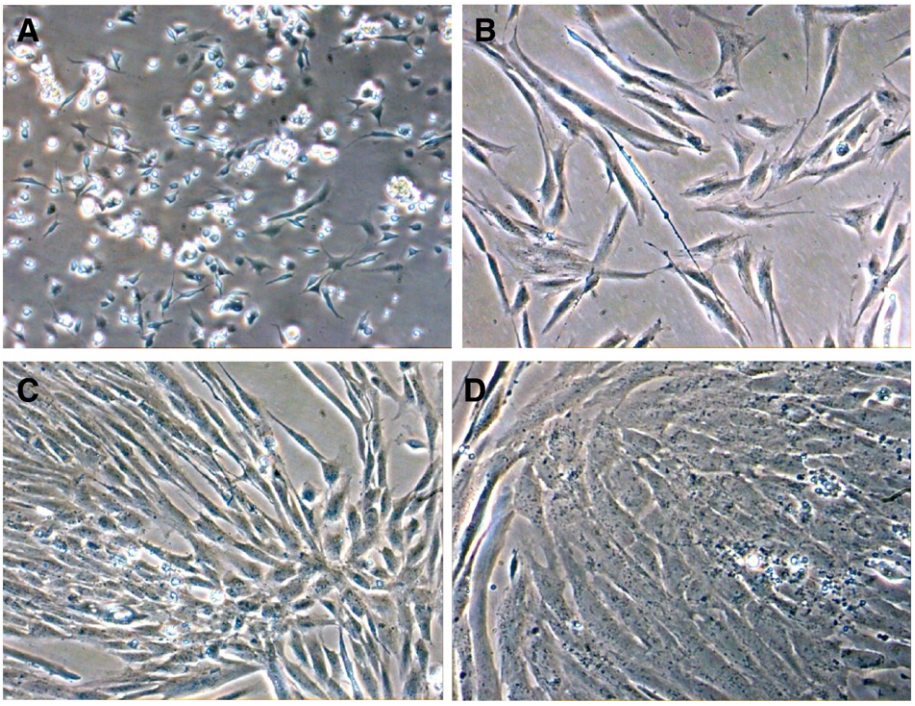

Fig. 1. Light micrographs of precursor cells isolated from salmon muscle tissue and their *in vitro* differentiation in an osteoblast-inducing medium during a 3-week cultivation period. (A) One day after plating. The cultures consist of spindle-shaped myosatellite cells and small, roundish to triangular cells. (B) One week after plating. The cultures consist of cells with a relatively heterogeneous morphology, with the majority of the cells showing an extended cytoplasm and a fibroblast-like and elongated morphology. Most cells have many cytoplasmic extensions and a polygonal shape. A few extremely thin and very elongated myosatellite cells are present. (C) Two weeks after plating. Many cells have changed their morphology from an elongated to a more compact shape. (D) Three weeks after plating. The cultures have reached confluence and consist mainly of cells with a round ("cobblestone") or ellipsoid shape. Original magnification: A, B, C: 100x, D: 200x.

(Fig. 3F, G). The expression of *mrf4* was heavily down-regulated during the fourth week whereas *mlc* showed a low but steady expression (Fig. 3H, I). Other muscle markers like *myoD1b, myogenin* and *myf5* showed steady down-regulation during the differentiation process. *Pparγ-short* was not detected.

3.4. Measurements of FA composition in media and in differentiating osteoblasts after adding different n-3/n-6 PUFA ratios

The fatty acid compositions of the cells mirrored the fatty acid composition of the medium in which they were cultivated (Table 2). The percentages of the longer chain n-3 FAs in the total osteoblast lipids were approximately two times higher in cells grown in the FS-enriched media (n-3/n-6 ratio of 2.9) and four times higher in cells grown in media containing FS + DHA (n-3/n-6 ratio of 4.1) than they were in the control media containing FBS (n-3/n-6 ratio of 0.86). The percentages of n-6 FAs in the total osteoblast lipids were approximately 1.5 times higher in the FBS group than they were in the FS and FS + DHA groups. The main contributors here were 18:2n-6 and 20:4n-6. Cells cultivated in a medium containing FBS + CLA contained approximately 4% CLA in their total lipids. Adding CLA to the media reduced the percentage of saturated FAs and increased the percentage of monounsaturated FAs compared to the control.

3.5. PGE_2 production

The FA composition of the cells affected the production of PGE_2 (Fig. 4). PGE_2 production in cells incubated with media rich in n-3 FA was lower than it was in cells incubated with media rich in n-6 FAs. It was 80% lower for cells incubated with FS, and it was 94% lower for cells incubated with a mixture of FS + DHA. Cells cultivated in CLA enhanced media resulted in a 35% reduction in PGE_2 production from the value of the control cells.

