Molecular pathology of temperature-induced vertebral deformities

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Introduction

In cold water aquaculture, an apparently efficient way of increasing rate of growth and development is to apply elevated temperatures. Unfortunately, in Atlantic salmon there is a clear relation between high temperatures in juvenile stages and the increased frequency of skeletal deformities. However, we still do not know fully why deformities develop, and the molecular pathways involved are still far from understood.

In order to elaborate the mechanisms involved in temperature-induced deformities, we designed an experiment where Atlantic salmon was exposed to two different temperature regimes, designated high and low temperature group. Our first aim was to analyse and compare normal (non-deformed) spinal columns from a high and low intensive temperature regime in order to see if there were any underlying differences between the two groups associated with higher risk of developing deformities. The second goal was to describe the fusion process in detail to get more knowledge of the underlying mechanisms involved.

Materials and methods

Fish from the high intensive and low intensive temperature groups were subjected to radiographic screening. Spinal columns of interest were sampled at two developmental stages (2 and 15g) and studied by a variety of histological and gene expression analytical techniques (Fig. 1).

Real time RT-PCR analyses and *in situ* hybridization (*ISH*) are two methods used to quantify and locate gene expression, respectively (Fig. 2).

Thus, these analyses give the ability to quantify and locate the level at which a particular gene is expressed within a cell, tissue or an organism.

Gene **expression** is the process by which information from a gene is used to make functional gene products (proteins). Gene **regulation** gives the cell control over which proteins that are to be made, hence structure, function, differentiation and the ability to adapt to stimuli. To find genes of interest we analysed pathways involved in bone and cartilage development that are known from mammalian studies. For example, the mesenchymal cells (MSC) are stem cells that can differentiate into osteoblasts (bone forming cells), chondrocytes (cartilage forming cells), adipocytes (fat cells) and myotubes (muscle cells). What kind of cell type they will turn into is decided by a number of regulatory proteins, e.g. the transcription factors.

Some of the key transcription factors in bone metabolism include Runx2 and Osterix, which are involved in differentiation and maturation of osteoblasts that express bone matrix (collagen1a) and matrix mineralising (osteocalcin and osteonectin) genes.

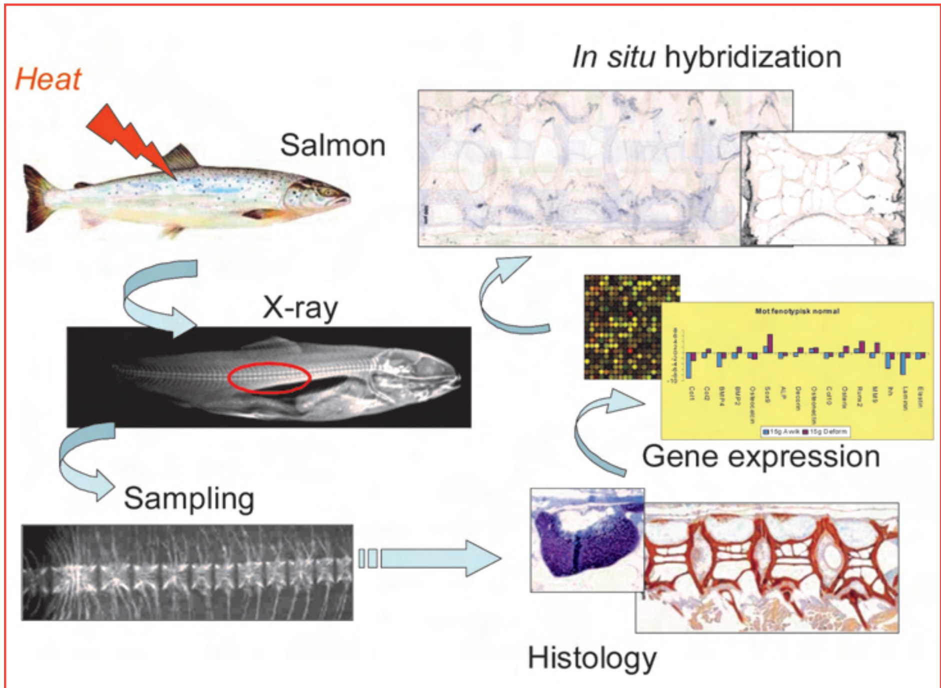

Figure1. Overview of the experimental pipeline used to study bone development and vertebral deformities in Atlantic salmon (*Salmo salar*). Fish was exposed to a high and low intensive temperature regime from fertilisation till 20g. During the experiment, fish were sedated and radiographed at 2g, 15g and 60g. From the live-radiography, normal, aberrant and deformed spinal columns were sampled and used for histological analysis, real time RT PCR and *in situ* hybridisation. This methodological approach has given new insight into the underlying mechanism of vertebral deformities in vertebrates.

