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Mechanisms of cartilage subdivision in the pectoral fin of teleosts

by

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1. General introduction

This introduction presents the current scientific understanding of cartilage development and how this eventually results in the formation of the adult endoskeleton. The first step in the formation of the adult endoskeleton is the appearance of chondrogenic cell condensations in a specific pattern. This initial template of cartilage condensations differentiates into cartilaginous elements, forming an embryonic skeleton. Although this embryonic skeleton already shows many features of the adult morphology, such as the positions, the number and the articulations of many of its elements, many other features of the adult skeleton only form through remodeling of the embryonic cartilages. The remodeling of the embryonic cartilages is a second crucial step that includes element growth, fusion, regression and, in the case of zebrafish, subdivision of elements. Together with the formation of the dermal skeleton, these modifications to the embryonic cartilages result in the adult skeletal morphology.

This doctoral thesis aims to understand the mechanisms that underlie the formation of cartilage subdivisions in the zebrafish skeleton. During cartilage subdivision, a specific region of a preexisting cartilage disappears, thereby subdividing a single cartilage into two separate elements. Although there are indications that cartilage subdivisions occur in various anatomical regions in zebrafish, I mainly focused my efforts on the characteristic subdivisions of the pectoral skeleton. In no anatomical region are cartilage subdivisions as obvious as in the pectoral skeleton, making it an ideal system to study the developmental events underlying this process.

1.1. Cartilage in Osteichthyes: form and function

The skeleton of all Osteichthyes or bony fish, including land dwelling tetrapods, is composed of three major types of elements: cartilages, endochondral and dermal bones (For a phylogenetic overview of the major osteichthyan taxa see Figure 1). While dermal bones develop directly from mesenchymal precursor cells, endochondral bone forms from a preexisting cartilage element that serves as a scaffold structure on which bone matrix is laid down. This requirement for cartilage as a precursor for endochondral bone explains why cartilage is the most common type of skeletal tissue in embryos, while being the rarest type in the adult skeleton of most osteichthyans. In adult Osteichthyes, cartilage tissue is usually restricted to the surfaces of articulating joints, regions of endochondral bone growth and some persistent cartilages that do not ossify.

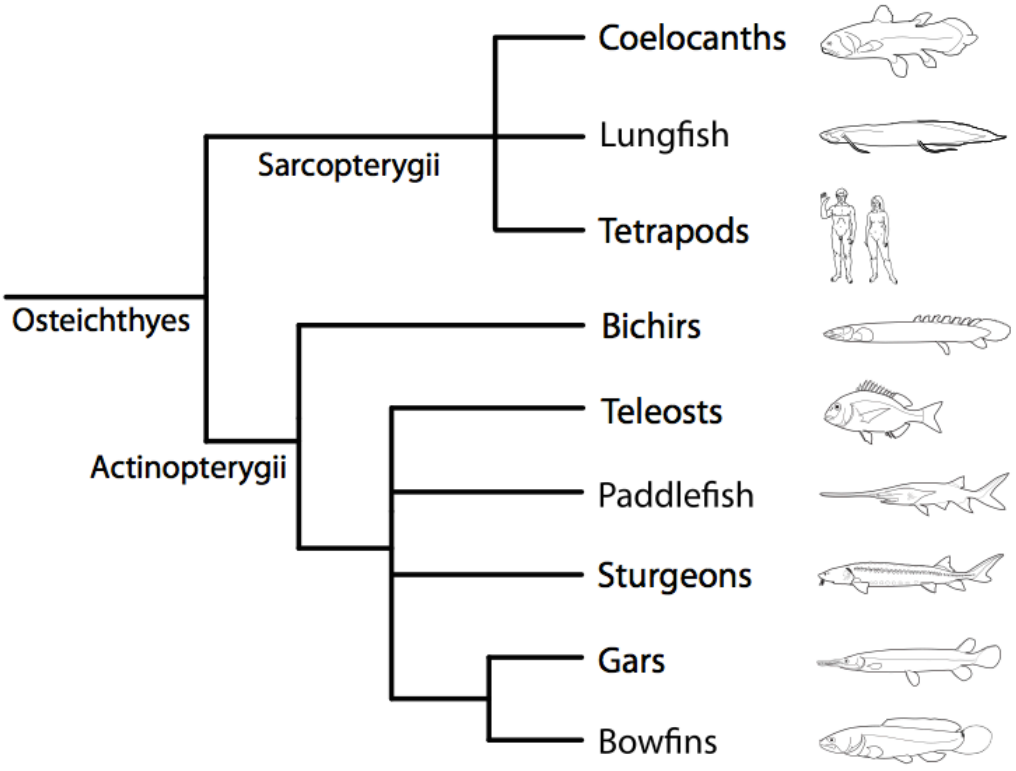

Figure 1
Consensus view of the phylogeny of extant Osteichthyes, inferred from phylogenetic analyses of molecular and morphological data.
Figure adapted from Suzuki et al. (2010)

In tetrapods, three major different types of cartilage can be distinguished at the histological level (Boyde and Jones, 1983). Fibrocartilage is found in some tendons, containing highly fibrous connective tissue, which is interspaced by elongated groups of cartilaginous cells, running parallel to the tendons' fibers (Benjamin et al., 2000). Unlike fibrocartilage, which appears as a blend between regular connective tissue and cartilaginous tissue, elastic cartilage only contains cartilaginous cells. However, the matrix of this often permanent type of cartilage contains various quantities of elastic fibers, absent in other types of cartilage tissue. In humans and many other mammals this type of cartilage can often be identified in the cartilages of the nose and ears. Hyaline cartilage is, however, the most common type of cartilage and forms all endochondral bones and the articulating surfaces of joints.

While these three distinct types can be distinguished in tetrapods, cartilage in actinopterygians can range through the entire spectrum of connective tissue types, containing various degrees of tensile or elastic fibrous and even adipose tissue (Witten et al., 2010). However, just as in tetrapods, hyaline cartilage is the most commonly observed. This introduction will therefore focus on the form, function and development of hyaline cartilage. The histology and the ultrastructure of this type of cartilage are well known (Boyde and Jones, 1983; Sheldon, 1983; Horton, 1993) and shown to be similar between tetrapods and actinopterygians (Huysseune and Sire, 1992; Huysseune, 2000; Witten and Huysseune, 2007). Hyaline cartilage tissue always consists of three major components: the chondrocytes, the extracellular matrix (ECM) and the perichondrium.

1.1.1. Chondrocytes

Mature chondrocytes are generally large, round cells with scalloped borders (Figure 2). Their main function is the production of the cartilage ECM. Their ultrastructure clearly shows this secretory function: the cell contains an elaborate rough endoplasmic reticulum (RER), large Golgi complexes and high numbers of cytoplasmic vesicles. The nucleo-cytoplasmic ratio of these cells is generally small, due to their large cytoplasmic region. Chondrocytes

are completely embedded in their matrix. Their cell bodies occupy matrix-free niches, called lacunae. Apart from blood, cartilage is the only connective tissue where cells show no cell-to-cell contacts, while other types of connective tissue actually require cell-to-cell contacts for proper tissue differentiation and homeostasis (Donahue et al., 2000; Jung et al., 2005; Yeung et al., 2005). Since the cartilage ECM encloses the chondrocytes, proliferation of these cells remains visible in the architecture of the matrix. Daughter cells are trapped in the original lacuna of the mother cell and recommence the production of ECM, creating a new matrix wall that separates them within their mothers' lacuna. This new matrix is often called the territorial matrix, as opposed to the inter-territorial matrix, situated between the individual mother cells.

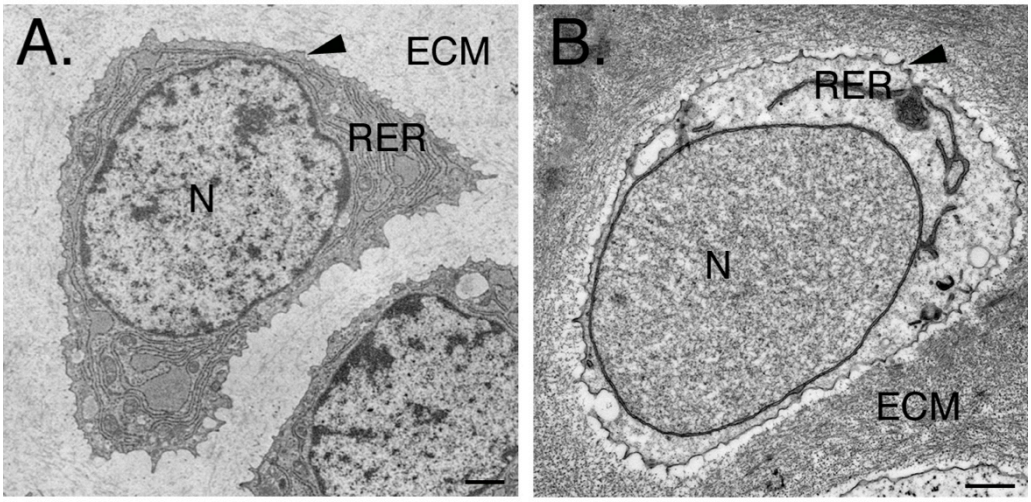

Figure 2
Transmission electron micrograph of a murine (A) and a zebrafish chondrocyte (B). (A) was adapted from Huntington (1983). Black arrowheads indicate the scalloped chondrocyte borders. Abbreviations: ECM: extracellular matrix, N: nucleus, RER: rough endoplasmic reticulum.
Scale bars = 1 μm

1.1.2. ECM

The ECM gives cartilage its supportive function. The ECM contains highly sulfated glycosaminoglycans (GAGs) (Gavaia et al., 2006), such as heparan-sulfate, keratan-sulfate, chondroitin-sulfate and dermatan-sulfate (Lash and Vasan, 1983). A core protein links large numbers of these GAGs,

forming an aggrecan proteoglycan. Large numbers of these proteoglycan complexes are again linked to a hyaluronic acid backbone, forming proteoglycan aggregates with very high molecular weight (Nap and Szleifer, 2008) (Figure 3). The negative charge created by the aggregation of a high number of sulfate groups in these large proteoglycan complexes gives the cartilage matrix the ability to retain water molecules in the matrix. These sulfate groups are also selectively stainable with alcian blue in acidic conditions, which is the most commonly used cartilage dye.

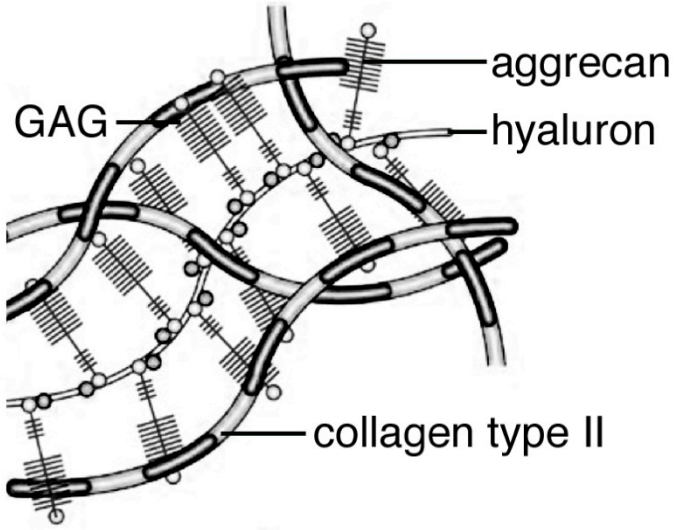

Figure 3
Schematic representation of the major sturctural components of cartilage extracellular matrix, adapted from Chen et al. (2006).

In addition to GAGs, the cartilage ECM contains a variety of structural proteins, collagens being the most important ones. Cartilage ECM comprises many types of collagens, but type II collagen is by far the most common. Collagen type II fibrils have a diameter of approximately 10-20 nm and form a fine structural meshwork. Like all fibrillar collagen, this cartilage collagen consists of triple-helix chains, called tropocollagen, organized in staggered arrangement. There are several types of α chains, which can be combined to form the triple-helix of fibrillar collagens, but, in collagen type II, three type 2 alpha1 chains (COL2A1) form the triple-helix building block (Mayne and von der Mark, 1983). The structural meshwork of collagen type II provides anchorage for the cells and other ECM components, such as the large proteoglycan complexes. The hydrostatic pressure resulting from the strong attraction of water by these proteoglycans counteracts the tensile strength of the collagen meshwork. This combination of hydrostatic pressure and tensile

strength gives cartilage its unique resistance to mechanical loading (Myers and Mow, 1983).

A large assemblage of proteins mediates the linkage between the collagen meshwork, the GAGs and the cells. These proteins thereby form the last important group of ECM components. Aggrecan, which was discussed above, is one example of such protein with an important linking function. Another well studied and versatile linker protein is fibronectin. This glycoprotein possesses attachment sites for collagens, GAGs and cell-surface receptors (Pankov and Yamada, 2002) and is involved in cross-linking various ECM components and anchoring chondrocytes to their ECM.

1.1.3. Perichondrium

The perichondrium is the tissue that surrounds a cartilage element. It can show a variety of forms and functions, ranging from a thin monolayer of flattened fibroblastic cells to a multilayered band of fibrous connective tissue. The fibroblastic cells of the perichondrium are often flattened tile-like cells. They have long cytoplasmic extensions that show abundant cell-to-cell contacts. Their nucleo-cytoplasmic ratio is high because of their small cytoplasmic region. It is also important to note that, in cartilaginous tissue, blood and nerve supply is always restricted to the perichondrium. Yet, in large cartilage elements, blood vessels and nerves may run through the element in canals, which are always lined with connective tissue continuous with the perichondrium (Blumer et al., 2005).

1.1.4. Growth and ossification

Cartilaginous elements can grow through several mechanisms. The first option is appositional growth, which is realized by perichondral cells added to the element along its outer borders. Once the production of cartilage matrix starts, the perichondral cells, now termed chondroblasts, gradually retract their cytoplasmic extensions. They lose their cell-to-cell contacts, thereby gradually transforming from fibroblast-like cells into chondrocytes that are incorporated in the element (Hinchliffe and Griffiths, 1983). Different

regions of the perichondrium can contribute chondroblasts at various rates, which can result in drastic alterations in the shape of the element (Thorogood, 1983). Active perichondral regions are always multilayered, with the innermost cells transforming into chondroblasts, while the outer regions remain fibroblastic connective tissue (Hinchliffe and Griffiths, 1983).

Secondly, cartilage elements can grow through interstitial growth, which, in contrast to appositional growth, is mediated by the chondrocytes of the element. Chondrocytes can influence growth by (1) increasing the ECM production, (2) by proliferation and (3) finally by increasing their cell size (Hinchliffe and Griffiths, 1983). Mature chondrocytes can differentiate further in order to become very large hypertrophic chondrocytes. Conventional tissue fixation techniques often prove inadequate to preserve these cells for histological examination, which long fueled the debate on the viability and function of these cells. However, alternative fixation methods have shown that most hypertrophic cells are certainly viable (Oi and Utsumi, 1980; Hunziker et al., 1984). Hypertrophic chondrocytes show an even lower nucleo-cytoplasmic ratio than mature chondrocytes and typically possess large intracellular vacuoles. The ECM of hypertrophic chondrocytes is also different from the ECM of hyaline cartilage. It contains less collagen type II but instead comprises large amounts of collagen type X (Schmid et al., 1990; Mizoguchi et al., 1997; Clement et al., 2008). Terminally differentiated hypertrophic chondrocytes even express osteoblast-related genes, such as *runt-related gene 2* and *osteocalcin*, which regulate the calcification of the ECM, starting in this region (Inada et al., 1999; Yagami et al., 1999; Simes et al., 2003; Gavaia et al., 2006; Hammond and Schulte-Merker, 2009).

In addition to their contribution to interstitial growth, hypertrophic chondrocytes are involved in the ossification of cartilage elements. In all Osteichthyes, the first sign of ossification is found in the perichondrium, which covers calcified hypertrophic cartilage. The cells in this region of the perichondrium differentiate into osteoblasts and start the production of the bone matrix. Once this process has started the perichondrium covers bone and is therefore termed periosteum (Bast, 1944). However, many skeletal

elements in Osteichthyes continue to grow after the first appearance of cartilage hypertrophy and perichondral bone (Hinchliffe and Griffiths, 1983). This growth is primarily realized by both appositional and interstitial cartilage growth in regions still free of perichondral bone.

This is most apparent in the long bones of tetrapods (Hinchliffe and Griffiths, 1983), where the perichondral bone collar forms in the center of the rod-like cartilaginous precursor element, a region commonly referred to as the bone shaft or diaphysis. The distal ends of the element, called the epiphysis, initially do not hypertrophy and remain free of perichondral bone. In mammals and birds secondary ossification centers appear in the epiphysis, which will eventually replace all epiphyseal cartilage by bone, thereby stopping long bone growth. The epiphyseal cartilage grows through the addition of chondroblasts at its outer periphery, which will produce new cartilage matrix. However, long bone growth is primarily accomplished by the epiphyseal chondrocytes nearest to the bone shaft. Here the mature chondrocytes of the hyaline epiphysis become highly proliferative and start a series of cell divisions. All resulting daughter cells organize their flattened cell bodies in columnar stacks parallel to the long axis of the bone. These flattened cells will again differentiate into chondrocytes, thus increasing their size and contributing to matrix production without disturbing the stacked organization. Once these chondrocytes differentiate into hypertrophic chondrocytes, their matrix will calcify and the perichondral bone collar will expand over this region, converting it into bone shaft. The combined effect of the directional cell divisions, matrix production and the enlargement of the stacked cells by their differentiation into hypertrophic chondrocytes is the major mechanism behind long bone growth. Therefore, the area that reaches from the proliferating to the hypertrophic chondrocytes is often referred to as the growth disk or metaphysis.

Although the zonation of proliferating and differentiating chondrocytes is most apparent in the metaphysis of long bones, very similar growth regions can be identified surrounding the first ossification centers in differently shaped elements. In general, all tetrapod bones that form from a cartilaginous

precursor rely mainly on appositional and interstitial cartilage growth to reach their mature size and form. This importance of appositional and interstitial growth is also apparent in the actinopterygian skeleton. In several teleost species, all the aspects involved in tetrapod bone growth have been identified, including growth centers that resemble long bone metaphyses (Haines, 1934; Haines, 1939; Haines, 1942). However, despite the apparent similarities in the mechanisms of early ossification and growth, one aspect of actinopterygian cartilage ossification shows considerable deviation from that in tetrapods.

The most important difference between endochondral ossification in tetrapods and actinopterygians can be found in the fate of the calcified, hypertrophic cartilage underneath the perichondral bone. In tetrapods, this hypertrophic cartilage is known to release a wide range of digestive enzymes capable of degrading many structural ECM proteins. In addition diffusible factors are released, such as Vascular Endothelial Growth Factor (VEGF), promoting the invasion of the hypertrophic cartilage by perichondrium derived tissue (Roach, 1999; Colnot and Helms, 2001; Nagai and Aoki, 2002). Osteo- or chondroclast cells lead this invasion, followed by periosteum-derived connective tissue, blood vessels and nerves. The walls of the invasive channels will immediately be used as scaffolds to lay down bone. Cavities form in the central region of the element, where bone resorption occurs faster than the production of new bone. In tetrapods, these cavities become occupied by hematopoietic tissue. Later in life the connective tissue in the bone cavities of most bones will be replaced by adipose tissue (Currey, 2003). As the entire cartilage element gradually becomes hypertrophic, all cartilage is eventually replaced by bone and other connective tissue, except for the articular surface, which remains hyaline cartilage. Because of this complete replacement of cartilage, such bones are also called endochondral bones. The final fate of hypertrophic chondrocytes has been a controversial subject in the field, partially because these cells are hard to preserve for histology, but it is safe to conclude that many hypertrophic chondrocytes undergo programmed cell death or apoptosis (Bronckers et al., 2000; Holmbeck et al., 2003) and are subsequently phagocytized by the invading chondroclasts

(Bronckers et al., 2000). However, evidence exists that some of these cells find a suitable microenvironment that allows their transdifferentiation into osteoblastic cells (Roach et al., 1995; Bianco et al., 1998; Holmbeck et al., 2003).