3.6. Effects of fatty acid composition on gene expression

The expression of *osteocalcin* was significantly up-regulated in differentiating osteoblasts treated with DHA or CLA (Fig. 5A and B). In contrast, the expressions of *bmp4* and *col1a1* were significantly down-regulated in cells treated with DHA, whereas that of *bmp4* was significantly up-regulated in CLA-treated cells. *Col1a1* was also up-regulated in CLA-treated cells, but the result was not statistically significant. *Alp* was insignificantly down-regulated in cells subjected to either treatment. The expression of *pparγ-long* was slightly higher in osteoblasts treated with CLA, while *pparγ-short* was not detected in these cells. The two treated groups were compared with a control cell culture treated with 10% FBS.

3.7. Effects of hyperthermia on gene expression

The temperature exposure study was performed with osteoblasts that had been differentiated in an osteogenic medium for 3 weeks. Osteoblasts were divided in two temperature groups. One group was kept at 12 °C (control), the other group moved to 16 °C. The cells in the two groups were tested for eventual differences in gene expression at 1 h, 6 h, 24 h and 48 h after initiation of the temperature study.

After 1h, the expression levels of *alp, col1a1* and *bmp4* were higher in osteoblasts at 16 °C compared to control cells kept at 12 °C (Fig. 6B). In addition, the expression levels of most marker genes, including *osteocalcin*, were down-regulated in the cells kept at 16 °C. A small up-regulation of *col1a1* and *bmp4* was detected 24 h after exposure to elevated temperature. Cells incubated at high temperature started to detach after 48 h. These cells were dead after 72 h.

4. Discussion

Osteoblast cultures have been established for several mammals: human, rat and sheep [5], and for gilthead sea bream [6]. We show

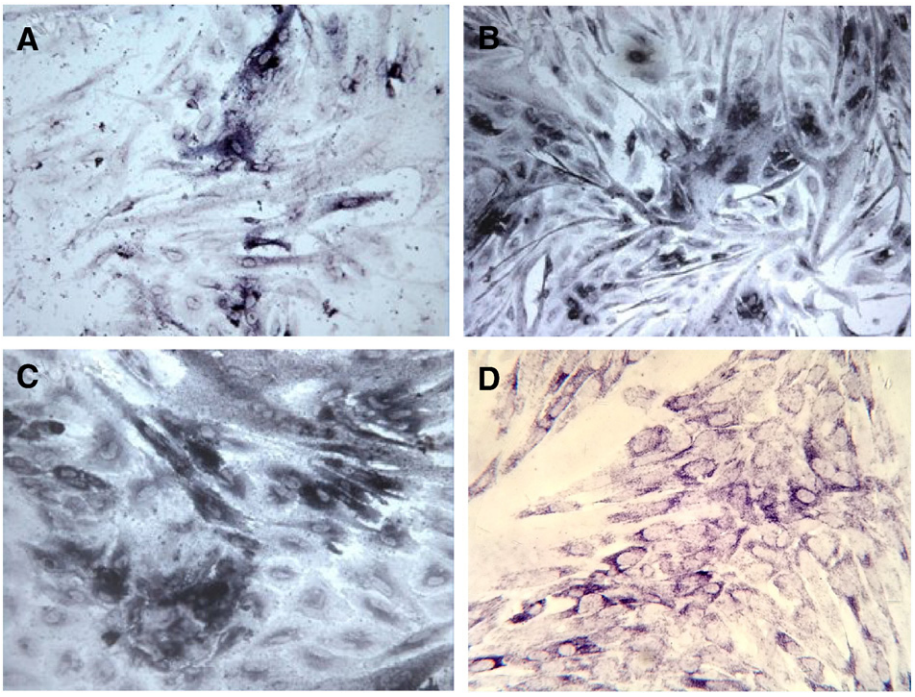

Fig. 2. Endogenous alkaline phosphate activity during a 3-week *in vitro* differentiation period of salmon muscle precursor cells in an osteoblast-inducing medium and *in situ* hybridization of osteocalcin 3 weeks after plating. (A) After 1 week in culture some of the cells stained positively for endogenous alkaline phosphatase activity. (B. A higher fraction of the cells was ALP-positive after 2 weeks in culture, the cells often being located in groups. (C) ALP staining after 3 weeks showed the same pattern as after 2 weeks with some groups of cells having an especially high ALP activity. (D) *In situ* hybridization of cultures 3 weeks after plating showed that col1a is expressed in the cell cytoplasm. Original magnification: A, B: 100×, C, D: 200×.

here that precursor cells isolated from Atlantic salmon muscle tissue can differentiate to osteoblasts *in vitro* when incubated in an osteogenic medium.