Mineralisation is the process where minerals are incorporated into the bone matrix, hence hardening of the tissue. In comparison, early chondrocyte differentiation is controlled by sox9, which regulates transcription of collagen2a, the major extracellular matrix (ECM) component of cartilage. Further, mef2c assures that chondrocytes further mature into collagen10a producing hypertrophic cells.

Both mineralised bone and cartilage is remodeled through the activity of osteoclasts (bone resorbing cells). These cells provide an acidic environment, through the expression of a number of proteins (cathepsins, TRAP (Tartrate Resistant Acid Phosphatase) and Mmps (Matrix metalloproteinases)), where mineralized matrix may be broken down. By analysing pathways like these, we ended up with a “molecular toolbox” containing 22 of the most important genes involved in skeletogenesis and a number of histological staining methods, including immunostaining of specific proteins.

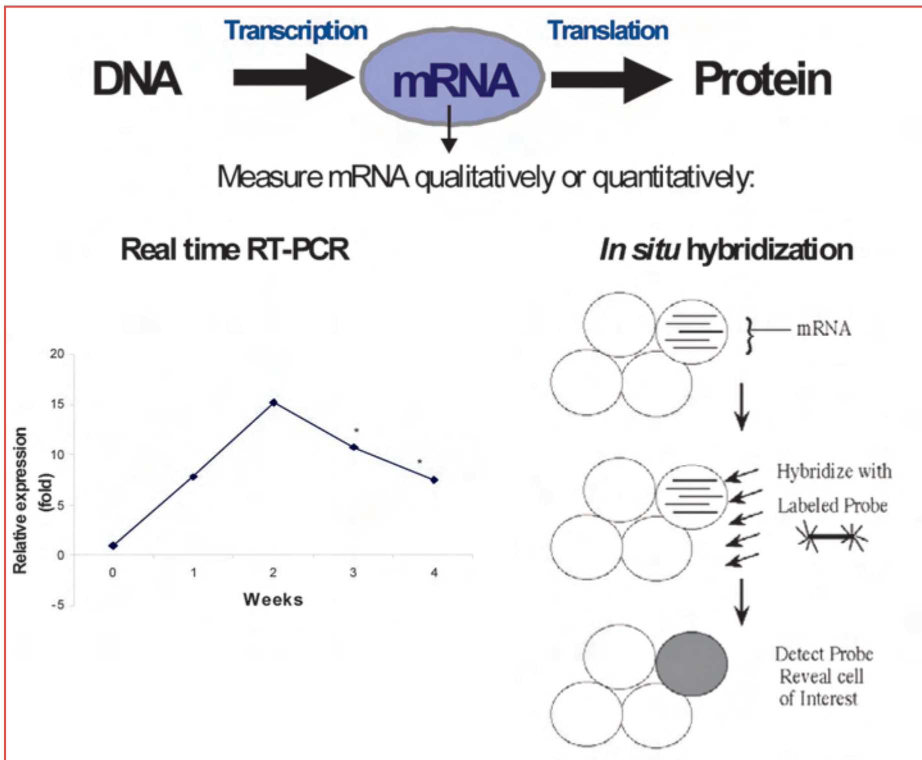

Figure 2. Genes from a DNA template are transcribed into mRNA, carrying the coding information that can be translated into proteins. Two methods used to measure mRNA transcription are real time RT-PCR and *in situ* hybridisation. Real time RT-PCR is used to quantify a target mRNA, whereas *in situ* hybridisation use labeled probes to localize the mRNA of interest in a tissue or a section of the tissue.

Results

Radiological comparison of non-deformed vertebral columns indicated that Atlantic salmon juveniles from the low intensive temperature group displayed denser and more regular vertebral bodies throughout the vertebral column than those from the high intensive temperature group. Correspondingly, a significant higher number of deformities were found in the high intensive temperature group. The number of deformities increased with time, at 2g size approximately 10% showed deformed spinal columns, at 60g size almost 30%.

Most deformities were of the fusion type. As expected, the fish reared at a high temperature regime grew much faster than those reared at low temperature, e.g. fish reared at high temperatures reached 2g in 6 weeks compared to 11 weeks at low temperature and 60g in 7 months compared to 10 months.

Molecular analysis of non-deformed vertebrae revealed that most genes were expressed differently in the high temperature group compared to the low temperature group.