In contrast to the swift and complete invasion and replacement of hypertrophic cartilage in tetrapods, many actinopterygian bones show a preserved cartilaginous core. Therefore, these bones are often referred to as perichondral bones. Although invasion of hypertrophic cartilage by chondroclasts does occur in actinopterygians, the replacement of the hypertrophic cartilage is often incomplete. The created digestive canals are also filled with adipose tissue, without or with a very limited amount of bone deposited onto the canal walls (Witten and Huysseune, 2007; Witten and Huysseune, 2009; Witten et al., 2010).

1.2. Cartilage development and its regulation

Most knowledge on cartilage development, differentiation and homeostasis is restricted to tetrapod models. However, zebrafish has recently gained importance in the field of cartilage biology, with various studies shedding new light on cartilage patterning, homeostasis and pathology (Kimmel et al., 1998; Crump et al., 2004; Clement et al., 2008; Carney et al., 2010; Eames et al., 2011). The large number of available mutants and the large array of research techniques will undoubtedly ensure the further use of zebrafish in the future. However, additional comparative studies between zebrafish and tetrapod chondrogenesis will certainly be required in order to establish zebrafish fully as a reliable model system to study chondrogenesis. In this section, I will summarize our current understanding of chondrogenesis, mostly obtained from tetrapod models. I will nevertheless highlight which aspects of chondrogenesis are similar to what is found during the development of zebrafish cartilages.

1.2.1. Distinct steps in cartilage development of tetrapods and teleosts

The availability of a sufficiently large population of precursor cells is a requirement for the development of cartilage. Chondrogenic precursor cells can either migrate towards or proliferate within the area of the future chondrogenesis. The embryonic origin of the cartilage precursors depends on the anatomic region in which the skeletal element forms. Elements from the splanchnocranium, such as the mandibular and hyoid arch in tetrapods (Santagati and Rijli, 2003) and the additional five branchial arches in actinopterygians (Knight and Schilling, 2006) are derived from migratory neural crest. Elements from the paired appendages on the other hand originate from local proliferation of lateral plate mesoderm (Mercader, 2007).

Despite these different origins of cartilage tissue, the principal events during chondrogenesis are always very similar. The first major step in cartilage development is the aggregation of the precursor cells into a cell condensation. These regions of densely packed cells can be easily

distinguished from the surrounding loosely packed mesenchyme. In the literature, a cell condensation can also be termed a blastema or primordium (Thorogood, 1983). The specific pattern in which different cell condensations form, combined with the internal organization of the condensed cells, will already predetermine many features of the adult skeleton (Hall and Miyake, 1992). The future position and number of cartilage elements is laid down by the pattern in which the cell condensations form (Hall and Miyake, 1992; Hall and Miyake, 1995; Hall and Miyake, 2000). The configuration of the polarized precursor cells within a condensation, on the other hand, predetermines important morphogenetic factors such as the directionality of cell proliferation and organized matrix secretion, thereby influencing the future shape and size of the elements (Thorogood, 1983; Huysseune and Sire, 1992; Grandel and Schulte-Merker, 1998; Kimmel et al., 1998).

The first important phase in chondrogenesis ends when the cells in the condensation center differentiate into chondrocytes, while the condensation periphery develops into the perichondrium. As the chondrogenic condensations differentiate, the embryonic cartilaginous skeleton is formed. This embryonic skeleton still has to go through several changes in order to form the adult skeleton, many of which will be discussed in section 3. In general, the embryonic cartilage will grow and ossify while joints and muscle attachments mature, thereby forming the articulated adult skeleton.

1.2.2. Developmental regulation during chondrogenesis

The establishment of cartilage condensations is the first crucial step in chondrogenesis. However, proper differentiation and growth of both chondrocytes and perichondrium are equally important for normal skeletogenesis. In general, proper chondrogenesis depends on a combination of processes, such as the secreted signaling molecules, the regulation of cell-cell adhesion and various cell-matrix interactions. All these processes are intertwined through numerous intracellular pathways, which are still far from completely understood (DeLise et al., 2000; Hall and Miyake, 2000).

Transcription factors

The group of SOX transcription factors is vital for a wide range of developmental processes and comprises key regulators of chondrogenesis. SOX9, SOX8, SOX6 and L-SOX5 are all involved in chondrogenesis and all these transcription factors, except for SOX8, directly regulate genes for cartilage ECM components, such as collagen type II and aggrecan (Ng et al., 1997; Lefebvre et al., 1998; Bell et al., 2000; de Crombrugghe et al., 2000; Sekiya et al., 2000). From these transcription factors SOX9 is located the most upstream in the chondrogenic pathway and the absence or ectopic expression of this factor results in either the complete loss of cartilage (Bi et al., 1999; Akiyama et al., 2002) or the formation of an ectopic element, respectively (Healy et al., 1999). The important role of SOX9 is a conserved feature in chondrogenesis. In zebrafish, this factor is represented by two paralogous genes, *sox9a* and *sox9b*. These paralogs are differently expressed in different elements of the zebrafish endoskeleton. Both paralogs appear to be absolutely required for chondrogenesis to occur in the elements where they are respectively expressed (Yan et al., 2005).

While *Sox 9* is vital for initial cartilage differentiation, its expression persists but is reduced in mature and resting hyaline cartilage of mouse epiphysis, where the synthesis of new collage type II is rather low (Davies et al., 2002). In addition to the lower levels of *Sox9*, the down-regulation of genes for matrix components in resting epiphyseal chondrocytes is also mediated by other transcription factors, such as, activating protein 2 (AP-2) and Zinc finger E-box-binding homeobox 1 **(**ZEB 1), which are expressed in this tissue and are known to reduce *collagen type II* expression (Davies et al., 2002). In proliferative chondrocytes of the growth disk on the other hand, both *Sox9* and *collagen type II* are again strongly up-regulated to accommodate the differentiation and increased matrix production of the proliferative cells (Davies et al., 2002).

In mice, the differentiation towards hypertrophic chondrocytes requires the activation of core binding factor α1 (CBFA1), also known as runt-related gene 2 (RUNX2) (Ueta et al., 2001), which is also an essential transcription

factor in osteoblast differentiation and the formation of dermal bones (Ducy et al., 1999). The important role of this transcription factor in the regulation of chondrocyte hypertrophy and thus endochondral bone formation appears to be a highly conserved feature in the development of endochondral bones, since chicken *RUNX2* (Eames et al., 2004) and zebrafish *runx2* (Eames et al., 2011) have been identified in similar settings.

Secreted signaling molecules

Fibroblast Growth Factors (FGFs)

In tetrapod limbs, FGFs, secreted at the apical ectodermal ridge (AER) of the limb bud, keep the embryonic mesenchyme in an undifferentiated and proliferative state (Niswander et al., 1993; Fallon et al., 1994; Vogel et al., 1996; Fischer et al., 2003; Nomura et al., 2006). In doing so, they inhibit the initial formation of mesenchymal condensations and subsequent chondrogenesis. However, this allows for the formation of a sufficiently large population of skeletal precursor cells. In later stages of chondrogenesis, after the initial acquisition of the cartilage phenotype, various FGF family members appear to be important regulators of chondrocyte proliferation. For example in mice, *Fgf1* is exclusively expressed in epiphyseal resting chondrocytes, while *Fgf3* expression matches the region of the proliferative chondrocytes (Koziel et al., 2005).

In addition, three FGF receptors (*Fgfr*) are differentially expressed throughout chicken and mouse chondrogenesis. Both in chicken and in mice, *Fgfr1* is expressed in the entire limb bud mesenchyme and is down-regulated in the condensation centers (Szebenyi et al., 1995; Li et al., 2005). In chicken, *FGFR2* is found during cartilage condensation, while only *FGFR3* is observed in differentiated cartilage (Szebenyi et al., 1995). In mice, *Fgfr2* is equally expressed in chondrogenic condensations, but early-differentiated and hyaline cartilage show no *Fgfr* expression. In hypertrophic cartilages, however, all three *Fgfr* genes are expressed (Hellingman et al., 2010). The consequences of the differential expression patterns of these receptors are not completely

understood, but it is safe to assume that they mediate different responses to FGFs in various stages of cartilage development (Hellingman et al., 2010).

In zebrafish, FGFs are equally important for proper fin bud outgrowth (Fischer et al., 2003; Norton et al., 2005). However, the involvement of FGFs in zebrafish cartilage differentiation has not been intensively studied. Nevertheless, some indications exist for FGF function during cartilage differentiation, such as the expression of *fgfr2* in cartilage condensations at 48 hours post-fertilization (Tonou-Fujimori et al., 2002).

Transforming growth Factor Beta (TGFβ) superfamily

Various isoforms of TGFβ (Merino et al., 1998; Chimal-Monroy and Diaz de Leon, 1999), but also activin, another member of the TGFβ superfamily (Merino et al., 1999b), have been shown to counteract the inhibitory effects of FGFs on early limb mesenchyme differentiation, thereby allowing condensations to form. *In vitro*, TGFβ signaling causes the production of more TGFβ, a basic auto-regulatory mechanism that creates a positive feedback loop once TGFβ signaling is activated. In the chicken autopodium both TGFβ2 and activin cause the up-regulation of Bone Morphogenetic Protein Receptor 1b (*BMPR1b*). This in turn allows cartilage condensations to respond to various BMP signaling molecules, such as *BMP2*, *4* and *7*, all of which are members of the TGFβ superfamily and are expressed in the limb/fin bud mesenchyme. The binding of BMP to its receptor leads to the assembly of a heterotetrameric complex of BMPRs, consisting of two type I and two type II receptors (Moustakas and Heldin, 2002). Several type I receptors and type II receptors have been identified in mammalian tissue and differential expression of these receptors mediates different responses to BMP signaling (Chen et al., 2004a). While the presence of *BMPR1b* expression results in chondrogenesis and digit formation in the chicken autopodium, the interdigital areas, which receive similar BMP and FGFs signals in absence of TGFβ signaling, undergo cell apoptosis (Merino et al., 1998; Merino et al., 1999b). Also in mouse limbs, *Bmpr1b* expression is essential for cartilage differentiation (Yi et al., 2000).

While BMPs may not be directly responsible for the initiation of chondrogenic condensations, they are vital in condensation growth (Duprez et al., 1996) and maturation (Enomoto-Iwamoto et al., 1998). Despite the different activation mechanisms for *BMPR1b* expression, the resulting BMP signaling mediates a strong up-regulation of *Sox* transcription factors, which important role in chondrogenesis is described above (Healy et al., 1999; Chimal-Monroy et al., 2003). However, ligand binding to BMBR1b equally enhances the expression of noggin, which binds and inactivates secreted BMP2, 4 and 7 (Zimmerman et al., 1996). Experimental over-expression of either BMPs or noggin results in oversized or undifferentiated cartilage elements in the limb, respectively (Brunet et al., 1998; Merino et al., 1998; Pizette and Niswander, 2000). This balancing act between positive and negative regulation of BMP signaling is just one example of the many auto-regulatory cascades involved in chondrogenesis. Expression data available for zebrafish strongly suggest that this BMP/noggin feedback loop is a conserved feature of vertebrate chondrogenesis (Bauer et al., 1998).

Growth and Differentiation Factor 5 (GDF5) is the last member of the TGFβ superfamily to be discussed. The expression of this signaling molecule exhibits a very similar pattern in both zebrafish (Crotwell et al., 2001; Crotwell and Mabee, 2007) and tetrapod chondrogenesis (Francis-West et al., 1999; Merino et al., 1999a; Storm and Kingsley, 1999). It is first expressed in the tissue surrounding chondrogenic condensations, while in the later stages it is found restricted to developing joints and the epiphyseal perichondrium. This factor has been shown to enhance condensation formation, just as other BMPs, and it stimulates appositional growth in the epiphyseal cartilage. However, in addition to its positive effects on epiphyseal growth, GDF5 is equally required in the development of the joint region where chondrogenesis is inhibited (Francis-West et al., 1999; Storm and Kingsley, 1999; Hartmann and Tabin, 2001; Crotwell and Mabee, 2007).

Hedgehog family

Indian Hedgehog (IHH) and Sonic Hedgehog (SHH) are both secreted proteins that are known to provide key signals in embryonic patterning in many organisms. Both IHH and SHH can bind to the same receptor, Patched (PTC), and ligand-binding results in the activation of GLI transcription factors, which can either activate (GLI1 and GLI2) or repress HH signaling (GLI3). In cartilage development, IHH plays a crucial role in the regulation of chondrocyte maturation. It is only expressed in pre-hypertrophic chondrocytes, which in long bones occupy the region between proliferating and hypertrophic chondrocytes (St-Jacques et al., 1999). Nevertheless, target tissue of Hedgehog signaling can be identified by the presence of *Ptc* and *Gli* expression in the perichondrium and in non-hypertrophic chondrocytes (St-Jacques et al., 1999; Koziel et al., 2005).

The production of parathyroid hormone-related protein (PTHrP) by the perichondral region facing non-hypertrophic cartilage is a well-studied response to IHH signaling. The PTHrP receptor is present in cartilaginous tissue from the moment condensational cells differentiate into chondrocytes (Shukunami et al., 1996) and PTHrP signaling regulates proper growth of endochondral bone (Karaplis et al., 1994; Lee et al., 1996; Vortkamp et al., 1996), mainly by preventing proliferating chondrocytes from hypertrophic differentiation (Chung et al., 1998). Interestingly, both Ihh and PTHrP are shown to be up-regulated by mechanical loading *in vitro*, inducing chondrocyte proliferation and inhibiting chondrocyte hypertrophy in response to these mechanical stimuli (Wu et al., 2001; Tanaka et al., 2005; Bian et al., 2012). While the IHH/PTHrP feedback loop regulates proper growth of endochondral bones by preventing proliferating cells from premature maturation, IHH signaling also mediates the transition from resting epiphyseal chondrocytes into stacked proliferative chondrocytes in a PtHrP-independent manner (Kobayashi et al., 2005; Koziel et al., 2005). Furthermore IHH signaling results in the ossification of the perichondrium (Long et al., 2004), making it one of the most versatile and important signaling molecules in tetrapod long bones (Maeda et al., 2007).

IHH is equally found in zebrafish, where it is expressed in pre-hypertrophic chondrocytes (Avaron et al., 2006). Just as in tetrapods, the IHH signal results in cartilage ossification (Hammond and Schulte-Merker, 2009). However, in the perichondral bones of small teleosts, IHH appears to exert this osteogenic effect not only on perichondral cells but also on the internal hypertrophic chondrocytes, which obtain many osteoblast characteristics (Gavaia et al., 2006; Hammond and Schulte-Merker, 2009).

Wnt family

WNTs are a large family of secreted molecules that transduce their signals through a number of different pathways (Gordon and Nusse, 2006). They are involved in a variety of patterning events during development, including patterning of the developing fin/limb buds (Mercader, 2007). Some evidence exists that they exert a crucial patterning function in zebrafish cartilage condensations by determining the polarization of the cartilage precursor cells (Clement et al., 2008).

WNTs are important regulators of chondrocyte differentiation. Especially the canonical or Wnt/β-catenin signaling pathway appears to control chondrocyte differentiation from mesenchymal progenitors (Day et al., 2005; Day and Yang, 2008). This pathway revolves around the presence of β-catenin in the cytoplasm. When WNT ligands bind to their co-receptors at the cell membrane, a sequence of events leading to accumulation of β-catenin in the cytoplasm is triggered. β-catenin is then translocated to the nucleus, where it serves as part of a transcriptional complex activating downstream target genes. In the absence of WNT ligands, β-catenin is phosphorylated and tagged for degradation. It is, however, important to note that in addition to the involvement in the canonical Wnt pathway, β-catenin is also required for proper cell-cell adhesion mediated by cadherins, which will be discussed below (section 2.2.2.) (Perez-Moreno and Fuchs, 2006). Conditional knock-out of β-catenin, inhibiting canonical Wnt signaling, leads to differentiation of ectopic chondrocytes in mouse mesenchymal condensations of endochondral elements, but also in the condensations of dermal bones (Day et al., 2005).

On the other hand, ectopic activation of the canonical Wnt pathway inhibits the differentiation of cartilage condensations, while osteoblast differentiation is enhanced (Rudnicki and Brown, 1997; Day et al., 2005).

WNT14 is a well-studied example of the involvement of Wnt during chondrogenesis. It is expressed specifically in those areas of the chondrogenic condensations that will form joint regions. This Wnt has the potential to block cartilage differentiation and even reverse the cartilage phenotype *in vivo*, while it has proven to be the most upstream factor during *in vivo* joint formation currently known (Hartmann and Tabin, 2001). Just like many other factors that regulate chondrogenesis, WNT14-mediated joint formation in the autopodium appears to function through an auto-regulated mechanism, which is capable of spacing the interphalangial joints. This was concluded from misexpression experiments, in which ectopic *Wnt14* induced in chondrogenic condensation resulted in ectopic joint formation, while misexpression near developing joints inhibited further joint development (Hartmann and Tabin, 2001).

Cell-cell interactions

Although cartilage is the only type of connective tissue that does not show cell-to-cell contacts in its differentiated form, cell-cell adhesion molecules are crucial in its development. Two different cell-cell adhesion molecules are well known to be involved in cartilage development, namely Neural-cadherin (NCAD) and Neural Cell Adhesion Molecule (NCAM).

NCAD, first discovered in neural tissue, is a member of the subgroup of the classical cadherins. These cadherins are all Ca^{2+}-dependent, single transmembrane glycoproteins, which mediate cell-cell adhesion by homotypic protein-protein interactions through their extracellular domain. In addition to the single transmembrane and the extracellular domain classical cadherins posses a highly conserved cytoplasmic domain responsible for binding to the actin cytoskeleton via the catenin molecules (see also above in section 2.2.2.) (Suzuki, 1996; Yonemura, 2011).

NCAD has proven to be vital in numerous developmental processes (Marrs and Nelson, 1996; Biswas et al., 2010; Miron et al., 2011; Piven et al., 2011; Redies et al., 2011) including cartilage development, in which its role appears to be highly conserved between tetrapods and teleosts. This cadherin is sparsely expressed in the mesenchyme of the early limb/fin bud. Its expression increases drastically in developing cartilage condensations, only to disappear again in differentiated cartilage (Hamburger and Hamilton, 1992; Oberlender and Tuan, 1994b; Oberlender and Tuan, 1994a; Liu et al., 2003). Interestingly, the periphery of the *N-cad* positive condensation remains strongly *Ncad* positive and will develop into the perichondrium (Oberlender and Tuan, 1994a). The crucial importance of NCAD cell-cell adhesion in the densely packed cartilage condensations is clearly shown when NCAD function is impaired. If NCAD function is blocked prior to condensation formation, chondrogenic condensations fail to form. However, if NCAD function is blocked in established condensations the differentiation of these condensations is unaltered (Hatta and Takeichi, 1986; San Antonio and Tuan, 1986; Liu et al., 2003). Concomitant with its involvement in the condensation phase of chondrogenesis, *Ncad* is up-regulated by signaling molecules, involved in ongoing condensation, like BMP2 and TGFβ (Chimal-Monroy and Diaz de Leon, 1999; Haas and Tuan, 1999). Interestingly, the subsequent loss of NCAD proves to be as important as its initial role in condensation establishment. In addition to being down-regulated, NCAD is shed from the cell membrane by the proteinase termed Disintegrin and Metalloproteinase domain-containing Protein 10 (ADAM10). In a human cell line, which is engineered to express a form of *Ncad* resistant to ADAM10-mediated shedding, the differentiation of chondrogenic condensations was inhibited (Nakazora et al., 2010).