Isolated salmon muscle precursor cells were stimulated to osteogenic differentiation by an incubation medium containing $CaCl_2$, β-glycerophosphate, L-ascorbic acid, dexamethasone and 1,25-dihydroxyvitamin D_3. These components direct the differentiation of various mammalian precursor cells towards the direction of osteoblasts [56–58]. The phenotype of the cells changed during the 3-week incubation period from small, spindle-shaped or triangular cells to become confluent cultures of large cells with a polygonal or cuboidal shape. The morphology of the cultured cells was similar to that of gilthead seabream osteoblasts, which were derived from vertebral explants [6]. The morphology of the osteoblasts we have grown here is distinctively different from that of salmon muscle cells, which in a myogenic medium develop to characteristic multinucleated, elongated myotubes [9]. The majority of cells retained the spindle or polygonal shape and did not fuse with other cells, as is otherwise common for precursor cells in a muscle cell differentiation medium [9]. We conclude that precursor cells isolated from salmon muscle are able to differentiate into the osteoblast lineage. Although not previously shown for teleost fish, it is well known from mammals that precursor cells from a specific tissue, like muscle or adipose tissue, are prone to differentiate to various cell types, among them osteoblasts [7,56,58–60].

A key question of osteoblast development is how progenitors progress from a primitive state to a fully functional matrix-synthesizing osteoblast. We found that the level of ALP increased during the differentiation process. Most cells expressed *alp* after 3 weeks of incubation. Increased ALP levels in mammals correlate with an increased rate of bone formation [61]. Strongly stained cells were often observed in clusters, where differentiation of cells seemed to occur in groups, a process known as condensation [62]. Although *alp* expression is strongly suggestive to that osteogenesis is taking place, this enzyme is not considered to be a specific marker for osteogenic differentiation. The expression levels of a repertoire of genes that are considered to be more osteoblast-specific were therefore examined.

The sequential expression of a combination of genes together with cell behaviour and cell morphology was used to identify an early osteoblast. Several osteoblast-related genes, including *alp, col1a1, osteocalcin* and the bone morphogenetic proteins *bmp2* and *bmp4,* were up-regulated during the first 2–3 weeks in culture, which agrees with results from previous studies on osteoblast development (review [3,48] and references therein). The levels of expression of most factors were significantly higher after 3 weeks. We suggest that the cultures stabilized or a positive feedback signalling was activated after 3 weeks in differentiation medium. The expression of *col1a1* expression has previously been shown to reach a stable level during osteoblast differentiation [63], as we observed. *Osteocalcin* has been shown to signal terminal differentiation of osteoblasts in rats [64]. Some experiments, however, describe *osteocalcin* expression prior to mineralization [65–67] while others describe its expression at the onset or after the beginning of mineralization [68,69]. Recent studies on Atlantic salmon jaw bones showed that osteocalcin mRNA is up-regulated in mature osteoblasts and odontoblasts, while early osteoblasts and odontoblasts do not express *osteocalcin* mRNA

Fig. 3. Expression level of osteoblastic: (A) *bmp2*, (B) *bmp4*, (C) *col1a1*, (D) *osteocalcin* and (E) *alp*, chondrocytic: (F) *col2a1* and (G) *sox9* and myogenic: (H) *mrf4*, (I) *mlc,* (J) *mrf5,* (K) *myogenin* and (L) *myoD1b* markers during 4 weeks of differentiation in culture analyzed by real time RT-PCR. *Alp* activity increased between week 1 and week 2. Gene expression studies showed that the bone-specific markers *col1a1* and *osteocalcin* and the expression of bone morphogenetic proteins *bmp2* and *bmp4* increased during the same period. Chondrocytic and myogenic markers decreased during the differentiation period. The transcription at week 0 is the control and has been set to 1. The following weeks are calculated relative to this level. Each dot at weeks 1, 2, 3 and 4 represents the mean of the normalized gene expression ratio, normalised with *ef1a*. Significant up- and down-regulations (p-value ≤ 0.05) are indicated with $*n = 4$.