The expression of genes involved in matrix production and mineralisation showed significantly less activity (down-regulation) in the high temperature group at both 2g and 15g. Expression of *col1a1, col2a1, osteocalcin, decorin*, and *osteonectin* were reduced in all individuals from the high temperature group. In addition, *ISH* with these genes showed a more restricted area of expression. The down-regulation of the genes encoding structural proteins taking part in the bone matrix supported the tendency of weaker radiodensity in the high temperature group and indicated that fast growth gives lower mineralisation of the vertebral tissue. Through *ISH* we also identified expressional similarities of the ECM components in Atlantic salmon to other vertebrates.

It is apparent that most of the factors and pathways that control bone formation are highly conserved in vertebrates. During optimal conditions, chondrocytes in the areas connecting the arches to the spinal column are lined up in three distinct and well organised bands. Detailed examination of these areas in fish from the high temperature group showed that the chondrocytes had a more distorted pattern. We also observed an increased expression of genes involved in the final maturation of chondrocytes, *mef2c* and *collagen10a*. Also from *ISH*, we observed an expanded area of *collagen10a*, indicating an increased area of hypertrophic chondrocytes in the cartilage. Chondrocyte hypertrophy is the final stage prior to endochondral ossification, a process where cartilage is replaced by bone. In addition, we did not find positive TRAP activity and real time expression data showed that *mmp9* and *mmp13* were both down-regulated. TRAP and Mmps are needed in bone remodeling by the osteoclasts to bring about the final steps in endochondral ossification. Our findings strongly indicates that temperature induced fast growth is severely affecting gene expression in osteoblasts and chondrocytes; hence change in the vertebral tissue structure and composition. The findings further indicated that bone in salmon developed at high temperature have a “softer” bone phenotype, and the higher percentage of deformities in this group may be linked to a reduced resistance to withstand mechanical pressure from the high muscle mass in farmed salmon.

To further understand the mechanisms involved in the development of vertebral deformities, we established a model for studying the pathogenesis of vertebral fusions in Atlantic salmon by using live radiography to identify fish at an intermediate and terminal stage of the fusion process, respectively. We confirmed by molecular tools that the calcification of the heterotrophic intervertebral cartilage and its subsequent remodeling into bone facilitates the fusion of vertebral bodies. Analyzing deformed spinal columns by *ISH* revealed an increased amount of cells expressing mixed signals of genes involved in both osteoblasts and chondrocytes, supporting the hypothesis that formation of chondroid bone (resembling both bone and cartilage) is facilitated during fast growth and pathological conditions.

This ectopic bone formation appears to be the basic mechanism in development of vertebral fusions. V*imentin*, producing a protein that makes chondrocytes more resistant to withstand mechanical pressure, was also down-regulated during the fusion process. In addition to having a "soft" bone phenotype, this low vimentin transcription may further reduce the ability to withstand mechanical forces during fast growth. Of the 22 genes we analyzed, structural genes (*osteocalcin, decorin, vimentin* and the collagens) and genes involved in differentiation *(pdgfrb, bmps* and the *hh*) were down-regulated in deformed spinal columns compared to non-deformed. Further, deformed spinal columns were characterized by an increased cell proliferation simultaneous with an increase in cell death, results reflecting a malignant potential of spinal fusions. The mechanisms behind these processes have not been described in detail.

Cells in culture:
A potential replacement for *in vivo* experiments

To study gene function, it is an advantage to isolate the systems of interest and thereby have a more simplified model to work with. Thus, we have started developing an osteoblast cell culture for Atlantic salmon. This cell culture system has subsequently been used to study the effects of factors that influence bone formation in Atlantic salmon. Studies have shown that differentiating osteoblasts are highly sensitive to increased temperature at early stages of differentiation, results that support our *in vivo* observations in the spinal column. In line with our findings above, a long-term heat exposure to the cells resulted in decreased expression of *alp*, *col1a1* and *osteocalcin*, indicating that the model system is suitable for study osteoblast biology.

Conclusion

Cultured Atlantic salmon is bred for rapid growth, and the industry will aim to obtain the best growth rate by the optimisation of inputs (e.g. diets) and environmental factors accordingly. Therefore, it is important to understand the molecular and cellular events in bone development in salmon, in order to deal with any problems with skeletal development that arise as a result of intensive rearing conditions.

By combining molecular tools with targeted radiography based sampling and histology, we are now able to describe a more complete picture of how spinal deformities in Atlantic salmon develop. 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 reliable correlation between defined skeletal markers and the risk of developing vertebral deformities found in our temperature experiments indicates that these genes can be developed as prognostic markers. Further, our skeletal tool-box can be used to investigate how the progression of skeletogenesis is modulated in response to other stimuli.