NCAM is a glycoprotein from the immunoglobulin superfamily (Chothia and Jones, 1997). Just as NCAD, it possesses a cytoplasmic, transmembrane and an extracellular domain, the latter of which is responsible for the homotypic binding that results in cell-cell adhesion (Rao et al., 1992). However, unlike cadherins, this adhesion is Ca^{2+} independent (Rutishauser,

1990). The expression of *NCAM* is similar to the expression of *NCAD*. Although the first appearance of *NCAM* occurs slightly later than *NCAD* (Tavella et al., 1994), *Ncam* reaches its maximal expression in chondrogenesis during the condensation phase. When the condensation starts to differentiate, *Ncam* is down-regulated, leaving matured cartilage completely devoid of *Ncam*, while the perichondrium retains high *Ncam* expression (Chuong, 1990). Unlike NCAD, NCAM is not strictly required for condensation formation. However, condensation size is significantly altered when NCAM function is impaired (Chuong et al., 1993; Widelitz et al., 1993).

The last method of cell-cell interactions to be discussed does not involve a mediator of physical adhesion between cells, but rather proteins that enable direct cell-to-cell communication. Members of the Connexin (Cx) proteins form hexameric groups that form closable channels in the cell membrane. In areas of cell-to-cell contacts the channels of neighboring cells connect, forming a direct bridge between their cytoplasm, which allows rapid exchange of ions and metabolites up to approximately 1 kD in size (Evans and Martin, 2002). Aggregations of these canals in areas of cell-cell contact, often stabilized by cadherins, are called gap junctions (Segretain and Falk, 2004). Cx43 is present in many tissue types and is also found in the superficial region of epiphyseal cartilage and in cultured chondrocytes. This led researchers to believe that these chondrocytes still exhibit cell-to-cell contacts (Schwab et al., 1998). However, recent research has confirmed that these differentiated chondrocytes lack physical cell-to-cell contacts and posses Cx43 hemichannels that connect the cytoplasm to the ECM fluids (Knight et al., 2009).

This implies that Cx43 will not mediate direct cell-to-cell communication in these chondrocytes, but rather perform a different function. There are good indications that these hemichannels are involved in the ability to sense and respond to mechanical stimuli (Knight et al., 2009). The ability to respond to mechanical stimuli has proven to be an important factor in connective tissue development and homeostasis (Eckes and Krieg, 2004; Chiquet et al., 2009), including cartilaginous tissue (Kelly and Jacobs, 2010). The importance of

mechanical stimuli is apparent when skeletal muscle movements are impaired in avian embryos by inducing paralysis or disturbing muscular development. This results in shorter long bones (Hosseini and Hogg, 1991; Wong et al., 1993), cartilage tissue with lower GAG content and weaker mechanical properties (Wong et al., 1993; Mikic et al., 2004). The chondrocytes' ability to respond to mechanical stimuli with an increase in matrix production has been confirmed on cultured chondrocytes that underwent several compression cycles (Angele et al., 2004; Miyanishi et al., 2006).

Cell-matrix interactions

ECM composition through chondrogenesis

The extracellular environment contains a large array of molecules, from collagens to various glycoproteins and proteoglycans. During chondrogenesis, these ECM components show a dynamic expression profile and many have been functionally implied in various stages of cartilage development. The matrix of pre-condensation mesenchyme is generally characterized by proteoglycans, collagen type I and fibronectin. During chondrogenesis, both collagen type I and fibronectin reach their maximum expression in cartilage condensation, only to be severely down-regulated when differentiation sets in (Kosher et al., 1982; Kulyk et al., 1989). At this point, collagen type I production is replaced by collagen type II, which becomes the most abundant collagen of the cartilage ECM (von der Mark et al., 1976; Dessau et al., 1980; Ede, 1983).

Fibronectin, on the other hand, remains present in differentiated cartilage but it is far less abundant and is present in a differently spliced isoform (Gehris et al., 1996; White et al., 1996; Gehris et al., 1997). While mesenchymal fibronectin contains both exon EIIIA and EIIIB exon (B+A+), cartilage fibronectin has the EIIIA exon spliced out (B+A–). Interestingly, the presence of exon EIIIA has proven to be required for condensation formation (Gehris et al., 1997). *In vitro*, the presence of fibronectin causes strong adhesion of pre-chondrogenic cells, which results in a spread-out cell shape (Peters and Msher, 1989). This strong adhesiveness appears to be crucial for

condensation formation, but inhibitory for chondrocyte differentiation (Zanetti et al., 1990).

In addition to alternative splicing, the presence of the glycoprotein tenascin (Jones and Jones, 2000) can explain how fibronectin can be present in differentiated cartilage despite its inhibitory effects on chondrocyte differentiation. Tenascin is found in undifferentiated cartilage and in newly differentiated regions of growing cartilage, but is absent in a fully differentiated matrix. *In vitro*, this ECM component inhibits the strong spreading of pre-chondrogenic cells on a fibronectin-rich surface, allowing them to attain a more rounded cell-shape, which in turn allows the condensational cells to differentiate (Mackie et al., 1987; Zanetti et al., 1990). In these differentiated nodules, the production of proteoglycans is strongly increased and their GAG chains become more heavily sulfated (Ede, 1983; Doege et al., 1991). In addition to retaining water, these GAG function as tethers of soluble signaling factors. For example, betaglycan can bind TGFβ thereby mediating the presentation of TGFβ to its type II receptor (Lopez-Casillas et al., 1993).

An extraordinary example of the importance of ECM composition during chondrogenesis was given in Hurle and Colombatti (1996). They discovered that ECM remodeling associated with wound repair after surgical manipulation in the interdigital space of a chicken's hind leg autopodium can induce an ectopic digit. The authors suggest that the interdigital spaces possess an ECM composition that naturally inhibits chondrogenesis, a composition that is altered during wound repair. They found that especially elastin fibers were missing in the wounded interdigital space and that intense deposits of tenascin were formed.

Mediators of cell-matrix interaction

It is well established that the influence of ECM components on developmental processes is mediated by binding to receptor proteins at the cell membrane. In addition to providing the physical adhesion of cells to the ECM, these receptors are capable of influencing numerous intracellular pathways through their intracellular domains.

A large and well-studied group of cell-matrix adhesion molecules is the integrins. In mammals, 18 alpha and 8 beta subunits combine to form 24 different heterodimeric complexes (Hynes, 2002). Each subunit is a glycoprotein with a large extracellular domain, a transmembrane domain and an intracellular domain. Different integrin subunits can bind to collagens, laminin or a specific tripeptide sequence, such as RGD-sequence, which is present in various ECM proteins, including fibronectin (Hynes, 2002). Extracellular binding between integrins and their ligands is translated to the cells' interior by the intracellular domain of integrins, influencing the organization of the actin cytoskeleton (Hynes, 2002; Humphries et al., 2004). Together with the aggregation of integrins at specific sites in the plasma membrane, the reorganized cytoskeleton can create a variety of adhesion complexes with different adhesive properties (Geiger et al., 2001). In addition to this integrin-mediated assembly of cytoskeletal linkages, ligation of integrins also triggers a large variety of signal transduction events that serve to modulate many aspects of cell behavior including proliferation, survival/apoptosis, shape, polarity, motility, gene expression, and differentiation (Geiger et al., 2001; Hynes, 2002).

The effects of integrin ligation are mediated by a complex community of intracellular proteins that link the internal domains of integrins with the cytoskeleton and the intracellular pathways (Boudreau and Jones, 1999; Geiger et al., 2001). Although further details on this topic are not within the scope of this introduction, I will present one example that has been shown to operate in chondrogenesis. Rho GTPases are well-studied regulators of integrin-associated changes in cell behavior (DeMali et al., 2003). RHOC in particular has proven to play an important role in chicken chondrogenesis. This member of the Rho family regulates the cells' cytoskeleton so that the cell acquires an elongated shape that is associated with strong matrix adhesion. It is therefore not surprising that RHOC is required for proper condensation formation, while being inhibitory to chondrogenic differentiation. The expression profile of this GTPase in chondrogenic condensations and perichondral tissue, including interphalangial joints suggests that it serves as

an inhibitory boundary for chondrogenesis, while its down-regulation allows differentiation to occur in the core of the elements (Montero et al., 2007).

In addition to integrins, other matrix receptors are present at the cell-surface. One example is the cell surface receptor CD44 for hyaluronan, which has proven to be crucial for proper assembly of the pericellular matrix (Knudson, 1993; Knudson and Loeser, 2002). Another example is syndecan-3, a transmembrane proteoglycan that is expressed in pre-condensation mesenchyme, chondrogenic condensations and later in perichondral tissue. This transmembrane protein has been shown to interact with tenascins and most likely functions as an inhibitory boundary in the perichondral regions of cartilage elements (Koyama et al., 1995; Koyama et al., 1996). Considering that a single cell type presents an array of different integrins and other ECM receptors at its cell surface, which all connect to different ECM components and interact with a diverse community of intracellular proteins in order to mediate a cellular response, it is not surprising that many aspects of cell-matrix interaction remain poorly understood. Nevertheless these interactions have significant importance in cartilage development and homeostasis. Moreover, recent studies suggest that the chondrocytes' ability to detect and respond to mechanical stimuli (previously discussed in section 2.2.2) equally depends on cell-matrix interactions (Bershadsky et al., 2003; Spiteri et al., 2010).

It is very likely that cell-matrix interactions are equally important in actinopterygian chondrogenesis, but until this day only limited knowledge exists on matrix composition and cell-matrix interactions during chondrogenesis in this clade. The presence of collagen type II and sulfated proteoglycans in differentiated cartilage of teleosts and more basal actinopterygians is well established (Huysseune, 1989; Huysseune and Verraes, 1990; Yan et al., 1995; Kimmel et al., 1998; Davis et al., 2004), but more detailed information is currently lacking. Despite this lack of information, zebrafish and its many mutant lines have already provided valuable insights in some cell-matrix interactions. For example, analysis of zebrafish mutant lines has shown that genes responsible for the synthesis and sulfation of cartilage

proteoglycans are essential for proper chondrocyte differentiation and maturation. The affected genes in these mutant lines are known to be involved in a variety of human disease-related physiological processes and the zebrafish mutant lines currently provide the only homozygous vertebrate model systems that allow the functions of these genes to be studied *in vivo* (Clement et al., 2008; Eames et al., 2011).

1.2.3. Catabolism in cartilaginous tissue

Since chondrocytes are completely embedded in their ECM, regulated catabolism is of major importance in many aspects of cartilage development. For instance, proliferating chondrocytes of the growth plate rely on ECM remodeling in order to align in axial rows, divide and change the composition of their ECM as they mature towards hypertrophic chondrocytes, all essential aspect for the proper growth of endochondral bones. Also chondrocytes residing in the stable ECM of permanent hyaline cartilage require the ability to remodel the ECM in order to proliferate or respond to mechanical stimuli. It is well established that the proper balance between synthetic and catabolic mechanisms in both hyaline and growth plate cartilage is of crucial importance for both maintenance of permanent cartilage function, as well as progression of endochondral bone formation (Yasuhara et al., 2010). Any imbalances in such homeostatic pathways can lead to a variety of pathological skeletal conditions that include skeletal dysplasias and osteoarthritis (AO), which is one of the leading causes of disabilities in the western world (Evans et al., 2004; Page-McCaw et al., 2007; Chun et al., 2008).

Cartilaginous ECM contains a variety of structural components and an equally diverse community of catabolic enzymes is known to enable chondrocytes to degradation the cartilage ECM. Hyaluronidases, cathepsins, matrix metallo-proteinases (MMPs) and the related families of proteinases, which contain many aggrecanases, the ADAMs (a disintegrin and metalloproteases) and ADAM-TSs (ADAMs with thrombospondin repeats) are all catabolic enzymes known to have an important role in cartilage ECM degradation (Zwicky et al., 2002; Nagase and Kashiwagi, 2003; Sugimoto et al., 2004; Cawston and

Wilson, 2006; Malemud, 2006). Although many questions remain on the exact physiological role of these catabolic enzymes, research of the most recent decade clearly shows that their functions far exceed mere ECM degradation. Many of these catabolic enzymes have been implicated in an enormous range cellular processes, such as the response to mechanical stimuli, proliferation, migration, metastasis, cell survival and cell death, and even ECM production (Page-McCaw et al., 2007; Spiteri et al., 2010; Tanimoto et al., 2010; Nebelung et al., 2012).

It is, however, important to note that several important catabolic events in cartilage development are not mediated by chondrocytes. Chondroclast, which degrade calcified hypertrophic cartilage, and macrophages, which form the cartilage canals of tetrapod epiphysis, also rely on these catabolic enzymes for their digestive function.

1.3. From endoskeletal patterning to morphology

In no embryonic system has chondrogenic pattern formation more rigorously been studied than in tetrapod limbs. The interest in the mechanisms that control pattern formation in the limb skeleton is not surprising, since both the origin and the diversity of the tetrapod clade are profoundly intertwined with variations in limb morphology. However, the evolutionary mechanism behind the fin-to-limb transformation can only be illuminated if we understand how the formation of complex skeletal patterns is regulated through development. This understanding can also provide an explanation for the incredible limb diversity between extinct and extant tetrapod taxa, which enabled tetrapods to acquire their many specialized locomotory strategies (Hinchliffe, 1994; Hinchliffe, 2002; Gatesy and Middleton, 2007; Kley and Kearney, 2007; Polly, 2007; Shapiro et al., 2007; Thewissen and Taylor, 2007).

Sadly, the interest in pattern formation within tetrapod limbs was never transferred to the actinopterygian lineage, the most successful group of vertebrates with the largest radiation (Nelson, 2006). Virtually all studies that used actinopterygian species aimed to assess whether or not a certain developmental process was specific to tetrapods or rather a conserved feature of osteichthyan development. In general, these comparative studies indicate that many aspects of chondrogenic pattern formation are indeed highly conserved among Osteichthyes. Therefore, I will first summarize the research on skeletal patterning in tetrapod limbs, which was excellently reviewed in Newman and Muller (2005), before addressing the major similarities with and differences from observations made in actinopterygian chondrogenesis. In all Osteichthyes the cartilaginous embryonic skeleton that results from the initial patterns of chondrogenesis will undergo extensive remodeling in order to complete the endochondral skeleton. The processes that accomplish this remodeling will be discussed in the final chapter of the introduction.

1.3.1. Patterning of chondrogenic condensations in tetrapods

In tetrapod limbs, cartilage condensations form in a proximo-distal direction as the limb bud continues to grow outward, with exception of urodelian limbs in which distal condensation form prior to more proximal elements. Shubin and Alberch (1986) subdivided the patterns in which these condensations are formed into three distinct categories, i.e. *de novo* formation, segmentation and branching or bifurcation. The authors hypothesized that the traction from cell movement on the extracellular matrix causes these discontinuous patterns to form during mesenchymal morphogenesis (Oster et al., 1983). Testing of this hypothesis showed, however, that limb mesenchyme does not produce traction on the ECM at least not *in vitro* (Markwald et al., 1990). Furthermore, patterns of *in vitro* condensations proved inconsistent with the predictions based on the mechanical-traction model proposed by Shubin and Alberch (Miura and Maini, 2004). Finally, it was shown that the number of skeletal elements in a specific section of the limb is already determined well before the first morphological signs of condensations (Wolpert and Hornbruch, 1990), suggesting that molecular processes pattern the limb mesenchyme rather than the mechanical forces of the morphogenic process. However, despite the lack of scientific support for the mechanical-traction model, the three condensation categories described by Shubin and Alberch (1986) proved to be universally applicable and remain in use until this day.

As an alternative to the mechanical traction model, it has often been hypothesized that the expression patterns of certain genes defined the major axes of the limb bud. The combination of these gene expressions would provide ‘coordinate’ information to the limb mesenchyme, which would in turn commit to form the skeletal element fitting to its position. Numerous studies have tried to identify morphogens capable of specifying the axis of the limb bud (Tickle, 2003). Many candidates, such as Shh (Wolpert and Hornbruch, 1981; Riddle et al., 1993), retinoic acid (Tickle et al., 1985), retinoic acid receptors (Maden et al., 1988), BMPs (Dahn and Fallon, 2000) and Hox genes (Morgan and Tabin, 1994; Wagner and Chiu, 2001), have been

proposed to perform such function. Although many of these factors proved vital in limb bud establishment, outgrowth and even patterning, further investigation always disproved simple axis-specific informational roles involved in chondrogenesis, for these factors (Noji et al., 1991; Wanek et al., 1991; Davis and Capecchi, 1994; Graham, 1994; Dudley et al., 2002; Sun et al., 2002; Ahn and Joyner, 2004).

Shh, for example, is expressed in a distinct region of the posterior portion of the limb bud, often referred to as the posterior zone of polarization (ZPA). When this zone is transplanted to the anterior region of a host bud, the limb skeleton distal to the stylopodium is duplicated in a mirror image, suggesting that different Shh concentrations result in the formation of different components of the limb skeleton (Wolpert and Hornbruch, 1981; Riddle et al., 1993). However, detailed examination of limb development in Shh null mutants shows that the role of this signaling molecule is far more profound than the specification of skeletal element identity. In the absence of Shh, initial stages of limb bud formation are unaffected and girdle and stylopod elements develop normally. However, the AER and the FGFs it produces are lost in later stages of limb bud outgrowth, resulting in strongly reduced limb buds in which zeugo- and autopodial elements are severely reduced and malformed. This indicates that Shh is required to maintain proper limb bud outgrowth, thus providing the precursors of the more distal elements (Chiang et al., 2001a). Nevertheless, Shh and more specifically its gradient distribution in the autopodium could aid in determining digit identity, but several lines of research have yielded contradictory results on this matter (Ahn and Joyner, 2004; Harfe et al., 2004).

The Hox genes are another example of well-studied candidates for limb patterning. In the limb bud, Hox genes belonging to the paralogous groups 9–13 of Hox A and D clusters are expressed in nested domains along the proximo-distal as well as antero-posterior axes, creating different combinations of Hox genes or different Hox codes in different regions of the limb bud. The nested expression patterns of these Hox genes appear to be regulated in three distinct phases, which apparently coincide with the three

proximo-distal regions of the limb skeleton (Morgan and Tabin, 1994; Wagner and Chiu, 2001). The last of these phases is limited to the region of the future autopodium, showing Hox genes expressed in the antero-posterior direction. Therefore, it was often speculated that this third phase was specific to tetrapod autopodium development and was required in order to establish digit identity (Hinchliffe, 1994; Sordino et al., 1995; Shubin et al., 1997; Hinchliffe and Vorobyeva, 1999; Wagner and Chiu, 2001; Hinchliffe, 2002; Spitz et al., 2003; Galis et al., 2005; Kundrát, 2009). More recent studies have shown, however, that both basal actinopterygians and teleost fins show a tri-phasic Hox expression pattern, including a final anterio-posterior phase in the distal-most portion of the fins (Davis et al., 2007; Ahn and Ho, 2008). As will be discussed in detail below, the fin skeleton differs considerably from the skeleton in tetrapod limbs. Thus the occurrence of a highly similar Hox-code pattern in these very different skeletal morphologies, i.e. the tetrapod autopodium and the distal part of actinopterygian pectoral fins, makes it unlikely that Hox-codes directly control the identity of individual skeletal elements, as was proposed for the autopodium digits. It rather suggests that these molecular patterns control the inherent properties of the limb and fin mesenchyme to generate discrete skeletal structures. This idea is very nicely illustrated by a recent study on HoxA 13 mutant mice, which show missing phalanx elements, fusions of the carpal/tarsal elements, and significant reductions in metacarpal/metatarsal length (Perez et al., 2010). This mutant phenotype appeared to be caused by increased levels BMP 2, which stimulates cartilage condensation growth, and decreased levels GDF 5, which regulates joint formation, thus suggesting that HoxA 13 biases limb mesenchyme precursors to aggregate in several small condensations rather than to create fewer larger condensations (Perez et al., 2010). Interestingly, in both fins and limbs the distal expression domain of HoxA 13 is consistent with the occurrence of more and smaller skeletal elements than in more proximal regions of fins and limbs. In general, it can be concluded that the factors involved in limb bud patterning do not directly result in certain skeletal patterns. Instead, these factors limit and refine other morphogenic molecules,

which in turn modulate the inherent properties of limb mesenchyme cells to generate discrete skeletal structures (Innis et al., 2002; Sun et al., 2002; Chen et al., 2004b; Harfe et al., 2004; Scherz et al., 2004; Zakany et al., 2004).