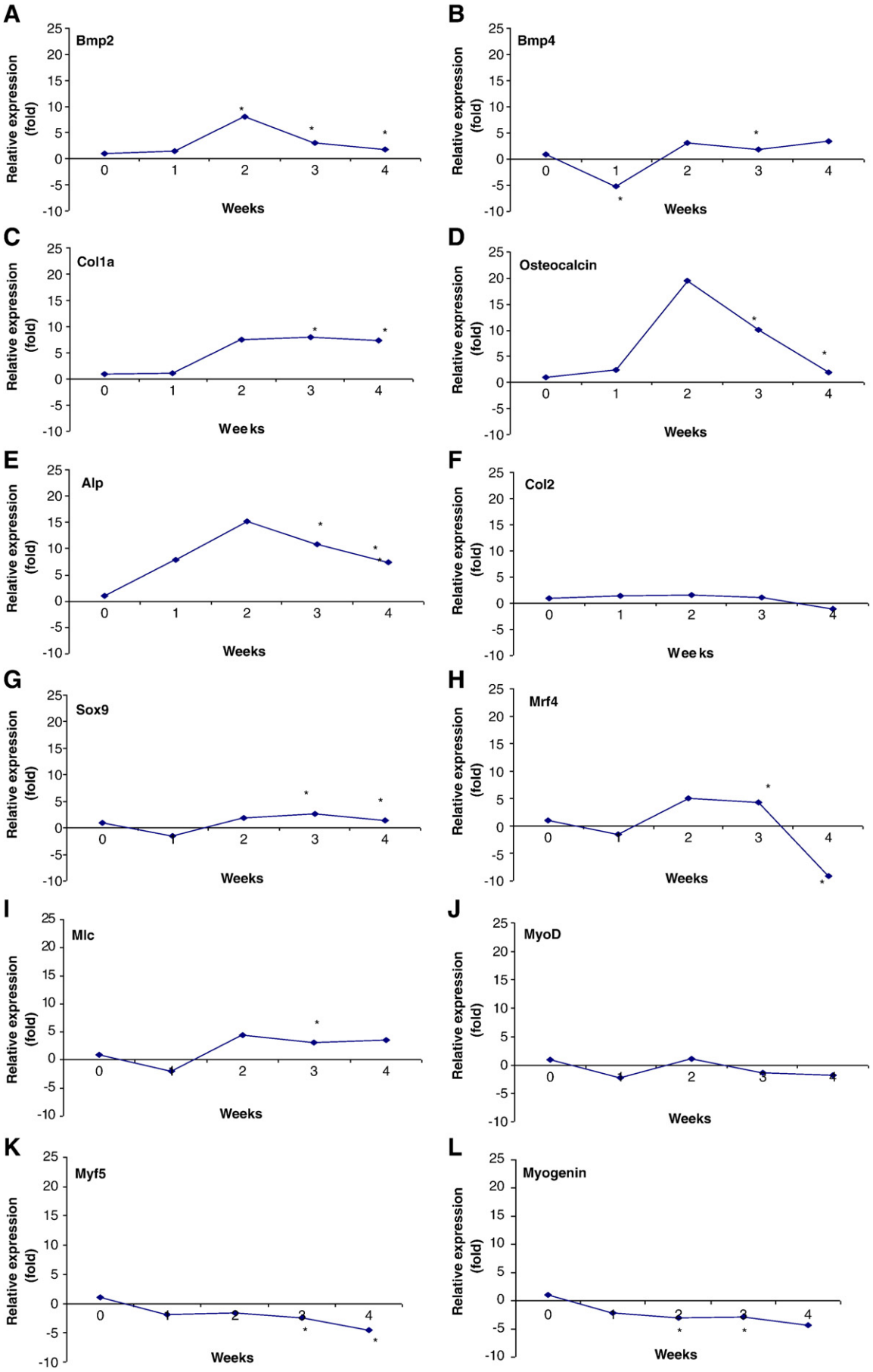
A
Bmp2
B
Bmp4
C
Col1a
D
Osteocalcin
E
Alp
F
Col2
G
Sox9
H
Mrf4
I
Mlc
J
MyoD
K
Myf5
L
Myogenin
Relative expression (fold)
Weeks

Table 2
FA compositions of the total lipid fractions of incubation media (10% FBS, 10% FBS + 0.05 mM CLA, 2.5% fish serum (FS), or 2.5% FS + 0.1 mM DHA) and differentiating osteoblasts incubated in the media for 72 h at 12 °C*.

Incubation media				Cells				
FA	FBS	FBS + CLA	FS	FS + DHA	FBS	FBS + CLA	FS	FS + DHA
14:0	1.1	4.2	1.3	1	2.7 ± 0.26^{b}	0.9 ± 0.59^{a}	1.1 ± 0.22^{a}	0.4 ± 0.08^{a}
16:0	19.6	13.9	15.7	12.4	15.5 ± 2.09^{c}	9.5 ± 0.96^{ab}	12.2 ± 1.26^{bc}	5.9 ± 1.27^{a}
18:0	11.5	7.6	4.4	3.7	8.8 ± 2.15^{b}	4.1 ± 0.84^{a}	4.4 ± 0.80^{a}	3.6 ± 0.60^{a}
16:1n-7	2.5	1.1	2.2	1.7	2.4 ± 1.18^{b}	0.2 ± 0.11^{a}	0.3 ± 0.12^{a}	0.5 ± 0.23^{ab}
18:1n-7	5.9	3.8	2.1	1.7	2.5 ± 0.13^{b}	2.5 ± 0.04^{b}	2.2 ± 0.21^{b}	1.5 ± 0.06^{a}
18:1 n-9	15.8	12.1	10.9	8.9	13.9 ± 1.94^{ab}	20.4 ± 5.34^{b}	10.6 ± 1.23^{a}	8.7 ± 0.91a
20:1n-7	nd	2	0.2	0.1	nd	1.6 ± 0.53^{b}	0.2 ± 0.14^{a}	0.4 ± 0.04^{a}
20:1n-9	0.7	0.6	2.1	1.7	2.9 ± 1.26^{ab}	7.5 ± 2.28^{b}	3.0 ± 1.79^{ab}	1.4 ± 0.58^{a}
20:1n-11	nd	0.9	nd	0.2	0.8 ± 0.39^{a}	0.5 ± 0.07^{a}	0.7 ± 0.42^{a}	1.7 ± 0.41^{a}
22:1n-9	nd	nd	0.3	0.2	0.3 ± 0.28^{a}	1.1 ± 0.35^{a}	0.7 ± 0.34^{a}	nd
22:1n-11	nd	nd	1.4	1.2	3.8 ± 1.54^{ab}	10.0 ± 3.21^{b}	5.2 ± 1.80^{ab}	1.9 ± 1.29^{a}
24:1n-9	nd	nd	0.5	0.4	nd	0.3 ± 0.22^{a}	1.4 ± 0.35^{b}	1.6 ± 0.13^{b}
16:2n-3	nd	nd	nd	nd	3.4 ± 1.00^{a}	0.6 ± 0.40^{a}	4.5 ± 1.83^{a}	5.2 ± 0.98^{a}
16:2n-6	nd	nd	nd	nd	0.7 ± 0.23^{a}	0.5 ± 0.33^{a}	0.4 ± 0.35^{a}	0.4 ± 0.13^{a}
18:2n-6	5.7	3.4	2.7	2.2	4.1 ± 0.76^{a}	6.2 ± 1.44^{a}	6.4 ± 1.48^{a}	5.6 ± 1.06^{a}
20:2n-6	nd	0.2	0.6	0.5	nd	0.1 ± 0.08^{a}	0.7 ± 0.49^{a}	0.1 ± 0.11^{a}
16:3n-4	nd	nd	0.1	nd	0.8 ± 0.28^{b}	0.1 ± 0.10^{a}	0.2 ± 0.16^{a}	0.1 ± 0.08^{a}
18:3n-3	nd	0.2	0.3	3	3.1 ± 1.56^{a}	0.7 ± 0.22^{a}	4.1 ± 1.65^{a}	4.4 ± 0.83^{a}
18:3n-4	nd	0.2	0.1	0.1	nd	1.9 ± 0.59^{b}	0.2 ± 0.11^{a}	0.3 ± 0.05^{a}
18:3n-6	nd	0.2	0.2	0.1	nd	0.1 ± 0.05^{a}	0.2 ± 0.13^{ab}	0.4 ± 0.11^{b}
20:3n-6	2.1	1.3	0.4	0.3	1.7 ± 0.30^{b}	0.7 ± 0.25^{a}	0.5 ± 0.18^{a}	0.8 ± 0.10^{a}
18:4n-3	nd	nd	nd	nd	2.0 ± 1.02^{ab}	0.5 ± 0.19^{a}	2.9 ± 1.19^{ab}	3.9 ± 0.07^{b}
20:4n-3	nd	0.5	0.3	0.4	nd	0.2 ± 0.16^{a}	0.3 ± 0.16^{a}	0.5 ± 0.11^{a}
20:4n-6	8.9	5.8	2.9	2.3	8.3 ± 1.69^{b}	3.8 ± 1.25^{a}	3.4 ± 0.66^{a}	3.0 ± 0.65^{a}
22:4 n-6	nd	nd	0.3	nd	2.8 ± 0.45^{c}	1.8 ± 0.59^{b,c}	0.1 ± 0.07^{a}	0.9 ± 0.16^{ab}
20:5 n-3	2.1	0.8	14	11.5	1.2 ± 1.16^{a}	4.5 ± 1.60^{a}	6.7 ± 2.53^{a}	3.9 ± 0.34^{a}
22:5 n-3	4.6	2.1	6.1	3.8	2.2 ± 0.43^{a}	1.4 ± 0.25^{a}	3.0 ± 0.62^{a}	2.2 ± 0.30^{a}
22:6 n-3	4	3.4	21.6	35.5	3.5 ± 0.76^{a}	5.0 ± 0.81^{a}	9.8 ± 3.46^{a}	24.9 ± 4.95^{b}
CLA	nd	26.9	nd	nd	nd	4.4 ± 0.42	nd	nd
Others	nd	4	nd	2.6	7.6 ± 1.71^{a}	3.9 ± 1.13^{a}	9.3 ± 2.63^{a}	9.2 ± 1.18^{a}
Σ n-3	10.7	7.1	42.6	54.2	15.4 ± 3.91^{a}	13.1 ± 3.04^{a}	31.3 ± 3.51^{b}	45.0 ± 4.57^{c}
Σ n-6	14.6	10.9	6.1	5.7	17.8 ± 1.73^{b}	13.1 ± 1.95^{ab}	10.8 ± 1.51^{a}	11.2 ± 0.68^{a}
n-3/n-6	0.7	0.7	7	9.5	0.9	1.0	2.9	4.0
Σsaturated	32.2	25.7	21.4	17.1	27.0 ± 4.00^{c}	14.5 ± 1.60^{ab}	17.8 ± 1.13^{b}	9.9 ± 1.79^{a}
Σmonosa t	24.9	20.5	19.7	16.1	26.5 ± 1.00^{a}	44.2 ± 0.38^{b}	24.5 ± 3.99^{a}	17.7 ± 3.15^{a}