All these findings suggest that a more integrated approach is needed in order to fully understand pattern formation in the limb endoskeleton. As mentioned in section 2.2. of this introduction, chondrogenesis is mediated by more than simple gene expression resulting from secreted signaling molecules. After activation of the chondrogenic pathway by for instance TGF β, many auto-regulatory mechanisms, which revolve around more than the control over gene expression, spring into action. These processes, such as the inhibitory binding of noggin to BMPs, the secretion and subsequent adhesion of pre-chondrogenic cells to fibronectin, and the up-regulation of cell-cell adhesion, play an equally vital role in chondrogenesis. Since these processes are not directly represented in the genetics of the cells, they are often referred to as epigenetic mechanisms of development. Attempts to integrate the effects of auto-regulatory genetic systems and important epigenetic factors through computer modeling have produced intriguing results.

These computer models are built around Turing's reaction-diffusion model, which uses partial differential equations to describe how a slow-spreading and self-enhancing ''activator'' (of any process or reaction) that induces, either directly or indirectly, the production of a faster spreading ''inhibitor'', gives rise to spatial patterning of the reaction product (Turing, 1952). Using this principle and applying it to chondrogenesis by creating differential equations for known processes in chondrogenesis, various authors have indicated that all three major patterns, i.e. *de novo* formation, segmentation (Meinhardt, 2001; Miura and Maini, 2004) and bifurcation (Newman and Muller, 2005) can be generated by these models, with parameters ranging well within physiological conditions.

It is, however, important to note that all these models are based on simplifying assumptions. Despite the current advances in the modeling power of computers, it remains impossible to modulate all the known auto-regulatory

processes involved in chondrogenesis. In addition, new auto-regulated events continue to be discovered. For example, hairy 1, the gene that regulates the molecular clock, which has proven to control the formation of new somites in vertebrate embryos (Andrade et al., 2007; Brend and Holley, 2009), has also been implicated in limb bud outgrowth (Vasiliauskas et al., 2003). Nevertheless, this line of research has shown that relatively simple auto-regulatory cascades, many of which prove to be conserved features of chondrogenesis (see section 2.2.), are themselves capable of producing all of the skeletal patterns observed in tetrapod limbs, depending on several factors such as limb bud size, shape and outgrowth (Newman and Muller, 2005).

1.3.2. Similarities and differences between patterns in fins and limbs

Until this day, no underlying developmental processes are known to explain the morphological differences between fins and limbs. Many processes that regulate chondrogenesis are found to be preserved features among Osteichthyes (discussed in section 2.2.) and virtually all factors known to regulate limb bud patterning, such as the ZPA or Hox codes, appear to be present in a similar fashion in fin buds, as shown by Mercader (2007). This is, however, not surprising since the factors involved in limb outgrowth and shaping can be traced back to ancestral metazoans where they were also (based on their functions in modern invertebrates) responsible for tissue outgrowth and shaping. The conclusions gathered from computer modeling of tetrapod limb chondrogenesis (discussed in section 3.1.) provide an interesting angle to explain how very similar developmental processes can result in considerably different skeletal morphologies. This line of research suggests that the many auto-regulatory events in chondrogenesis could produce diverse skeletal patterns, which heavily depend on subtle differences in limb bud size, shape and outgrowth. In this chapter, I will discuss in which aspects fin development and the formation of the fin endoskeleton differ from or are similar to the observations on tetrapod species.

The common origin of fins and limbs

It is commonly believed that the ancestral paired fin of all extant Chondrichthyes and Osteichthyes is a rather broadly based fin that possesses three basal cartilaginous or endochondral elements, which articulate with the girdle. From anterior to posterior these elements are called the propterygium, the mesopterygium and the metapterygium. These elements articulate distally with a series of increasingly smaller elements called the proximal and distal radials. The distal radials are flanked by a ventral and a dorsal series of fin rays (lepidotrichia) that extend into the fin fold. As the sarcopterygian and the actinopterygian lineage diverged, they retained different aspects of this ancestral fin (Figure 4).

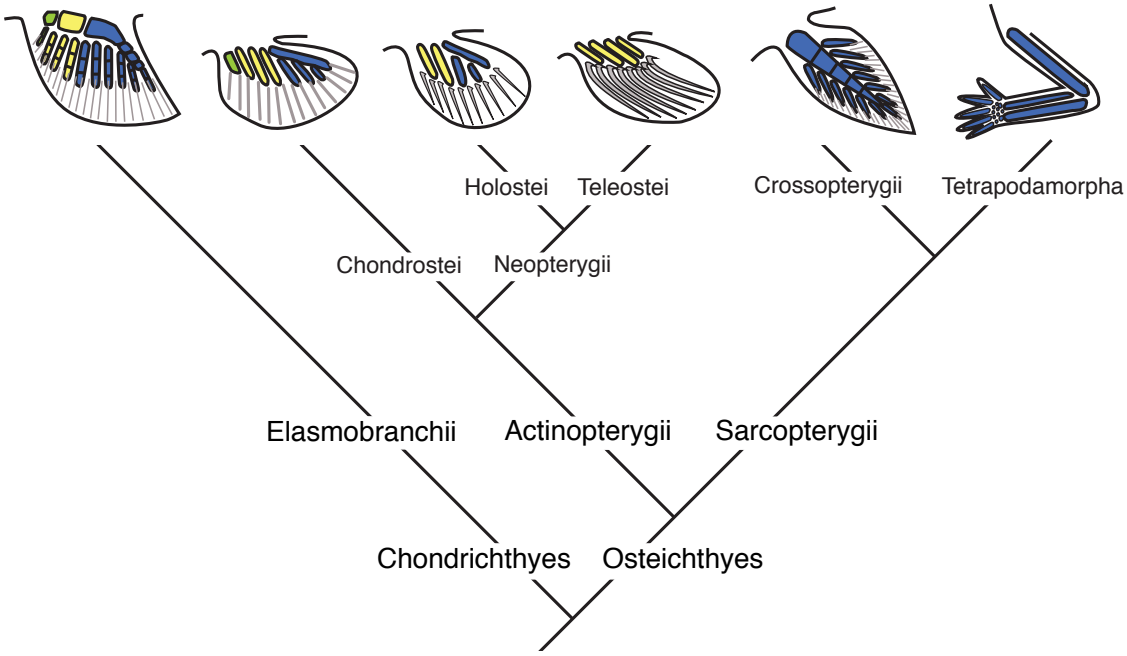

Figure 4
Schematic representation of the pectoral skeleton of the major extant gnathostome taxa. For comparative purposes all fins were depicted at the same horizontal level, despite the fact that they represent fins from species of different taxonomical level.The endoskeleton is represented in green, yellow and blue for the pro-, meso- and meta-pterygium respectively. Collagenous or bony fin rays are shown in grey.

Although ancestral sarcopterygians also started out with a broad-based tribasal fin (Zhu and Yu, 2009), extant sarcopterygian species generally possess a slender-based fin in which the endoskeleton represents only the

most posterior portion of the ancestral fin, i.e. the metapterygium. In this lineage, the metapterygial axis is subdivided, creating a series of proximo-distally articulating elements. In extant sarcopterygian species such as Dipnoi and Crossopterygians, the elements of the metapterygial axis articulate with numerous radials, both anterior and posterior to the main fin axis, called pre- and post-axial radials. These radials support the bony fin rays that continue into a fin fold. In tetrapods, which form a derived clade within the sarcopterygian lineage, the pectoral skeleton was further reduced and reorganized during the transition from aquatic to terrestrial locomotion. These morphological changes are well documented in paleontological records, but are still not completely understood (Wagner and Chiu, 2001; Coates and Ruta, 2007; Wagner and Larsson, 2007). All tetrapod limbs show a single proximal element, the stylopodium, which distally articulates with only two elements, the zeugopodium. The most distal section of the tetrapod limb is the autopodium. Depending on the taxon, the latter comprises a variable number of carpals/tarsals, metacarpals/tarsals and digits, each consisting of several phalanges. However, the most important derived character of tetrapod limb is the loss of the fin fold. It has been proposed that the AER of tetrapod limbs is homologous to the earliest anlage of the fin fold in other Osteichthyes (Grandel and Schulte-Merker, 1998). In fins an epithelial thickening at the distal edge of the fin bud grows outwards into an epithelial fold. This fold is then invaded by mesenchymal tissue and eventually it will develop the bony fin rays. It is important to note that the fin fold is never invaded by myogenic tissue and never contains cartilaginous elements. All fins are thus composed of a proximal section that contains the endoskeleton and musculature and a distal fin fold section in which only dermal bones form. The loss of AER outgrowth in tetrapods results in the complete absence of this later section. Recent research has shown that the loss of two genes that encode a structural collagen of the fin ray, actinodin 1 and 2, which support the early stages of the fin fold, could have contributed to the loss of the fin fold and even to the emergence of the autopodium in tetrapods (Zhang et al., 2010).

Paired fins in the actinopterygian lineage

Just as in tetrapod limbs, the endoskeleton of actinopterygian fins forms in a proximo-distal direction as long as the fin bud grows outward. Furthermore, the pattern in which chondrogenic condensations form can equally be considered as either de novo formations, segmentations or bifurcation events, as described by Shubin and Alberch (1986). However, in the actinopterygian lineage, the three distinct portions of the ancestral fin are still represented in various taxa with a more basal phylogenetic position. The fin buds of these basal actinopterygians are generally more anterio-posteriorly elongated than the slender and proximo-distally elongated buds of sarcopterygians and the developing endoskeleton possesses developmental characteristics of both tetrapod limbs and the fins of more derived actinopterygians.

Similarities between pectoral endoskeletal development of basal actinopterygians and tetrapod limbs are found in the most posterior regions of the fin during the period in which the fin bud continues to grow outward. Just as tetrapod limbs, the fins of basal actinopterygians are characterized by a long period of proximo-distal outgrowth. In these fins, a metapterygial element articulates proximally with the girdle, and runs along the caudal border of the fin towards its distal margin. The fin bud grows outward along its entire distal border in a region also referred to as the distal rim mesenchyme. This growth region contains undifferentiated precursor cells situated just proximally from the apical fin fold, similar to the undifferentiated mesenchymal cells underneath the AER in limb buds. However, only in the posterior metapterygial region new radials are added to the growing fin field. Just as in tetrapod limbs, these new radials form by bifurcations at the growing distal edge of the metapterygial element (Davis et al., 2004) (Figure 5).

As proximo-distal outgrowth slows down, the distal edge of each radial will form a separate element, i.e. the distal radial (Davis et al., 2004). Although the development of these radials was never described in detail, it is possible to deduce from published figures and from our own observations that these elements are formed as subdivisions of the proximal radial condensations.

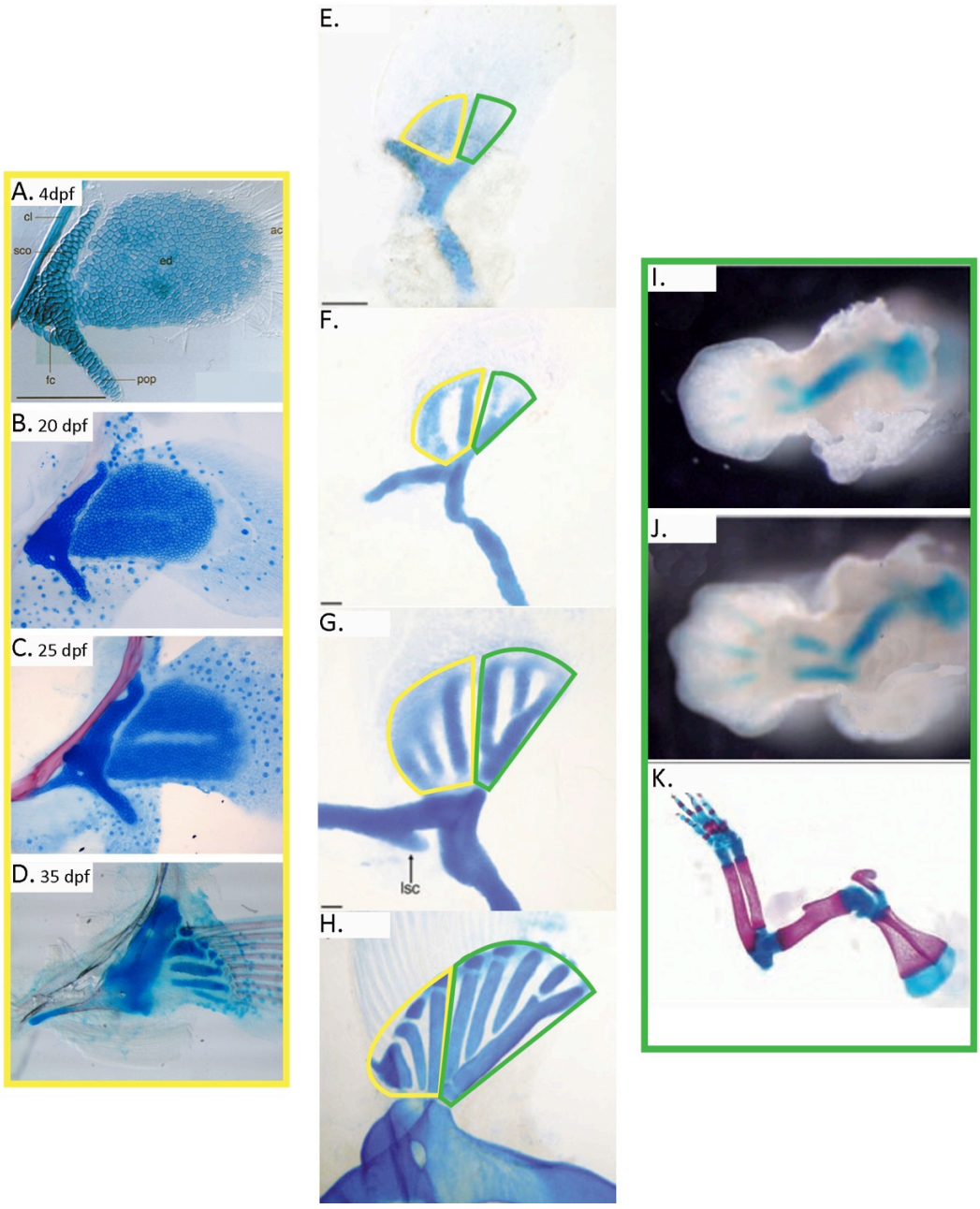

Figure 5
This figure shows the development of the pectoral endoskeleton in a derived actinopterygian, zebrafish (A-D), a basal actinopterygian, paddlefish (E-H), and a derived sarcopterygian, mouse (I-K). In addition, this figure shows that the development of bony fin rays in actinopterygians coincides with the formation of distal radials (D and H). Yellow shows the formation of multiple elements out of a continuous anlage, typical for actinopterygians, while green shows the formation of new elements by bifurcation, typical for tetrapods. (A) adapted from Grandel and Schulte-Merker (1998). (E-H) adapted from Davis et al. (2004). (I-K) adapted from Boulet and Capecchi (2004).

These subdivisions bear a striking resemblance to the subdivisions that form the digital phalanges in the tetrapod autopodium. However, in actinopterygians these subdivisions occur concomitant with the formation of the bony fin rays in the fin fold, both of which are lost in tetrapods (Figure 5).

Apart from these features of actinopterygian fin chondrogenesis, recognizably similar to tetrapod limbs, other aspects appear to be unique to actinopterygian fin chondrogenesis. The most important difference can be found in the earliest anlage of the fins' endoskeleton. The earliest sign of chondrogenesis in actinopterygian fin buds is the formation of a single chondrogenic condensation from which the future cartilaginous part of the girdle as well as the radials of the external fin will form. In the external part of the fin bud, this condensed pre-skeletal tissue forms as a disk-shaped condensation that spans the entire muscularized portion of the fin bud, ending at the proximal margin of the fin fold (Grandel and Schulte-Merker, 1998; Davis et al., 2004). As the fin bud expands along its distal margin, separate cartilaginous fin radials are formed out of this continuous condensation. The individualization of these radials appears to be accomplished by the continued differentiation of the radial tissue, in contrast to a loss of pre-skeletal characteristics in the interradial areas (Grandel and Schulte-Merker, 1998; Davis et al., 2004) (Figure 5).

This is contrary to observations in tetrapods, where the limb elements are formed by the emergence of several discrete condensations in loosely packed mesenchymal tissue. It is, however, important to note that the formation of multiple elements from a single condensation is not limited to actinopterygian fins, but is a widespread phenomenon. In Chondrichthyes (Grandel and Schulte-Merker, 1998) and even in some lissamphibian species (Shearman, 2008), the condensations of the pectoral girdle are also continuous with the condensations of the external elements. Likewise, as mentioned earlier, individual phalanges in the tetrapod autopodium are formed out of a continuous digit condensation (Casanova and Sanz-Ezquerro, 2007), just as the different elements of the same branchial arch in the splanchnocranium differentiate from a single condensation, both in

actinopterygians (Kimmel et al., 2001) and in tetrapods (Miyake et al., 1996). One could argue that it becomes impossible to distinguish between *de novo* formation and segmentation events due to the densely packed nature of both pre-skeletal and non-skeletal cells. Even in tetrapods, where condensations are identified as separate entities by histology, faint alcian blue positive zones can be observed in the regions that will develop into joints connecting the early elements in a continuous alcian blue positive structure. (Figure 5)

In all actinopterygian species, proximal fin radials form from a single skeletal condensation that expands as long as the fin bud grows outward. The distal fin radials form at the edge of the endoskeletal fin disk when proximo-distal outgrowth has stopped and bony fin rays or lepidotrichia start to form. Only in teleosts a distal radial forms at the base of each lepidotrichium, resulting in several distal radials for each proximal element. However, the development of the proximal radials also shows remarkable differences between various actinopterygian species. Grandel and Schulte-Merker (1998) reviewed that the level of tissue differentiation in the continuous fin disk prior to proximal radial individualization differs significantly between actinopterygian taxa. While in *Acipenser ruthenus* and *Acipenser stellatus* the disk-shaped anlage is composed of compacted mesenchyme, this anlage has been described as 'procartilaginous' in *Acipenser sturio* and *Amia calva* or 'cartilaginous' in *Polyperus senegalus, Lepidosteus* species and teleosts. In all these taxa, proximal radials individualize when the interradial regions loose their skeletal nature. However, when the interradials are already composed of cartilaginous tissue this event includes the loss of apparently differentiated cartilaginous tissue and can thus be considered as a remodeling event of the embryonic cartilage skeleton. Interestingly, both *Polyperus senegalus* (Bartsch et al., 1997) and teleost species (Thorsen et al., 2004; own observations) actively use their pectoral appendages prior to the subdivision of the continuous skeletal disk. It is very likely that these actinopterygian taxa have evolved the possibility to produce a premature, functional larval pectoral fin by prolonging the disc-phase during pectoral development (Grandel and Schulte-Merker, 1998).

It is, however very important to note that teleost and polypterid species (also called Bichirs, see Figure 1) exhibit considerable phylogenetic distance. While teleosts are considered the crown group of most derived actinopterygians, polypterids occupy a very basal phylogenetic position (Hurley et al., 2007; Suzuki et al., 2010). This large phylogenetic distance is also seen in other aspects of their pectoral development. While polypterids exhibit an extensive period of fin bud outgrowth resulting in a considerably large contribution of cartilaginous elements to the pectoral fin skeleton (Bartsch et al., 1997), the pectoral fin bud outgrowth is strongly reduced in more derived actinopterygian species, like *Amia calva* and *Lepidosteus* species, resulting in a strong reduction of cartilaginous elements in the pectoral fin skeleton (Goodrich, 1958; Jarvik, 1980). In teleost species, this reduction of fin bud outgrowth is the most extreme and only four proximal radials remain in the pectoral fins, while the main portion of their fins is supported by bony fin rays. It is, therefore, highly likely that the establishment of an early disk-shaped pectoral skeleton and its subsequent subdivision into proximal radials in both teleosts and polypterids is an example of convergent evolution.

1.3.3. Remodeling of the embryonic cartilage skeleton

In all Osteichthyes chondrogenic condensations differentiate to form an embryonic cartilage skeleton that will serve as a template from which the adult skeleton can be forged. Remodeling of this embryonic skeleton in order to achieve adult skeletal morphology is a common feature of osteichthyan skeletogenesis. It is, however, important to stress that remodeling is often used to address bone rejuvenation in response to mechanically induced micro fissures, by the formation of secondary osteons. In this sense bone remodeling distinguishes from bone modeling, which indicates shape and density adaptations of bony tissue in response to loading conditions (Currey, 2003). However, in this report remodeling is not used to distinguish between these aspects of bone development, it is rather used to encompass all

fundamental morphological modifications to the cartilaginous embryonic skeleton as it matures to its final adult morphology.

In tetrapods, this remodeling is often achieved during or after endochondral bone formation, which, as mentioned before, occurs relatively earlier in tetrapods than in fish. Growth and especially allometric growth between different parts of the skeleton is one important process by which the early embryonic skeleton can be modified. The digits supporting bat wings present a striking example. While they are initially not different from the short digits of mice, excessive growth in the metaphyseal growth plates makes them develop into the very long and slender digits needed to support the bats' wings (Sears et al., 2006). Just as in the evolution of bat wings, the amount of metaphyseal growth has played an important role in the evolution of specialized limbs in many tetrapod taxa (Hinchliffe, 1977; Hinchliffe and Griffiths, 1983; Müller, 1991). In actinopterygians, metaphyseal growth contributes less to changes in the embryonic skeleton, because endochondral ossification appears later in development when most of the skeletal elements have reached adult proportions (Cubbage and Mabee, 1996; Bird and Mabee, 2003; Davis et al., 2004).

The fusion of skeletal elements is a second mechanism by which the embryonic skeleton can be modified. In many mammalian taxa the metacarpal and metatarsal bones of the autopodium are fused to various degrees, especially in ungulates. Avian metatarsals fuse as well and, in this case, it is well described that these fusions occur after the elements have ossified (Namba et al., 2010). In zebrafish, fusion between skeletal elements is a known phenomenon. For example the cleithrum, which is a dermal bone of the pectoral girdle, and the coracoid, an endochondral portion of the girdle, fuse when an extension or apolamellae of the coracoid perichondral bone connects to the cleithrum (Grandel and Schulte-Merker, 1998). Note that again this fusion takes place after ossification.

Finally, the embryonic skeleton can be modified by the loss or regression of specific cartilaginous elements or regions. In tetrapods, regressions of skeletal elements during limb development are rare (Shubin

and Alberch, 1986) and detailed observations on such events are not available. It is, however, important to note that the term regression is also used for the evolutionary reduction of the skeleton. For example, in the cetacean lineage hind limbs are severely reduced, but this reduction is accomplished by a premature arrest in hind limb development and not by the loss or regression of specific cartilaginous elements during hind limb development (Thewissen et al., 2006). Other reports of skeletal regression do correspond to the actual loss of a skeletal structure during development. However, this does not necessarily mean that skeletal tissue is actually lost. For example, in avian wings the anlage of digits I and V seemingly disappear shortly after they are formed. In this case, the anlage of digits I and V never fully develop, while digits II, III and IV continue to grow and gradually incorporate the anlagen of digit I and V (Galis et al., 2003). A highly similar event is known in zebrafish, were the post-coracoid process was thought to regress some weeks after its formation (Grandel and Schulte-Merker, 1998). However, our own investigation showed that the cartilage tissue in this process is not lost but rather becomes inconspicuous by appositional growth at other sites of the cartilage girdle (Chapter 2).

Nevertheless some examples of cartilage loss during skeletal development do exist. In amphibians, the hyoid arch radically decreases in diameter during metamorphosis (Rose, 2009). In the mouse endoskeleton, three separate regions are known to regress during development, i.e. (1) the calvarial cartilages of the cranium, (2) the grove of Ranvier situated next to the metaphysis of long bones, and (3) the posterior section of the Meckelian cartilage, which transforms into sphenomandibular ligament (Harada and Ishizeki, 1998; Holmbeck et al., 1999; Holmbeck et al., 2003). It is, however, safe to conclude that remodeling of the embryonic skeleton by the loss or regression of cartilage tissue is an uncommon mechanism in skeletal development that is very poorly understood. The formation of the proximal radials in teleost pectoral fins is by far the prime example of how cartilage loss can contribute to normal skeletal development.

Before this doctoral research, the only available information on this event was the loss of sulfated proteoglycans in the interradial regions, suggesting the loss of cartilage ECM. Therefore, these regions were called matrix decomposition zones. However, no attempts were made to discover how the ECM is degraded and which cell types are responsible. I will investigate the involvement of various cell types known to degrade cartilaginous tissue, as well as the involvement of some of the digestive enzymes or proteinases that are capable of degrading the structural components of the cartilage ECM, such as acid phosphatases, matrix metalloproteinases and cathepsins. In general, this doctoral research aimed to understand the cellular mechanism behind this loss of cartilage that is essential for proper pectoral development in over 30% of all vertebrate species, i.e. teleosts.

2. Aims and Outline

Teleost fish are characterised by an unusual architectural transformation of their pectoral fin endoskeleton. In this transformation, a continuous cartilage element that comprises both the girdle, internal to the body wall, and a disk-shaped cartilage, called the fin disk cartilage, external to the body wall, subdivides into the scapulocoracoid and four rod-like proximal radials (Grandel and Schulte-Merker, 1998; Dewit et al., 2011).

In this doctoral thesis, I investigated which cellular mechanisms underlie the loss of cartilage tissue during the development of the pectoral fin endoskeleton in zebrafish (*Danio rerio*), a teleost species commonly used in developmental research.

First, I asked myselves whether the composition of extracellular matrix (ECM) in the fin-disk cartilage would represent unusual features, related to the type of transformation observed. The presence of certain ECM components, such as collagen type I and II, tenascin, elastin and fibronectin, can reliably distinguish different types of connective tissues, as well as different developmental stages in cartilage development (Hall, 2005). In other actinopterygian taxa, the pectoral fin endoskeleton equally forms out of a continuous anlage, but in the majority of more basal actinopterygians the subdivision occurs prior to cartilage differentiation. By examining the ECM composition I aimed to test two separate hypotheses. First, that differences in ECM composition between presumptive interradial tissue and radial cartilages could foreshadow the process of subdivision in the fin disk. Second, that the fin disk cartilage is not composed of fully matured cartilaginous tissue prior to

subdivision, as it is the case in more basal actinopterygians. For the latter I compared the ECM composition of the fin disk cartilage with the cartilages of the splanchnocranium and the median fins. (Chapter 1).

Second, I aimed to understand how the ECM in the interradial regions is resorbed and which cells are responsible for this resorption. According to the literature, the degradation of chondrocytes and their elaborate ECM can only be accomplished by a limited number of cellular mechanisms. Specialized resorption cells, such as macrophages (Blumer et al., 2008) or chondroclasts (Vu et al., 1998), can resorb cartilaginous tissue. The resident chondrocytes can undergo programmed cell death, apoptosis, and be resorbed secondarily by the surrounding tissue or the previously mentioned specialized resorption cells (Bronckers et al., 2000). Finally, chondrocytes could de-/trans-differentiate, arresting the production of new ECM components and resorbing their previously secreted ECM (Yocum et al., 1995; Holmbeck et al., 2003). I aimed to evaluate the involvement of each of these processes by using a variety of techniques, such as (tartrate-resistant) acid phosphatase detection, acridin orange / propidium red staining and a detailed analysis of the fin disk cartilage ultrastructure during its transformation (Chapter 2).

Third, I questoned whether proteinases would be involved in the ECM degradation during the subdivision of the pectoral fin cartilage. Several members of the matrix metalloproteinases (MMPs) family are known to be essential in both normal and pathological loss of cartilage tissue in tetrapod models (Imai et al., 1997; Holmbeck et al., 1999; Ishiguro et al., 2002; Murphy et al., 2002; Holmbeck et al., 2003; Kuroki et al., 2005; Malemud, 2006). Using immunohistochemical detection and both broad and narrow spectrum pharmacological inhibition, I tested the contribution of these MMPs to the subdivision process (Chapter 3).

Finally, collaboration with the laboratory of Dr. Winkler (Singapore University) allowed us to study the involvement of another proteinase, cathepsin K, during the cartilage subdivision in the medaka (*Oryzias latipes*), another model teleost species only distantly related to zebrafish. First, I

wished to investigate if cathepsin K is expressed during the subdivision process, by using both a transgenic reporter line and specimens hybridized *in situ* for cathepsin K. Second, I took advantage of the abundant expression of cathepsin K in chondro/osteoclast cells (To et al., 2012) to test whether medaka and zebrafish behave similarly with respect to the (lack of) involvement of chondroclasts in the subdivision of the pectoral fin cartilage (Chapter 4).

3. Results

3.1. The distribution of fibronectin in developing zebrafish (*Danio rerio*) cartilage

by

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Reference:

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3.1. The distribution of fibronectin in developing zebrafish (*Danio rerio*) cartilage

Abstract

The extracellular matrix (ECM) plays a complex and vital role throughout the process of cartilage formation. Fibronectin is a large ECM glycoprotein with an important role in various developmental processes, including skeletogenesis. Taking advantage of the known sequence of cartilage development in zebrafish and using an immunohistochemical stain for collagen type II to identify differentiation phase cartilage, we evaluate the distribution of fibronectin in various cartilaginous elements of the zebrafish (elements of the splanchnocranium, and of the dorsal, caudal, pelvic and pectoral fins). Contrary to what is observed in tetrapods, our data on zebrafish indicate the apparent lack of fibronectin during the condensation phase of cartilage development. This lack is possibly linked to the high developmental rate of the zebrafish and the small size of the condensations, which brings different needs for the extracellular environment to ensure cell survival. Furthermore, the fin disk cartilage of the pectoral fin develops an ECM with a strong fibronectin signal, whereas other cartilage elements show only a weak fibronectin signal in early differentiation, which gradually disappears. Thus, the pectoral fin disk cartilage is unique not only because of its specific way of development (subdivision of a continuous plate into four elements, the proximal radials), but also because of its strong fibronectin-positive ECM.

Introduction

The development of skeletal elements can be subdivided into four transient stages. The first is the migration of precursor cells to their future site of skeletogenesis. The second stage is the signaling interaction between epithelium and mesenchyme, leading to the formation of a skeletogenic condensation. This condensation of cells, which all contribute to further skeletal development, is seen as a third phase of skeletogenesis. Differentiation, i.e. the establishment of a specific cell phenotype, is the fourth and final phase (Hall and Miyake, 2000).

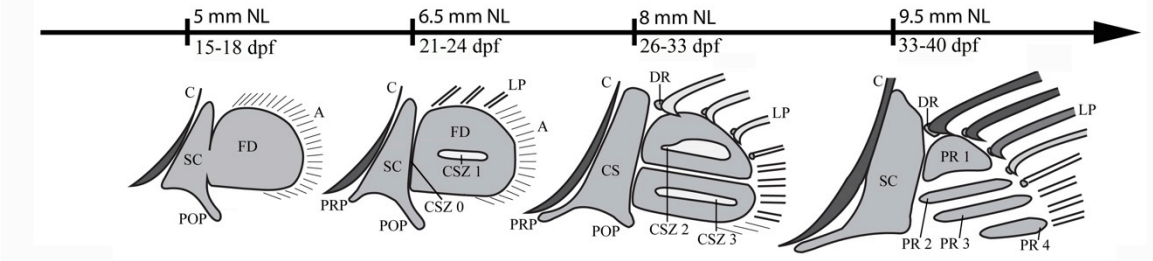
Most elements of the vertebrate endoskeleton develop as cartilage, which is later surrounded by perichondral bone and replaced by endochondral bone. However, in smaller teleost species, such as zebrafish, endochondral bone formation is a rare event. Here, cartilage that is surrounded by perichondral bone usually persists, or is replaced by adipose tissue (Verreijdt et al., 2002; Witten and Huysseune, 2007; Witten and Huysseune, 2009; Witten et al., 2010). Throughout the process of cartilage formation, the extracellular matrix (ECM) plays a complex and vital role. In fact, a considerable portion of our understanding of cartilage development has been acquired through the detection of various ECM components during this process in tetrapod models (Hall and Miyake, 1995).

One ECM component with a supposed important role in chondrogenesis is fibronectin. Fibronectin is a large glycoprotein that displays complex interactions with cell surface receptors and with other ECM components (Pankov and Yamada, 2002). Fibronectin has been found to be of vital importance in numerous developmental processes, such as neural tube, somite and vascular development (George et al., 1993). During tetrapod cartilage development, fibronectin is strongly up-regulated at the condensation stage compared to the uncondensed surrounding mesenchyme (Kulyk et al., 1989). Yet, the role of this versatile protein is far from completely understood. For instance, when chicken wing and leg bud cell cultures were treated with a monoclonal antibody directed against the amino-terminal heparin-binding domain of fibronectin, condensation formation was inhibited and

chondrogenesis was reduced in wing buds. Surprisingly, condensation formation in leg bud cell cultures remained unchanged (Downie and Newman, 1995). Similarly, *in vivo* injection of a monoclonal antibody, directed against the alternatively spliced exon IIIa of fibronectin, had no effect on leg buds, but inhibited chondrogenesis in wing buds (Gehris et al., 1997).

This variation between different cartilage condensations in the chick inspired us to gather data on fibronectin distribution in the zebrafish (*Danio rerio*). The zebrafish has become a widely acknowledged model to study molecular aspects of cartilage formation, in particular in the splanchnocranium (Kimmel et al., 2001), but until now no data are available on the ECM composition in the various phases of cartilage development throughout the body.

An additional incentive to gather data on ECM components, like fibronectin, in zebrafish, is the teleost-specific pectoral development. In tetrapod limbs, the initial pattern of cartilage condensation can be modified by fusion, growth and, in rare cases, regression of the cartilage elements (Hinchliffe, 1977; Hinchliffe and Griffiths, 1983; Shubin and Alberch, 1986; Müller, 1991). In zebrafish, on the other hand, most of the pectoral girdle and fin endoskeleton arises as a continuous cartilaginous element. This continuous anlage is formed during the second and the third day post-fertilization and consists of the girdle element and a cartilaginous disk supporting the functional larval fin. This cartilaginous disk subdivides into the four proximal radials during the third and fourth week post-fertilization, when the interradial tissue looses its cartilaginous character and forms so-called matrix decomposition zones (MDZ) (Grandel and Schulte-Merker, 1998; Witten and Huysseune, 2007) (Figure 1).



In this study, we evaluate the distribution of fibronectin during the condensation and differentiation phase of cartilaginous elements of the splanchnocranium and of the dorsal, caudal, pelvic and pectoral fins. Taking advantage of the known sequence of cartilage development in zebrafish, and using an immunohistochemical stain for collagen type II to ascertain the timing of differentiation of the cartilage, we show that fibronectin is absent in zebrafish cartilage condensations. Furthermore, the pectoral fin disk cartilage is unique not only because of its specific way of development, but also because of its strongly fibronectin-positive ECM.

Material and Methods

Zebrafish specimens used for this study ranged from 48 h post-fertilization (hpf) to 45 days post-fertilization (dpf). Animals were raised at a standard temperature of 28.5°C in E3 medium (Kimmel et al., 1995). They were fed with dry food starting from 5 dpf until 13 dpf, after which they were fed with freshly hatched Artemia. For staging purposes, age was used for specimens up to 1 week old; for later stages, notochordal length (NL) was used. To study the condensation phase, we selected the branchial arch cartilages (48-60 hpf), the pectoral girdle and fin disk (35-70 hpf), the pelvic fin cartilages (5.4-7.0 mm NL) and the distal radials of the pectoral fin (6.0-8.0 mm NL), which are formed during the subdivision of the pectoral fin disk. The developmental sequence of these elements was described in detail (De Beer, 1937; Kimmel et al., 1995; Cubbage and Mabee, 1996; Schilling and Kimmel, 1997; Grandel and Schulte-Merker, 1998).

Figure 1
Schematic overview of the reorganization of the zebrafish pectoral fin endoskeleton. Abbreviations: A: actinotrichia, C: cleithrum, CSZ 0-3: cartilage subdivision zone 0-3, DR: distal radial, FD: fin disk cartilage, LP: lepidotrichia, PR1-4: proximal radial 1-4, SC: scapulocoracoid cartilage, POP: postcoracoid process, PRP: precoracoid process.

We used a mouse anti-collagen type II antibody (II-6II3, Hybridoma Bank) to determine the exact moment of commitment to a cartilaginous phenotype or differentiation stage, for each of the elements studied (Yan et al., 1995), i.e. to identify the developmental stage of a certain specimen in the well-known developmental sequence of the zebrafish endoskeleton. This allowed to link fibronectin data even to the condensation stage of chondrogenesis, which is not yet characterized by collagen type II. It is, for example, safe to assume that in Figure 2 (a-c) the fourth branchial arch is in condensation phase, since collagen type II is present in all pharyngeal arches except arch four which normally develops only a few hours after the third branchial arch. The distribution of fibronectin can thus be linked to a specific phase of cartilage development, by means of a double immuno-histochemical detection of both collagen type II and fibronectin.

Specimens used for light microscopy were fixed in a mixture of paraformaldehyde (PFA) and glutaraldehyde, decalcified in 0.1 M EDTA added to the fixative, and processed for embedding in epon as described in Huysseune and Sire (1992). Subsequently, semi-thin serial sections of 2 μm were prepared and counterstained with toluidine blue.

Specimens used for immunohistochemistry were fixed in 4% PFA for 24 h at 4°C, dehydrated and stored in methanol at -20°C. Prior to staining, embryos or larvae were rehydrated. For larvae larger than 5.0 mm NL, the head, and the caudal, dorsal and pectoral fins were dissected to allow optimal tissue orientation under the confocal laser scanning microscope (CLSM). The embryos, larvae or dissected parts were then permeabilized using a 15 min treatment with 0.1% proteinase K in PBS at 25°C and a 20 min treatment with 2% hyaluronidase in PBS. After rinsing and blocking (1% BSA and 1% DMSO in PBS), they were incubated overnight at 4°C with the primary antibodies (polyclonal rabbit anti-fibronectin (Sigma-Aldrich), mouse anti-collagen II (Hybridoma Bank), both diluted 1 / 200 in blocking solution). After rinsing, the samples were incubated at 25°C for 2 h with the secondary antibody (Alexa anti-rabbit 488, Alexa anti-mouse 595 (Invitrogen), both diluted 1 / 300 in

blocking solution). After final rinsing the specimens were placed in a 80% glycerol solution and observed using the CLSM.