The quantity of each fatty acid is given as the percentage of total fatty acids. The values given are means ± SEM ($n = 3$). Different letters indicate significant differences ($p \leq 0.05$) between the different treatments.
*The quantity of each FA is given as a percentage of the total quantity of FAs. Data are presented as means ± SEM ($n = 4$).

[55,70]. However, an *osteocalcin in situ* probe gave a positive signal in early osteoblasts situated on the endbones of Atlantic vertebral bodies ([47], submitted). Moreover, *osteocalcin* transcription increased strongly during the osteoblast differentiation experiments and peaked after 3 weeks, which is the time point at which the majority of cells stained positive for ALP. This indicates that *osteocalcin* is expressed both during the early steps in the differentiation process and in mature osteoblasts of Atlantic salmon. In summary, the increased level of osteoblastic marker transcripts after 3 weeks in culture strongly indicates the osteogenic nature of the cultured cell population. Earlier experiments have shown that all of these osteoblast-associated markers are up-regulated prior to the cessation of proliferation in osteoblast precursors (for review see [71]).

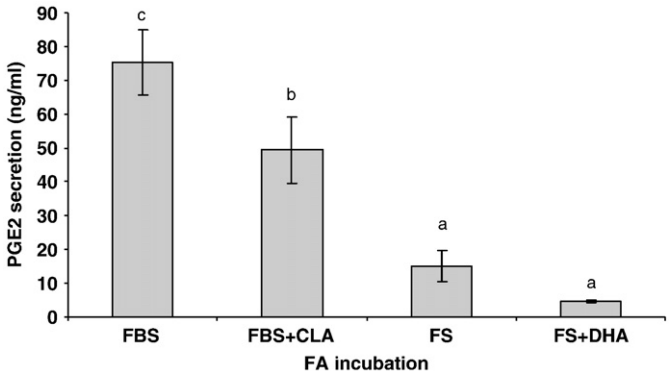

Fig. 4. Production of PGE_2 in cells differentiated in an osteblast-inducing medium for 3 weeks. Differentiated cells were incubated for 72 h in an FA-free medium supplemented with either 10% FBS (control), 10% FBS + 0.05 mM CLA, 2.5% fish serum (FS), or 2.5% FS + 0.1 mM DHA. The culture medium was sampled and analyzed for eicosanoids as described. Data are means ± SEM, $n = 3$. Different letters indicate significant differences ($p \leq 0.05$) between the different FA-treatments.

Gene expression revealed that *sox9* and *col2a1* were down-regulated simultaneously during the fourth week of differentiation. Sox9 directly regulates the expression of *col2a* in chondrocytes [17], and c*ol2a* is a recognised marker gene for chondrocytes [72,73]. These results indicate that the cultures did not differentiate in the direction of cartilaginous cells. Furthermore it is unlikely that further muscle cells formed, since *mrf4*, a marker for early muscle differentiation [19], was almost undetectable after 4 weeks in the osteogenic medium. The stable level of the expression of *mlc* may be the result of some muscle cells being present in the culture [22,23], which is in agreement with our finding that a minor proportion (<5%) of the cells had the morphology resembling that of myotubes. The down-regulated transcription of *myoD1b, myogenin* and *mrf5* indicated however, that the majority of the cells did not differentiate into muscle cells, which is in agreement with Rudnicki et al. [20]. The adipocyte pathway was neither activated, since *pparγ*-short was not expressed [27].

4.1. The n-3/n-6 PUFA ratio affects gene expression and PGE2 production in differentiating osteoblasts

The production of PGE_2 was significantly influenced by the FA composition of osteoblasts. Interestingly, the production was reduced

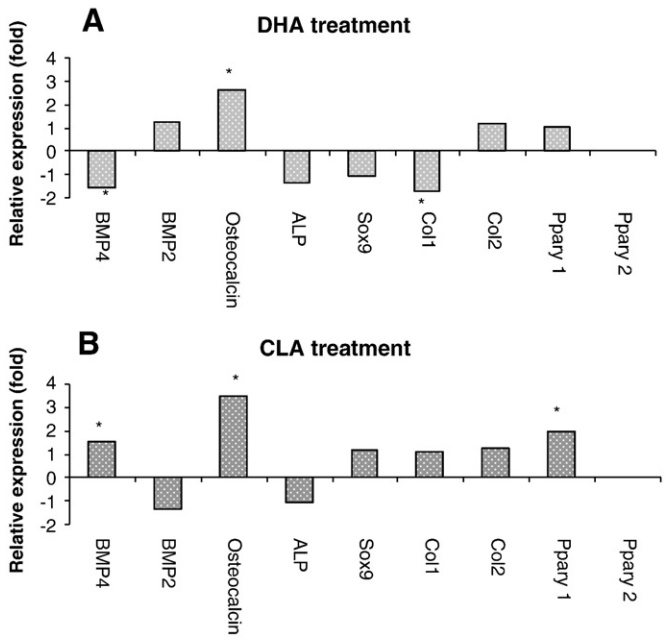

Fig. 5. Relative expression levels of *bmp4, bmp2, osteocalcin, alp, sox9, col1a1, col2a1, pparγ-short* (2) and *pparγ-long* (1) in cells cultivated in a media containing (A) DHA and (B) CLA. Values are calculated relative to cells cultivated in a media containing FBS (control). The level of expression of *osteocalcin* was higher in both treatment groups. The levels of expression of *pparγ-long* and *bmp4* were significantly up-regulated in CLA-treated cells, while the levels of *col1a1* and *bmp4* were significantly down-regulated in DHA-treated cells. Each bar is the mean of the normalized gene expression, normalised with *ef1a*. Significant up- and down-regulation (p-value ≤ 0.05) are indicated with $*n = 3$.

by 80% in cells incubated with FS and by 94% in cells incubated with a combination of FS and DHA compared to the level in cells exposed to FBS, which produced considerable amounts of PGE_2. PGE_2 has been shown to be involved in bone formation in rats *in vivo* [39,40] and to inhibit bone formation at high concentrations *in vitro* [41]. Our results agrees with results from Watkins et al. [74], who describe experiments with osteoblast-like cell lines in which the presence of n-3 PUFAs reduced PGE_2 production. Our *in vitro* findings further correlate with *in vivo* results obtained in a recent Atlantic salmon feeding study carried out by our group ([75], in press). The level of PGE_2 in blood plasma was lower in fish fed a fish oil-based diet than in fish fed a soybean oil-based diet; whereas the bone of fish fed the soybean oil diet was less mineralized. These results indicate that PGE_2 may be one of the factors involved in regulation of bone formation in Atlantic salmon. Dietary PUFAs is known to influence vertebral malformations in sea bass [42,43], but it remains to be determined whether the n-3/n-6 PUFA ratios affect bone mineral density in this species. Studies with mammals however, have shown that n-3 PUFAs have beneficial effects on bone mineral density [35,37,76]. Further, results from mammalian studies [37] have shown that bioactive fatty acids such as CLA have effects that are similar to those of n-3 PUFAs. Cusack et al. [77] showed that increasing the CLA concentration led to down-regulation of the biosynthesis of PGE_2 in human osteoblast-like cells, which agrees with our findings, where CLA reduced PGE_2 production by 35%. Addition of CLA to a diet for juvenile Atlantic salmon causes a significant increase in whole body levels of calcium and phosphorus [78], and this increase can be expected to have a positive effect on bone mineralization. Both DHA and CLA induced the expression of the *osteocalcin* gene much more strongly than FBS did. This agrees with results reported by Cusack et al. [77] and by Watkins et al. [36], who showed that incubation with CLA increases *osteocalcin* levels. Different mechanisms, however, may be operating. It is therefore possible that the addition of specific bioactive fatty acids counteracts the negative effects of a low n-3/n-6 ratio in diets for Atlantic salmon. Whereas CLA increased the level of *bmp4* transcription, DHA-treated cells surprisingly showed a down-regulated expression of *bmp4* and *col1a1*. Currently, no explanation for these results exists and the findings need to be more studied. In addition, we analyzed cells for the adipocyte marker *pparγ-short,* an isoform of *pparγ* that is induced during adipocyte differentiation in Atlantic salmon [27]. In osteoblasts treated with CLA and DHA *pparγ-short* was not present, while the expression of *pparγ-long* was slightly, but significantly, higher in osteoblasts treated with CLA. *Pparγ-long* is induced in the early phase of cultivation and repressed at later stages of differentiation [27]. The pattern of expression of this subtype in the osteoblasts suggests that the effects of PUFAs on osteoblasts are mediated by the activation of *pparγ-long*. Further work is required to confirm or refute this.