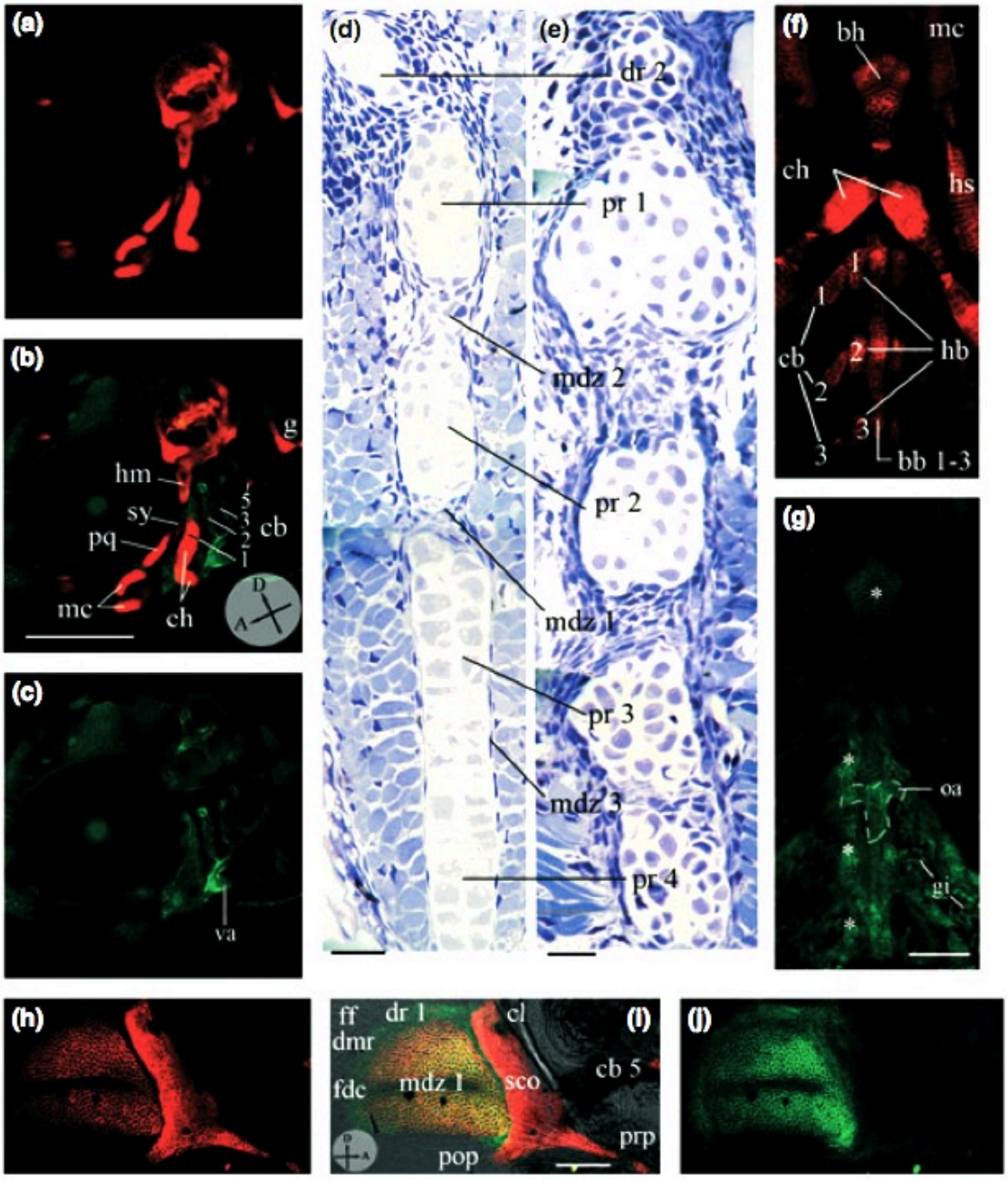
For staining controls the same protocol was applied, without the use of a primary antibody. The double staining protocol was controlled by using each antibody separately on two specimens and comparing these signals with a double stained specimen.

Results

Fibronectin in cartilage condensations

Immunostaining for collagen type II showed that, during the second and third day post-fertilization, the elements of the splanchnocranium arise in a specific sequence, consistent with that described in the literature (De Beer, 1937; Kimmel et al., 1995; Cubbage and Mabee, 1996; Schilling and Kimmel, 1997). Having established collagen type II immunostaining as a tool to distinguish negative cartilage condensations from positive differentiation phase cartilage, we used a double immunostaining for fibronectin and collagen type II to assess if fibronectin was present in cartilage condensations (Figure 2). We found that the fibronectin signal in the cartilage condensations of the splanchnocranium could not be discriminated from background staining (Figure 2a-c, compare to f-g). This unspecific background staining was also observed in the condensation stage of the pectoral girdle and fin disk cartilage, which arises between 35 and 70 hpf, and in the condensations of the pectoral distal radials and the pelvic fin elements, which arise a few weeks later, at approximately 6.5 mm NL.

Staining for fibronectin above the level of background staining was observed starting at 50 hpf in the successive branchial arches (Figure 2c). However, this signal did not coincide with collagen type II staining. Detailed observation of the fibronectin-positive structures showed that the structures from each branchial arch connect medio-ventrally and medio-dorsally, thus strongly reminiscent of the arrangement of the splanchnocranial blood vessels at that time in development, described in Isogai et al., (2001). In later stages the vessels of the splanchnocranium grow and their lumen becomes apparent



(data not shown). Simultaneously, the fibronectin signal extends into the forming gills, which remain stained in all later stages tested (Figure 2g).

Fibronectin in differentiated cartilage

Shortly after acquiring collagen type II staining, the cartilage elements (i.e. splanchnocranium elements, dorsal, caudal and pelvic fin cartilages and the pectoral girdle) showed a fibronectin signal, which was faint when

Figure 2
(a-c) show a ventro-lateral view of the splanchnocranium at 60 hpf, with immunostain for (a) collagen type II and (c) fibronectin, figure (b) shows the merged image of (a) and (c). Orientation arrows point to anterior (A), and dorsal (D). Both left and right Meckels' cartilage (mc) and hyomandibula (hm) are visible; 1st ceratobranchial (cb 1) is located underneath the ceratohyal (ch) and is indicated by a black line. (d-c) are semi-thin sections of the pectoral fin of an 8 mm (d) and 10 mm (e) NL larva, stained with toluidine blue. Lines connect similar structures. (f-g) show a ventral view of the splanchnocranium at 18 dpf, 6.2 mm NL, with immunostain for (f) collagen type II, (g) fibronectin. In (g) the asterisks show the fibronectin-positive basihyal and hypobanchial 1-3 and the dashed line indicates the opercular artery (oa). (h-j) show a confocal optical section of dissected pectoral fin of a 6.2 mm NL larva, with immunostain for (h) collagen type II, (i) merged image of (h) and (j), supplemented with transmitted light, and (j) fibronectin. Orientation arrows point to the larvas anterior (a) and dorsal (d) side. Other abbreviations: bb, basibranchial; bh, basihyal; cb 1-5, ceratobranchial 1-5; ch, ceratohyal; cl, cleithrum; dr 1-2, distal radial 1-2; dmr, distal mesenchymal rim; fdc, fin disk cartilage; ff, fin fold; g, girdle; gi, gill blood vessels; hb 1-3, hypobanchial 1-3; hs, hyosymplecticum; mc, Meckelian cartilage; mdz 1-3, matrix decomposition zone 1-3; pop, postcoracoid process; pq, palatoquadrate; pr 1-4, proximal radial 1-4; prp, precoracoid process; sco, scapulocoracoid; sy, symplectic. Scale bars in (b) for (a-c), in (g) for (f-g) and in (I) for (h-j) = 100 μm. Scale bars in d and e = 10 μm

compared to the signal observed in the developing branchial vessels and gills. However, approximately one week after the cartilage elements attained their differentiated phenotypes, this signal was reduced to the perichondral areas. For example, the cartilages in all seven branchial arches had a fibronectin-positive matrix at 5 and 7 days post-fertilization (data not shown). At 15 dpf and beyond only the perichondrium retained the signal, which appeared to be strongest in the joint areas. Between 15 and 20 dpf, only the basihyal, hypobranchial 1, 2 and 3 and hypobranchial 1-3 retained a positive signal within the cartilage matrix (Figure 2g-f).

In contrast to these cartilages, which lose their weak fibronectin signal shortly after their differentiation, the fin disk cartilage maintained a positive signal until 9.5 mm NL, i.e. when the four proximal radials are completely formed. Between 15 and 20 dpf, at approximately 5.5 mm NL, i.e. a few days prior to the start of the subdivision of the fin disk into the four proximal radials,

the first of two rounds of chondrocyte divisions occurs in the disk. At this time, the fibronectin signal increased in intensity, reaching its peak when the first matrix decomposition zone (MDZ 1) appears and starts the subdivision process (6.0 mm NL). This MDZ 1 is then clearly visible as a zone negative for both collagen type II and fibronectin (Figure 2h-j). During the completion of the second and third MDZ, however, the cartilage matrix of the disk itself gradually loses its strong fibronectin signal, leaving the individualized proximal radials negative for fibronectin, with the exception of their perichondrium.

The cartilage elements of the dorsal, caudal and pelvic fins showed a fibronectin signal in their matrix, similar to what was observed in the splanchnocranium. The signal appeared only after the cartilage attained differentiation and was hardly distinguishable from background staining (data not shown). Their perichondral areas retained a positive signal until the formation of perichondral bone, which is clearly indicated by the loss of collagen type II staining.

The negative controls showed weak and unspecific fluorescence for both the fibronectin and collagen type II antibody. Staining with one antibody revealed no difference in either fibronectin or collagen type II distribution compared to double staining.

Discussion

Fibronectin in cartilage condensations

Our observations concerning the distribution of fibronectin indicate the apparent lack of this protein during the condensation phase of zebrafish cartilage development. This is in contrast with observations in tetrapods, where fibronectin has been reported to be strongly up-regulated at this phase of chondrogenesis (Kulyk et al., 1989). This difference in the distribution of fibronectin could be related to the significant difference in developmental rate, which is remarkably fast in zebrafish, even compared to other teleosts, but relatively slow in tetrapods, especially in mammals.

In the fast developing zebrafish, most of the cartilage elements arise as a stack of pre-chondrogenic cells organized in tube- or plate-like fashion. Such a stack is initially only one cell wide in cross section. Later, this specific cell organization can be lost due to proliferation of the chondrocytes, but these divisions generally do not affect the overall shape of the element (Kimmel et al., 1998). Pre-condensation mesenchyme is probably capable of establishing itself as a small but well defined cartilage condensation just by altering cell-cell adhesion, without requiring any condensation specific ECM. One possibility is the switch to N-CAM and N-cadherin based cell-cell adhesion (Liu et al., 2003; Baker, 2005), which is also an important factor in the establishment of tetrapod condensations (Hall and Miyake, 1995; Hall and Miyake, 2000). However the fact that shortly after the appearance of collagen type II and thus element differentiation, a transient presence of fibronectin was detected in all the studied elements, suggests that fibronectin is still part of the cartilage developmental program in the zebrafish.

In the tetrapod cartilage condensations that have been studied so far, on the other hand, usually expand until they are many cells wide in cross section. The cells inside these large condensations may therefore require a more elaborate, condensation specific and fibronectin rich ECM to ensure cell survival. Thus, we propose that the distinctive distributions of fibronectin in zebrafish and tetrapod condensations can be caused by the difference in developmental rate, which is linked to the development of smaller condensations, thereby resulting in different needs for the extracellular environment.

Fibronectin in differentiated cartilage

The second major observation of this study is that fin disk cartilage develops an ECM with an intensive fibronectin signal, whereas other cartilage elements show only a weak fibronectin signal in early differentiation, which gradually disappears. The fibronectin signal in the fin disk increases in strength during the third week post-fertilization, when fin disk reorganization starts with a first round of chondrocyte division. However, once the four

proximal radials are individualized by the formation of three MDZs, the signal disappears. This disappearance of fibronectin staining in the proximal radials coincides with the disruption of the typical double-layer stack of chondrocytes and the appearance of a more conventional chondrocyte arrangement, as in the other differentiated cartilages examined (compare the proximal radials (pr) between Figure 2d,e).

These data indicate that the fin disk cartilage is different from the other studied cartilaginous elements of the zebrafish endoskeleton, not only because of its specific development (subdivision of a continuous plate into four elements), but also because of its strong and sustained fibronectin-positive staining of the ECM. Our observations suggest that the pectoral fin disk cartilage is retained in an early differentiation phase until the functional, but premature larval fin is remodeled into its adult morphology.

Interestingly, Yan et al. (2002; 2005) found evidence for the different nature of the pectoral fin disk cartilage by examining the two *sox9* paralogs present in zebrafish. They showed that these important cartilage transcription factors, which are only represented by one orthologous gene in tetrapods, are differentially expressed in the zebrafish endoskeleton. Whereas *sox9a* is expressed in the splanchnocranium and pectoral girdle cartilage, *sox9b* shows expression in the mesenchyme surrounding the splanchnocranium and the pectoral fin disk cartilage. When sox9a activity is compromised by mutation or morpholino injection, the only cartilage element that appears unaffected is the pectoral fin disk cartilage (Yan et al., 2002; Yan et al., 2005). First, this differential regulation of the pectoral fin disk is consistent with our data on sustained fibronectin presence and prolonged stack configuration, suggesting that the fin disk cartilage is unique within the zebrafish skeleton. Second, the major differences in pectoral development between tetrapods and derived actinopterygians, such as the zebrafish, could be explained by this differential use of these duplicated regulatory genes.

In conclusion, our fibronectin data provides interesting perspectives to study the influence of developmental rate on cartilage development and the evolution of pectoral fin development.

Acknowledgements

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3.2. The Mechanism of Cartilage Subdivision in the Reorganization of the Zebrafish Pectoral Fin

by

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The Mechanism of Cartilage Subdivision in the Reorganization of the Zebrafish Pectoral Fin

Abstract

A cartilaginous pectoral fin endoskeleton in zebrafish (*Danio rerio*) develops early, after which the cartilage of the larval fin endoskeleton undergoes a complete transformation into the adult morphology. This transformation includes multiple subdivisions of a single cartilaginous disk. The type of cartilage subdivision is unique to teleost fish. In this study, we present the timing and the developmental features of these subdivisions and we discuss variation in this process, caused by differences in growth rate. We establish that the cartilage subdivisions are developmentally linked to the formation of lepidotrichia in the fin fold. At the cellular level, we show that neither apoptosis nor resorption by chondroclasts and/or macrophages contributes to the cartilage subdivision. Ultrastructural observations show dedifferentiation of chondrocytes in subdivision zones. Different from forelimb development in other vertebrates, dedifferentiation is an important mechanism in the development of the adult pectoral fin skeleton. We here provide further support for the idea that the phenotype of skeletal tissues is not terminal and that plasticity of differentiated connective tissues can play an important role in various developmental and homeostatic processes.

Introduction

The development of the pectoral appendages is highly conserved among vertebrates. From the fins of sharks to the wings of birds, these appendages first form as a bud of lateral plate mesoderm. In all vertebrates, the epithelium overlaying this bud forms an apical ectodermal ridge, which stimulates the outgrowth of the bud (Mercader, 2007; Witten and Huysseune, 2007). In the center of these buds the cartilage endoskeleton forms, separating a dorsal and a ventral population of muscle precursors that originate from the paraxial mesoderm (Neyt et al., 2000).

In chondrichthyans and basal actinopterygians, three basal skeletal elements articulate proximally with the girdle and distally with a large number of cartilaginous radials. In sarcopterygians, only the caudalmost basal element, called the metapterygium, persists. In tetrapods, the associated distal cartilages form in a highly conserved pattern, with only two elements in the middle section and a variable number of digits in the most distal section, the autopodium. In derived actinopterygians, such as teleosts, the endoskeleton of the pectoral fin is also reduced. Only four cartilaginous radials remain, believed to be homologous with the distal elements of the central basal element of the ancestral fin, the mesopterygium (van Eeden et al., 1996; Grandel and Schulte-Merker, 1998).

Although the endoskeleton in teleost pectoral fins is reduced compared with more basal actinopterygian fins and even tetrapod limbs, its development shows some unique characteristics. These features are believed to be related to the fast development of teleost embryos into actively feeding larvae, which use their pectoral fins for locomotion very early in life (Thorsen et al., 2004). In zebrafish, the cartilage elements of the pectoral fins start to form together with the splanchnocranium at about 48 hours post-fertilization (hpf) (Kimmel et al., 1995). The larval pectoral fin endoskeleton is different from the four radials seen in adult pectoral fins. Similar to all other teleost larvae, early pectoral fins are supported by a single cartilaginous disk, which is continuous with the early girdle cartilage (Grandel and Schulte-Merker, 1998). Only when the vertebral bodies and the skeleton of the median and pelvic fins start to form, do these

larval pectoral fins transform into their adult morphology. In this process, four cartilage subdivision zones (CSZs) are formed which separate the disk from the girdle and individualize the four radials (Figure 1).

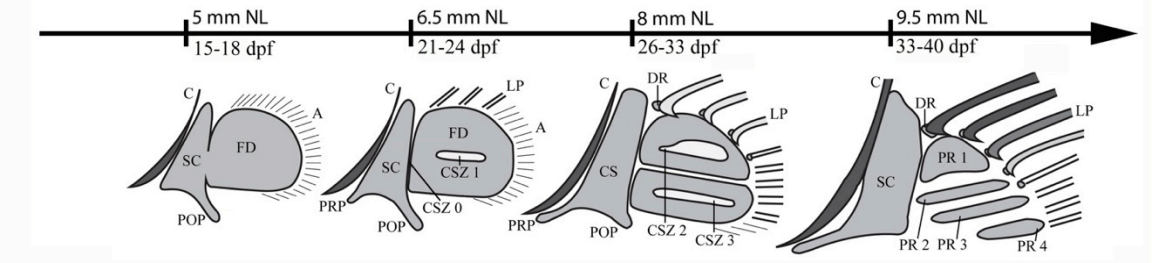


Figure 1
Schematic overview of the reorganization of the zebrafish pectoral fin endoskeleton. Abbreviations: A: actinotrichia, C: cleithrum, CSZ 0-3: cartilage subdivision zone 0-3, DR: distal radial, FD: fin disk cartilage, LP: lepidotrichia, PR1-4: proximal radial 1-4, SC: scapulocoracoid cartilage, POP: postcoracoid process, PRP: precoracoid process.

Subdivision of cartilaginous skeletal elements is common in vertebrates. A preskeletal disk subdivides to form the pectoral radials in basal actinopterygians (Cohn et al., 2002; Davis et al., 2004). In tetrapods, the different phalanges in the autopodium individualize through subdivisions of the digit at the level of the future interphalangeal joints (Casanova and Sanz-Ezquerro, 2007). However, in all these cases, subdivisions occur during the condensation phase, i.e. when a group of cartilage precursors is established from mesenchyme (Hall and Miyake, 2000). In contrast, the teleost pectoral fin subdivisions occur in differentiated cartilage (Mabee and Trendler, 1996; Grandel and Schulte-Merker, 1998; Dewit et al., 2010). How this is achieved at a cellular level is currently not known.

The aim of this study is to characterize the transformation of the simple but fully functional larval pectoral fin endoskeleton into the differentiated adult fin endoskeleton. Moreover, we attempt to elucidate the cellular mechanisms that are responsible for the process of cartilage subdivision, by using a variety of whole-mount staining techniques, enzyme histochemistry and conventional light and transmission electron microscopy (TEM). We observe that the cartilage subdivisions, which constitute a major step in the reorganization

process, are developmentally linked to the formation of lepidotrichia in the fin fold. Apoptosis and cartilage resorption by chondroclasts or macrophages, processes that are responsible for the replacement of cartilage by bone during long bone development in tetrapods and in teleost fish (Bronckers et al., 2000; Witten and Huysseune, 2009), are not involved. In contrast, all evidence suggests that chondrocyte dedifferentiation is responsible for subdividing the cartilage. Overall, this article sheds new light on the events that facilitate this skeletal reorganization and serves as a basis for further research on the molecular and epigenetic mechanisms that control this subdivision process.

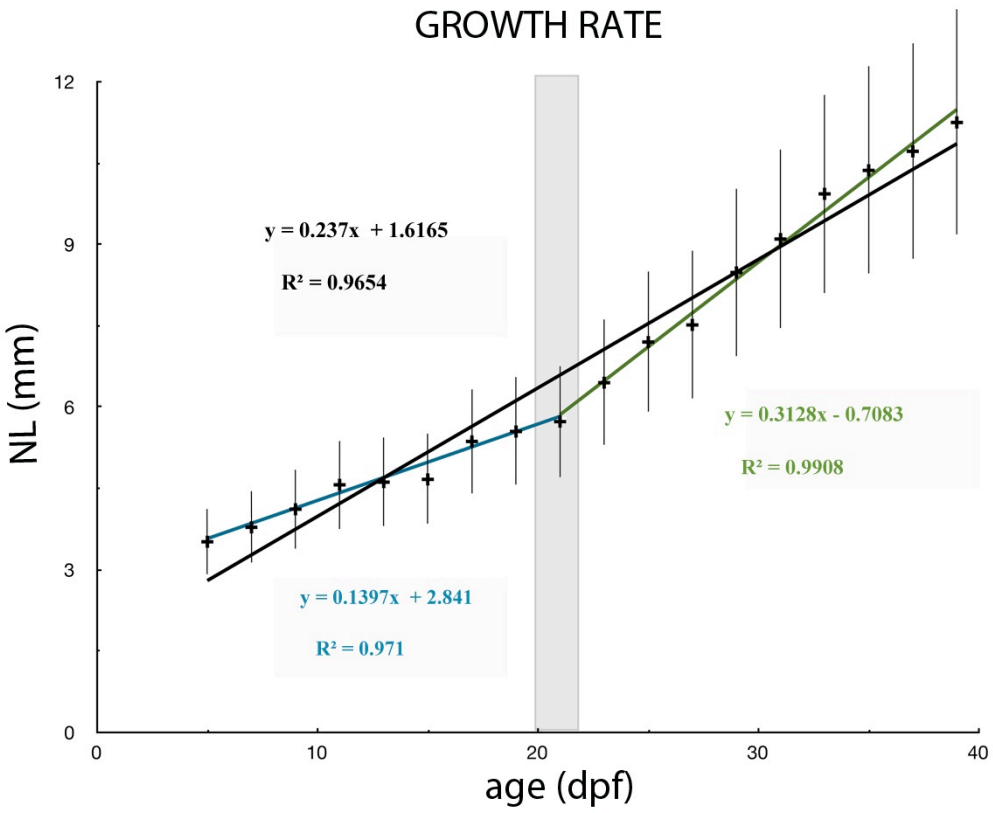
Material and Methods

Wild type zebrafish (*Danio rerio*, Cyprinidae, Teleostei) embryos were raised in E3 medium (Brand et al., 2002) at a standard temperature of 28.5°C. After 6 days post-fertilization (dpf), the larvae were transferred to permeable containers, suspended in a large aquarium filled with previously aerated tap water at 26°C. From 5 dpf until 14 dpf, the larvae were fed with dry food (ZM 000) (ZMsystems, Winchester, UK) twice a day. At 12 dpf, freshly hatched *Artemia salina* nauplii replaced the dry food and were given twice a day for the remainder of the rearing time.

Before fixation all larvae were euthanized with ethyl 3-aminobenzoate methanesulfonate salt (MS222; Sigma-Aldrich, St. Louis) and their standard length, respectively notochordal length (NL), was measured (Cubbage and Mabee, 1996). Since fish of equal age can show considerable developmental variation, NL measurements provide a more accurate staging of the larvae (Grandel and Schulte-Merker, 1998). The measurements were taken with an ocular micrometer on a MZ APO stereomicroscope (Leica, Heerbrugg, Switzerland). An average growth curve was established by collecting NL measurements from ten larvae randomly sampled every 2 days starting from 5 dpf to 39 dpf. This data was then plotted in a graph with age on the x-axis and the average NL on the y-axis (Figure 2).

Acidic-free whole mount alcian blue/alizarin red staining was performed according to Walker and Kimmel (2007). Specimens (n=120, between 4.8 and

12.0 mm NL / 12-50 dpf) were fixed in 4% buffered paraformaldehyde (PFA) for 24hr at 4°C. Subsequently, specimens were rinsed with demineralized water and dehydrated in 50% ethanol for 10 min. The ethanol was replaced by the staining solution that consists of a part A and B mixed just before staining with relative proportions of 100/1. Part A contains: 5ml of 0.4% alcian blue in 70% ethanol, 70ml of 95% ethanol, 200nM $MgCl_2$, and 25ml of demineralised water. Part B is 0.5% alizarin-red S dissolved in demineralized water. Staining occurred overnight, at room temperature. Specimens were subsequently rinsed with 70% ethanol and gradually rehydrated. Then specimens were rocked in bleach solution (1% KOH and 25% glycerol), which was replaced every 3 days until the specimens turned transparent. For detailed observation and photo documentation of the fin endoskeleton the pectoral fins were dissected and mounted in glycerin between two coverslips, sealed with paraffin. Pictures were taken using a light microscope (AXIO Imager Z1, Zeiss, Göttingen, Germany) fitted with an Axiocam camera. Series of partially focused images were merged with Helicon Focus software (version 4.6).



Specimens (n=10, between 4.8-12.0 mm NL / 12 - 50 dpf) used for light microscopy were fixed in a mixture of 2% paraformaldehyde (PFA) and 2.5% glutaraldehyde, decalcified in 0.1 M EDTA added to the fixative, and processed for embedding in epon as described in Huysseune and Sire (1992). Subsequently, semi-thin serial sections of 2 µm were prepared on a HM 360 microtome (Micron, Walldorf, Germany) and counterstained with toluidine blue (0.5% in 1% borax).

Specimens (n=10, between 4.8-12.0 mm NL / 12-50 dpf) for transmission electron microscopy (TEM) were fixed overnight at 4˚C in 2% paraformaldehyde, 2.5% glutaraldehyde, 0.5% $CaCl_2$ and 0.1 M EDTA in 0.2 M sodium cacodylate buffer. Specimens were further processed as described in Bert et al. (2003). After fixation they were rinsed for 8 h in 0.134 M sodium cacodylate buffer. Postfixation took place in reduced osmium, a mixture of 1 ml OsO_4 (4%), 3 ml Na cacodylate (0.134 M) and 66 mg $K_3Fe(CN)_6$, for 36 h at pH 7.4. After rinsing with double distilled water the specimens were dehydrated in 50%, 75%, 90% and absolute ethanol to which $CuSO_4$ bars were added to remove any remaining water. The specimens were subsequently infiltrated with a low-viscosity embedding medium (Spurr, 1969). Ultrathin (50-90 nm) sections were cut on a Reichert UltracutS ultramicrotome (Leica, Vienna, Austria) with a diamond knife (Diatome Ltd., Biel, Switzerland) and mounted on formvar-coated single slot copper grids (Agar Scientific, Stansted, UK). The sections were stained (EM stain, Leica) with uranyl acetate and lead citrate and viewed with a Jeol JEM-1010 (Jeol Ltd, Tokyo, Japan) transmission electron microscope operating at 60 kV. Photographs were digitalized using the DITABIS system (Pforzheim, Germany).

Figure 2
Notochordal length (NL) as a function of age; measurement of 180 larvae (5-39 dpf, ten larvae sampled every 2 days). Average at each age is marked by a +. The black curve and equation represent growth rate over the entire data set, whereas the blue and green curve and equations represent the larval and juvenile growth rates, respectively. The period in which the pectoral fin endoskeleton starts to transform and the vertebral bodies and median fins first appear, is marked by the grey box. Vertical lines represent standard deviations.

Specimens for BrdU proliferation assay were selected to represent the entire period of lepidotrichia formation (n=9). They were submerged tree times in 0.5% BrdU, in E3 medium (Brand et al., 2002), for two hours with six hours interval. After the third BrdU incorporation specimens were fixed in 4% PFA and processed for paraffin embedding. After rinsing the specimens were dehydrated in 30%, 50%, 70%, 80%, 90% and absolute ethanol. Subsequently specimens were submerged in two 6 h parasolve baths, before two paraffin impregnations at 60˚C, of 8 h each. Specimens were then properly orientated and the paraffin was allowed to harden. Sections of 5 µm were made, mounted on uncoated slides and dried at 40˚C.

Detection of the incorporated BrdU starts by deparaffinating the sections. Sections are heated to 70˚C for 5 min, allowed to cool down for an other 5 min and heated again for 5 min, before submersion in two parasolve baths for 15 and 10 min. Subsequently sections were rehydrated in 100%, 90%, 80%, 70% and washed 3 times in PBS-D (1% DMSO in PBS). Then chromatin was precipitated by a 30 min HCL (2M) bath. This reaction was stopped by a 5 min incubation in borax (1,89 dilution of 0.1M $B_4Na_2O_7.10H_2O$ solution). Sections were washed with PBS-D and submerged in block solution (3% BSA, 1% milk powder in PBS-D) for 2h. Then the primary antibody, rat anti-BrdU (Abcam, Cambridge, UK), diluted 1/100 in block solution was put on over night at 4˚C. Subsequently sections were washed tree times for 5 min with PBS-D, before the secondary antibody, Alexa 488 anti-rat (Invitrogen, Carlsbad, USA) was put on, diluted 1/200 in block solution. Sections were rinsed and counterstained with DAPI (1/300 dilution of 5µg/ml stock solution) (Invitrogen, Carlsbad, USA). After final rinsing slides were cover slipped with vectashield (Vector Laboratories Inc., Burlingame, USA) and photographs were made using an epifluorescent microscope (AXIO Imager Z1, Zeiss, Göttingen, Germany) fitted with an Axiocam camera.

Similar to the situation in mammals, acid phosphatase activity can be used as a marker to detect the activity of chondroclasts and macrophages in teleost fish (Witten et al., 1998; Witten et al., 2001). Detection of the activity of these enzymes was performed on specimens (n=15, between 5.5-8.8 mm NL

/ 17-35 dpf) fixed in cold 100% acetone, which were subsequently stored at -20˚C. Specimens were processed according to Witten and Villwock (1997). They were rehydrated, decalcified in 10% buffered EDTA for 48 h, rinsed, dehydrated in graded acetone solutions, and impregnated with methacrylate monomer solution (2-hydroxyethyl-methacrylate 80 ml + 200 ppm p-methoxyphenol + ethylene glycol monobuthyl ether 12 ml + benzoyl peroxide 270 ml) for 60 min. For the second step of impregnation a monomer solution was used for 24 h. For embedding 2% catalyst (N,N- dimethylaniline 1 ml + polyethylene glycol-200, 10 ml) was added to the monomer solution. Specimens were then embedded in polyethylene jars with tight snap-on lids. Polymerization took place at 4°C for 24 h and was completed within another 24 h at room temperature. Sections of 5 µm were made, mounted on uncoated slides and dried at 20˚C.

The first step in the acid phosphatase and tartrate-resistant acid phosphatase activity detection is a preincubation of 30 min at 20°C in 0.1 M acetate buffer at pH 5.5, with 50 mM L(+) di-sodium tartrate dihydrate added to detect only tartrate-resistant acid phosphatase activity. The acid phosphatase-activity was demonstrated with an artificial substrate, naphthol AS-TR phosphate (N-AS-TR-P) and a color reagent, hexazotized pararosaniline (PRS). The latter was prepared by dissolving 1 g PRS (chloride) [C I. 42500] in 19.25 ml demineralized water, adding 5.75 ml of 32% HCl, heating, and storing in the dark at 4°C. For hexazotation, 2 ml 4% $NaNO_2$ (0.58 M) was added to 1 ml of prepared PRS-solution at 20°C. The incubation solution was prepared by adding 1 ml hexazotized PRS, 600 µl 2% MgCl solution and 2 ml enzyme substrate solution (2 mg N-AS-TR-P dissolved in 2 ml N,N-dimethyl-formamide) to 30 ml 0.1 M acetate buffer pH 5.5, with an additional 100 mM di-sodium tartrate dihydrate for tartrate-resistant acid phosphatase-detection. After an incubation of 30 min at 20°C, slides were rinsed in demineralized water. Slides were subsequently counterstained with Mayers hematoxylin for 10 min, rinsed in running tap water for 15 min, flushed with demineralized water, dried at 40°C, and mounted with DEPEX. Several controls were preformed: 1) heating of the sections at 90°C for 10 min prior to

incubation, 2) incubation without substrate, 3) adding NaF (10 mM/l) to the incubation solution, which completely blocks all enzyme function. As a positive control for this protocol, observations were made on the haemal arches, which are present in all studied sections and are known areas for bone resorption and thus acid phosphatase activity (Witten et al., 2001).

Apoptosis was detected by exposing anesthetized larvae (n=15, between 5.5-8.8 mm NL / 17-35 dpf) for 15 minutes to a double stain solution, containing acridine orange (6.6 µg/ml) and propidium iodide (1µg/ml). Larvae were then mounted on their side and imaged with a confocal laser-scanning microscope, D-eclipse-C1 (Nikon, Melville N.Y., U.S.A.). Acridine orange quickly penetrates all living cells and accumulates in acid organelles (Landex and Kayser, 2004). Propidium iodide on the other hand only penetrates apoptotic cells, staining their nucleus red (Jones and Senft, 1985). These dyes were selectively visualized by exciting the same optical section separately with a 488 nm and a 543 nm laser. This makes detailed observation of vital and apoptotic cells possible. This protocol was successfully validated by examining the development of the olfactory pit at 36 hpf, an area known for its specific aggregation of apoptotic cells (Cole and Ross, 2001). After imaging, the larvae were fixed and stained with alcian blue, as described above, to determine the exact stage of cartilage subdivision.

Results

The early formation of the girdle elements and of the pectoral fin endoskeleton in the zebrafish has been well described (Grandel and Schulte-Merker, 1998) and thus only the most prominent features are briefly recapitulated below. The cartilaginous girdle or scapulocoracoid forms as a small aggregation of cells, caudal to the cleithrum. Ventrocaudally, a single row of cells protrudes into the body wall, forming the postcoracoid process. Distally, the girdle condensation extends into the external fin bud, where the cartilage precursors form a single sheet of cells. These precursors differentiate into the fin disk (FD) cartilage, which is continuous with the now

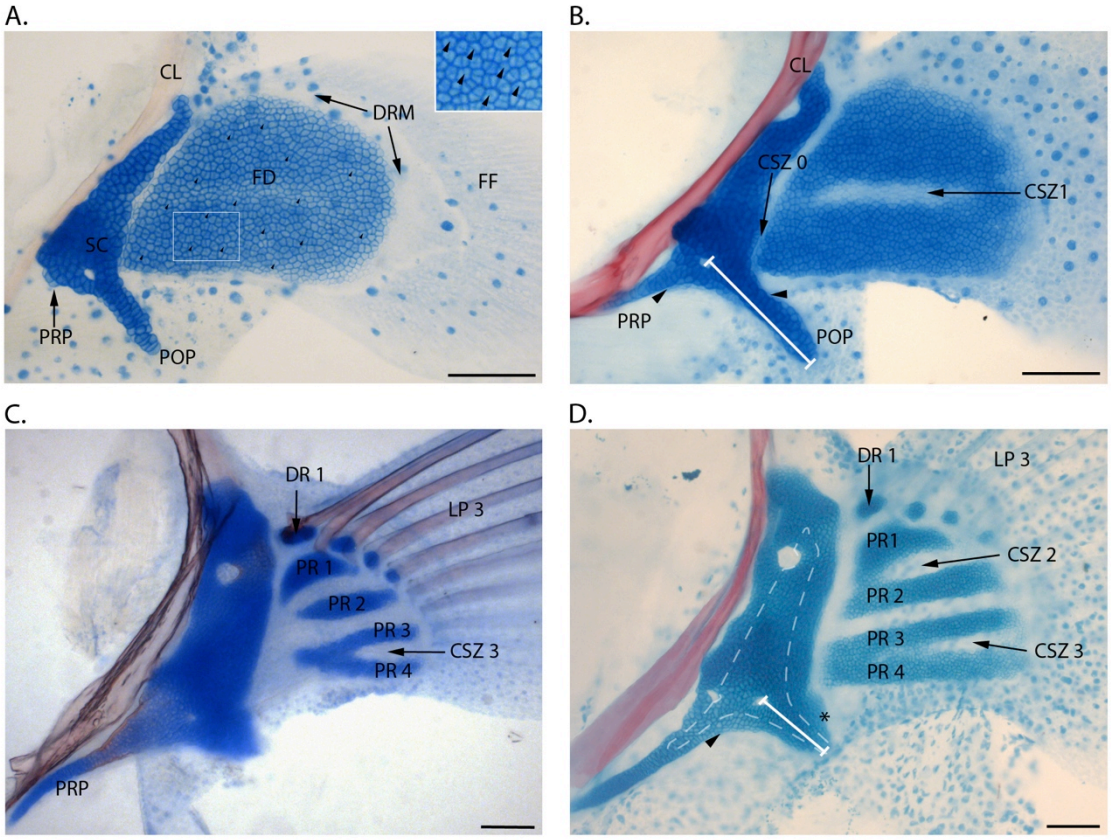
cartilaginous girdle element. The larval pectoral fin skeleton is completed at 1 week post-fertilization (wpf), when the single row of FD chondrocytes undergoes a first round of chondrocyte divisions. This coordinated round of chondrocyte divisions, described by Grandel and Schulte-Merker (1998), sweeps through the FD, creating a dorsal and a ventral row of perfectly superimposed daughter chondrocytes.

Timing and development of cartilage subdivision

Cartilage subdivisions are an essential step in the reorganization of the larval pectoral fin, but various other morphological changes contribute to this process. Below we present the development of cartilage subdivisions together with the morphological changes that precede or coincide with them.

The first morphological sign of the reorganization process is the start of appositional growth in the scapulocoracoid (Figure 3A). The addition of perichondral cells to the rostroventral side of the scapulocoracoid (5.2-5.6 mm NL / 15-18 dpf) forms the anlage of the precoracoid process. Similar cell additions result in the rostral outgrowth of this process and in an overall shape change of the scapulocoracoid (Figure 3 and 4A). At the ventral and the ventrocaudal surface of the scapulocoracoid, appositional growth (and not cartilage regression, cf. Grandel and Schulte-Merker 1998) causes the disappearance of the postcoracoid process, which was already present in the earliest anlage of the larval scapulocoracoid.

Simultaneously with the first signs of appositional growth in the scapulocoracoid, the FD grows by interstitial cell division. These chondrocyte divisions can be observed by the appearance of a new matrix septum inside the lacuna of each mother cell. These new matrix septa appear simultaneously throughout the FD, suggesting that a synchronized round of cell divisions has taken place (white arrowheads in Figure 3A). These are the first cell divisions in the FD after the initial, equally synchronized, round of chondrocyte divisions that transformed the larval, single-rowed FD into a double row of exactly superimposed chondrocytes at 1 wpf. In addition to being similarly synchronized, the second round of cell divisions (5.2-5.6 mm



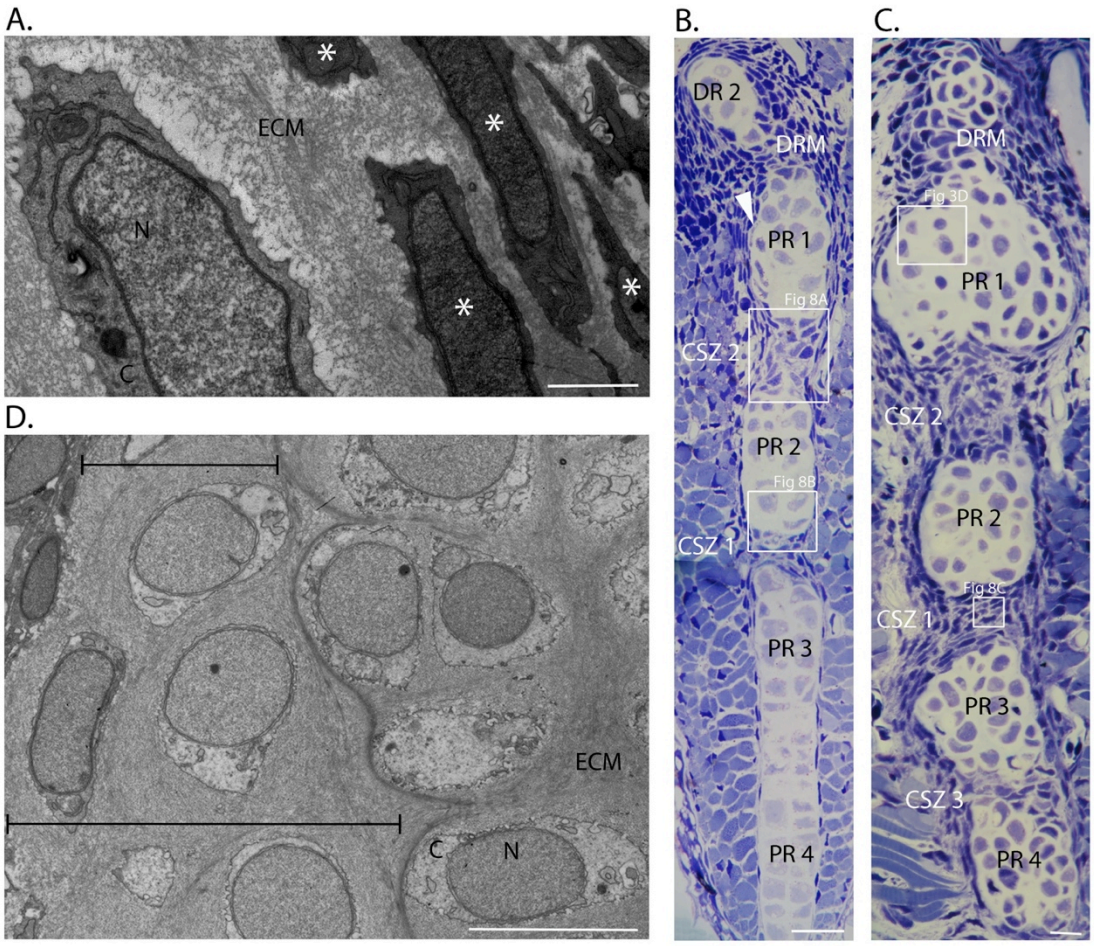
NL / 15-18 dpf) also shows uniform directionality of the cell divisions. Yet, instead of division planes being parallel to the FD plane, as in the first round, all cleavage planes are now perpendicularly oriented with regard to the FD plane. This orientation preserves the double-rowed appearance of the cartilage of the FD and results in the expansion of the disk without any thickening.