4.2. Transcriptional activity of differentiating osteoblasts affected by hyperthermia

Several studies have shown that fish that grow at elevated temperatures exhibit increased levels of vertebral deformities [44,46,79,80]. The mechanisms of this are poorly understood, but it is generally accepted that changes of transcriptional processes due to temperature in bone-forming and cartilage-forming cells are involved [44,81,82]. We have recently shown that rearing Atlantic salmon fry at an elevated temperature (16 °C) reduces the transcriptional activity of extracellular matrix genes such as *col1a1*, *osteocalcin*, *osteonectin* and *decorin* ([47], submitted), which may suggest a defect in the late maturation of osteoblasts to become bone producing cells [83–85]. In line with these findings, our long-term 16 °C heat-exposed cells showed a decreased expression of most genes, including *alp*, *col1a1* and *osteocalcin*. Surprisingly, the cells exposed to elevated temperature died after 72 h, even though the temperature to which they were exposed was within the normal temperature range of Atlantic salmon. This might be due to the method applied in this experiment. Instead of slowly raising the temperature to 16 °C so that the cells are able to adapt, the cells were exposed to a heat shock when transferred directly to 16 °C. However, the effects of high temperatures during the differentiation of osteoblasts in cell culture are controversial: some experiments in mammals have shown that heat shock has a positive effect on osteoblastogenesis [86], while other experiments have shown that heat shock leads to apoptosis and cell death [87]. Nørgaard et al. [86] suggested that a 1-h heat shock of 42.5 °C had a positive effect on mammalian cells when inducing them to differen-

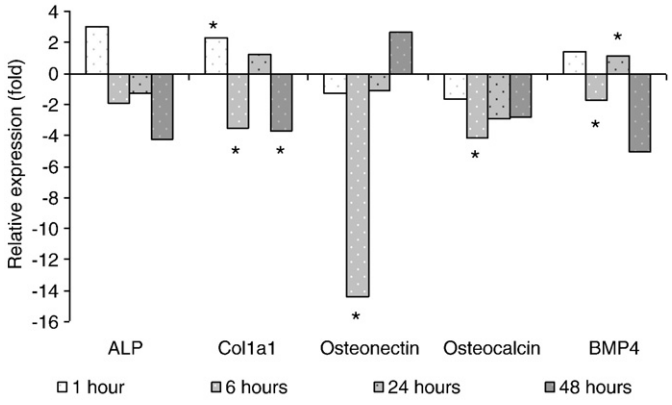

Fig. 6. Relative gene expression of *alp, col1a1, osteonectin, osteocalcin* and *bmp4* in cells incubated at 16 °C after 1 h, 6 h, 24 h and 48 h. Values are calculated relative to control cells incubated at 12 °C. The expression levels of *alp, col1a1* and *bmp4* were increased after 1 h at 16 °C. After 2 days, *osteonectin* was the only marker showing an up-regulated level of expression. Each bar is the mean of the normalized gene expression ratio, normalised with *ef1a*. Significant up-regulation and down-regulation (p-value ≤ 0.05) are indicated with $*n = 4$.

tiate into osteoblasts. Previous studies have demonstrated that exposure to mild heat shock stimulates the proliferation and osteogenic differentiation also of human mesenchymal stem cells, resulting in enhanced mineralization, whereas long-term treatment results in inhibited proliferation [88]. However, the mechanisms involved are still not known and appropriate functional studies are needed to elucidate these questions. The expressions of *alp, col1a1* and *bmp4* were up-regulated after 1 h of heat treatment in salmon muscle precursor cells and suggest that a short heat shock stimulates differentiation into osteoblasts in Atlantic salmon. This is the opposite of the effect of long-term exposure to elevated temperatures. In conclusion, our *in vitro* results suggest that Atlantic salmon osteoblasts are highly sensitive to long-term exposure to heat during the early stages of differentiation.

5. Conclusions

This is the first report that shows that unspecialised precursor cells isolated from salmon muscle tissue are able to differentiate to osteoblasts-like cells *in vitro*. These cells appear to be a suitable model system for the study of osteoblast biology *in vitro*. Their morphology has several osteoblast-like characteristics, and alkaline phosphatase is present in the cells. Furthermore, the cells express several osteoblast-related markers such as *osteocalcin, col1a1, bmp2* and *bmp4*. The production of PGE_2 decreased and the level of expression of *osteocalcin* increased when CLA was added to the growth medium of osteoblast culture and when the n-3/n-6 PUFA ratios was raised, suggesting that n-3 PUFAs and CLA have a beneficial effect on bone formation in fish. Further, 1 h heat exposure increased the expression levels of gene markers for osteoblastogenesis, while long-term heat exposure induced cell death. MSC are available at relatively high levels in fish tissue at early life stages. These primary stem cells provide a valuable tool compared to cell culture of differentiated osteoblast, since they still possess the ability to differentiate into osteoblasts, myotubes, chondrocytes and adipocytes. The plasticity of these cells in culture will be of great value in future studies of for instance the influence of nutritional factors influencing osteogenic lineage determination.

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