In the wake of the second proliferation round, the first two cartilage subdivisions appear. The few cells bridging the FD and the scapulocoracoid lose their affinity for alcian blue. Simultaneously with this separation from the scapulocoracoid, the central region of the FD also looses alcian blue staining (5.3-5.7 mm NL / 16-18 dpf). This is indicative for a loss of highly sulfated proteoglycans (Figure 3B). Previously, we reported that these regions of subdivision also loose collagen type II, another cartilage-specific extracellular matrix (ECM) component (Dewit et al., 2010). Although we found no differences between the events in the FD-to-girdle border and the central region of the FD, only the latter region was named matrix decomposition zone

Figure 3
Whole mount alcian blue/alizarin red staining of the pectoral fins of larvae between 5.2 and 8.2 mm NL. In (A), the pectoral fin skeleton, at 5.2 mm NL and 16 dpf, shows the first signs of reorganization. The precoracoid process (PRP) is only three cells large at this point and thin matrix septa, indicative for the second proliferation round, become visible (black arrowheads and inset). In (B) at 6.0 mm NL and 18 dpf, CSZ 0 at the girdle-to-disk border and CSZ 1 in the centre of the FD are clearly visible; also note the grown PRP. The braced white line indicates the measurement taken to assess the possible regression of the postcoracoid process. The black arrowheads point to newly added chondrocytes encircled by lighter staining ECM. In (C), the pectoral fin skeleton of a slow growing larva, at 7.3 mm NL and 32 dpf, shows CSZ 3 still in progress while CSZ 2 has been completed and the dorsal lepidotrichia (LP) are already calcified. In (D) on the other hand, the pectoral fin endoskeleton of a fast growing larva, at 8.2 mm and 24 dpf, shows both CSZ 2 and 3 still in progress and no signs of calcification in the lepidotrichia. The white braced line on the former postcoracoid process shows where the measurement was taken to determine possible regression. The dashed line demarcates the boundary of the girdle element at 6 mm NL, indicating how apposition of cells has contributed to the growth of the girdle. The black arrowhead points to a small, newly added chondrocyte embedded in lighter staining ECM. The asterisk marks the location of the TEM micrograph shown in Figure 4A, which confirms the occurrence of appositional growth. Dorsal is to the top and rostral is to the left in all figures. Further abbreviations: CL: cleithrum, DR1: distal radial 1, DRM: distal rim mesenchyme, FD: fin disk, FF: fin fold, SC: scapulocoracoid, POP: postcoracoid process, PR 1–4: proximal radial 1–4; CSZ, cartilage subdivision zone.
Scale bars =100 μm.

by Grandel and Schulte-Merker (1998). In this article, we will refer to these regions as CSZ 0, and CSZ 1 respectively in order to maintain the numbering proposed by Grandel and Schulte-Merker (1998). The progression of CSZ 1 eventually divides the FD into a dorsal and a ventral half. Each half is later similarly divided by the formation of another CSZ (CSZ 2 and 3, respectively; see below). In this way, three CSZs transform the FD into four rod-like proximal radials, with the first proximal radial being the most dorsal and the fourth proximal radial the most ventral (Figure 3C and D).

Before the appearance of the CSZs 2 and 3, several other events are initiated which contribute to the changes in the pectoral fin skeleton. First, the chondrocytes inside the remaining FD start another (i.e. third) round of cell divisions (6.4-6.7 mm NL / 21-23 dpf). Unlike the first two proliferation rounds,



the daughter cells resulting from this proliferation round do not show a preferential orientation. The cell divisions appear first proximodorsally in the FD and spread gradually to ventrodistal areas, as judged from the progressive loss of the stacked arrangement of chondrocytes in this direction. This was observed on stained and cleared as well as on sectioned specimens. This new series of cell divisions results in expansion and thickening of the remaining FD cartilages (Figure 4B and C).

Second, the distal rim mesenchyme at the border between the FD and the fin fold (indicated in Figure 4B and C) also shows an increase in cell numbers. This increase can be observed on semi-thin sections, first in the proximodorsal area of the FD border (6.4-6.7 mm NL / 21-23 dpf), spreading more distally along the FD border, finally reaching the ventral area of the FD. This increase in cell numbers, and its progression along the FD border, was confirmed by a BrdU proliferation assay. Slightly before the formation of

Figure 4
Light (B and C) and transmission electron micrograph (A and D) of the pectoral fin at the level of the scapulocoracoid perichondrium of a 8-mm NL larva (A) and at the level of the second distal radial (DR 2) of a 8.0-mm NL (B) and a 10.0-mm NL larva (C, D), respectively. (A) TEM image of the region indicated by an asterisk in Figure 3D, showing the appositional growth of the ventrocaudal region of the scapulocoracoid. The white asterisks point to newly added cells in a state of chondroblast to chondrocyte transition. (B) The chondrocytes of the first proximal radial (PR 1) have already divided during the third round of chondrocyte divisions while the cells in proximal radial 4 (PR 4) have not. A white arrowhead marks an area of appositional growth. (C) During the third round of proliferation, the chondrocytes divide in various directions, resulting in the loss of the double-rowed arrangement and the thickening of the radials. Also shown is the extensive thickening of PR 1 by appositional growth, which has already started in (B). (D) TEM image of the region indicated in (C), which shows the ultrastructure of PR 1. In the region of formerly stacked chondrocytes the ECM fibrils still surround several daughter cells. The region indicated with shows the ultrastructure of PR 1. In the region of formerly stacked chondrocytes the ECM fibrils still surround several daughter cells. The region indicated with black brackets on the other hand is added by appositional growth and does not show signs of earlier chondrocyte proliferation. Top of all figures points dorsally. Boxed areas in (B) and (C) refer to TEM micrographs as indicated. C: cytoplasm, CSZ 1–3: cartilage subdivision zone 1–3, DRM: distal rim mesenchyme, N: nucleus, ECM: extracellular matrix.
Scale bars in B and C=10 μm, in A and D= 5 μm.

skeletal elements in the distal rim mesenchyme and fin fold, the only proliferating cells are located proximodorsally (Figure 5; 5.3 mm specimen, region A vs. regions B and C). Then several alcian-blue-positive stripes develop in the fin fold, representing the anlagen of the lepidotrichia or bony fin rays. These start to form in the dorsal region of the fin fold just distal to the distal rim mesenchyme and grow out into the fin fold, thereby replacing the actinotrichia (Figure 3C and D). Once the anlage of a lepidotrichium has appeared, a distal radial forms as a round alcian-blue-positive element in the distal rim mesenchyme, specifically between the two half rays of the lepidotrichium (Figure 3C and D). This distal radial forms the articulation between the lepidotrichium and the proximal radial. As the formation of the lepidotrichia continues, the distal rim mesenchyme that is located more distally and ventrally along the FD shows increased cell proliferation (Figure 5, 6.7mm specimen, region C). Simultaneously, proliferation diminishes in areas where lepidotrichia have already formed (Figure 5, compare regions B and C

in the 6.7-and 8.3-mm specimen). Only the first distal radial develops slightly differently, as previously described by Grandel and Schulte-Merker (1998). Although it originates next to the FD in the dorsal distal rim mesenchyme, it expands proximally to meet the growing scapular region of the cartilaginous girdle. Where these two elements meet, the scapular cartilage forms an articulation with the distal radial 1 (compare Figure 3C and D). Furthermore distal radial 1 fuses with its lepidotrichium, which is most likely responsible for the higher mitotic index observed in this region (Figure 5, 8.3 mm specimen, region A).

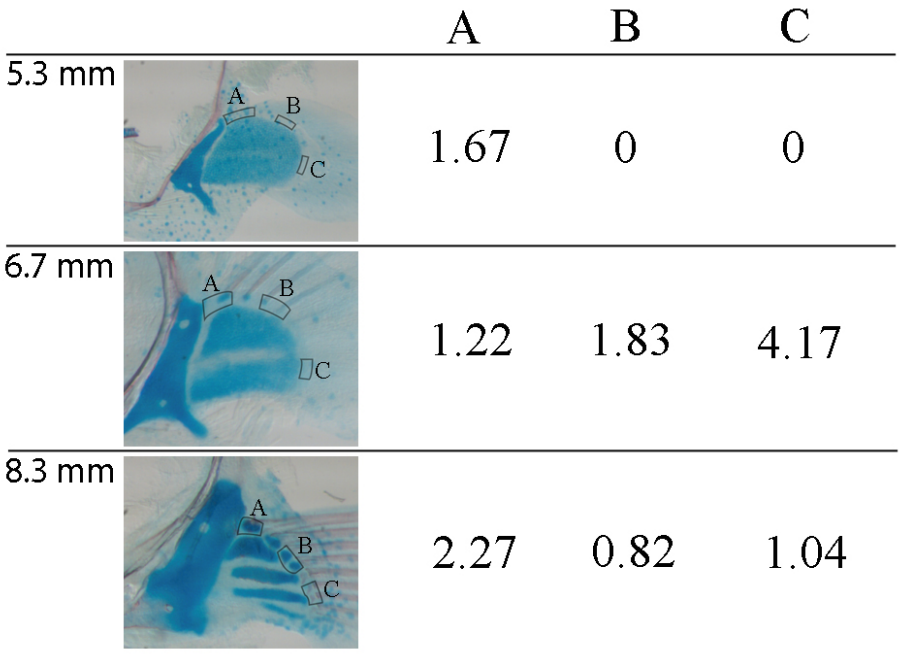


Figure 5
BrdU proliferation assay of three distinct regions of the distal rim mesenchyme at three stages of pectoral fin remodeling: immediately before remodeling (5.3mm NL), during lepidotrichia formation (6.2 mm NL) and just after all lepidotrichia have formed (8.3 mm NL). Region A reaches from the scapulocoracoid perichondrium to the distal border of distal radial 1, region B from the proximal border of distal radial 3 to the distal border of distal radial 4, and region C from the proximal border of distal radial 6 to the distal border of distal radial 7. The table contains the mitotic index of each region for the given stages (percentage of BrdU-positive cells over the total cell number).

Third, the FD enlarges by appositional growth in the outer rim adjacent to the newly forming distal radials. Appositional growth also occurs at the dorsal region of the FD, corresponding to the future proximal radial 1, resulting

in significant thickening of the FD in this area (Figure 4B and C). The appositional growth is easily identifiable in TEM micrographs, because of the different organization of the collagen fibrils in the newly added cartilage. In stacked chondrocytes, most of the ECM fibrils originate from before the third proliferation round and thereby surround several daughter cells. This is not the case for newly added chondrocytes, which are surrounded individually by matrix, and also have considerably larger ECM spaces (Figure 4D).

Simultaneously with the appositional growth of the FD and the formation of distal radials and lepidotrichia, the last two CSZs form. The timing of formation of CSZ 2 and 3 shows considerable variation between specimens with different growth rates. In slow growing specimens, the CSZs appear at the distal edge of the FD, progressing proximally (Figure 3C). The proximal radial 4 is often reduced in size or can even be absent. In contrast, fast growing larvae form CSZ 2 and 3 in the center of the remaining disk halves and proximal radial 4 has the same size as proximal radial 3. In these fast growing specimens, CSZ 2 and 3 develop simultaneously (7.9-8.3 mm NL / 26-30 dpf) (Figure 3D), while in slow growers the formation of CSZ 2 in the dorsal FD half (6.8-7.0 mm NL / 27-30 dpf) (Figure 3C) always precedes the formation of CSZ 3 in the ventral FD half (7.2-7.5 mm NL / 30-34 dpf). In general, these two CSZs were never observed before the appearance of the anlage of the lepidotrichium that forms at the same dorsoventral level. In fast growing larvae, successive lepidotrichia appear fast (starting at 7.0 mm NL / 23-24 dpf), resulting in swift replacement of actinotrichia throughout the fin fold before CSZ 2 and 3 start to form in the FD. In slow growing specimens, the anlage of new lepidotrichia and distal radials appears later (6.5 mm NL / 26-27 dpf), and spreads much slower toward the ventral region of the fin. In these specimens, CSZ 2 was never observed before the anlage of lepidotrichium 4, while CSZ 3 appears only later when the anlage of lepidotrichium 7 can be detected. The slower formation of successive lepidotrichia in slow growers is shown by the differentiation and calcification of the earliest dorsal fin rays before the anlagen of the last-appearing ventral-

most rays can be detected. In contrast, fast growers show the anlage of all fin rays well before the first rays start to calcify (compare Figure 3C and D).

Cellular mechanisms of cartilage subdivisions

During the reorganization of the pectoral fin endoskeleton, cartilage disappears in four regions: the girdle-to-FD border (CSZ 0) and the three CSZs of the FD cartilage (CSZ 1-3). Here we present data on how these cartilage subdivisions occur at the cellular level.

First, we checked for resorbtive activity by chondroclasts and/ or macrophages through the visualization of acid phosphatase and tartrate-resistant acid phosphatase activity. A distinct signal was found in areas known to undergo bone resorption, such as the haemal arches and the tooth-bearing fifth ceratobranchial (Witten et al., 2001) (Figure 6A and B). Furthermore, enzyme activity was detected in the cartilage anlage of the Weberian apparatus, indicating the resorption of this cartilage (Figure 6A). However, the four cartilage subdivision areas under investigation did not show any signal for acid phosphatase or tartrate-resistant acid phosphatase activity at any point of development (Figure 6C).

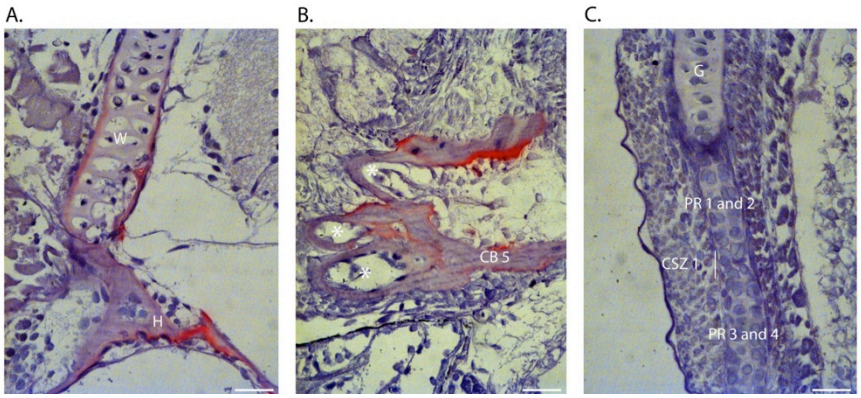


Figure 6
Acid phosphatase detection in control sections (A and B) and CSZ 1 (C). All sections shown are taken from a specimen at 25 dpf and 7.3 mm NL. (A) Detail of section with cartilage of the Weberian apparatus (W) and bone of the haemal arches (H) showing areas of acid phosphatase activity in red. (B) Detail of section showing positive staining in the fifth branchial arch (CB 5) and the attachment bone of teeth (marked with white asterisks). (C) Detail of the pectoral region in the same section as in (A) showing CSZ 1 without any detectable signal. Top of all figures points dorsally. G: girdle, PR 1 and 2: proximal radial 1 and 2, PR 3 and 4: proximal radial 3 and 4, CSZ: cartilage subdivision zone. Scale bars = 10 μm.

Second, acridine orange and propidium iodide double staining was applied to detect the presence of apoptotic cells in the CSZs (Figure 7). This technique was validated by confirming the presence of apoptotic ectodermal cells during the development of the olfactory pit at 36 hpf (Cole and Ross, 2001). The 488 nm laser visualized acridine orange and showed all viable cells in green. A red signal could be seen in the nucleus of apoptotic cells

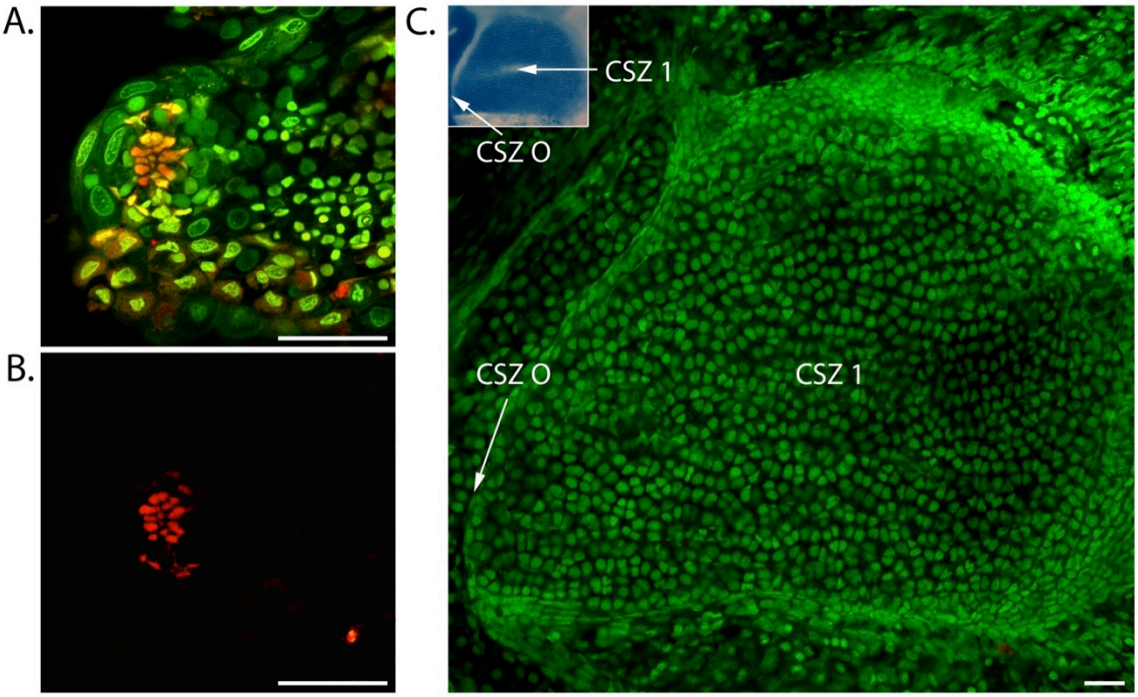
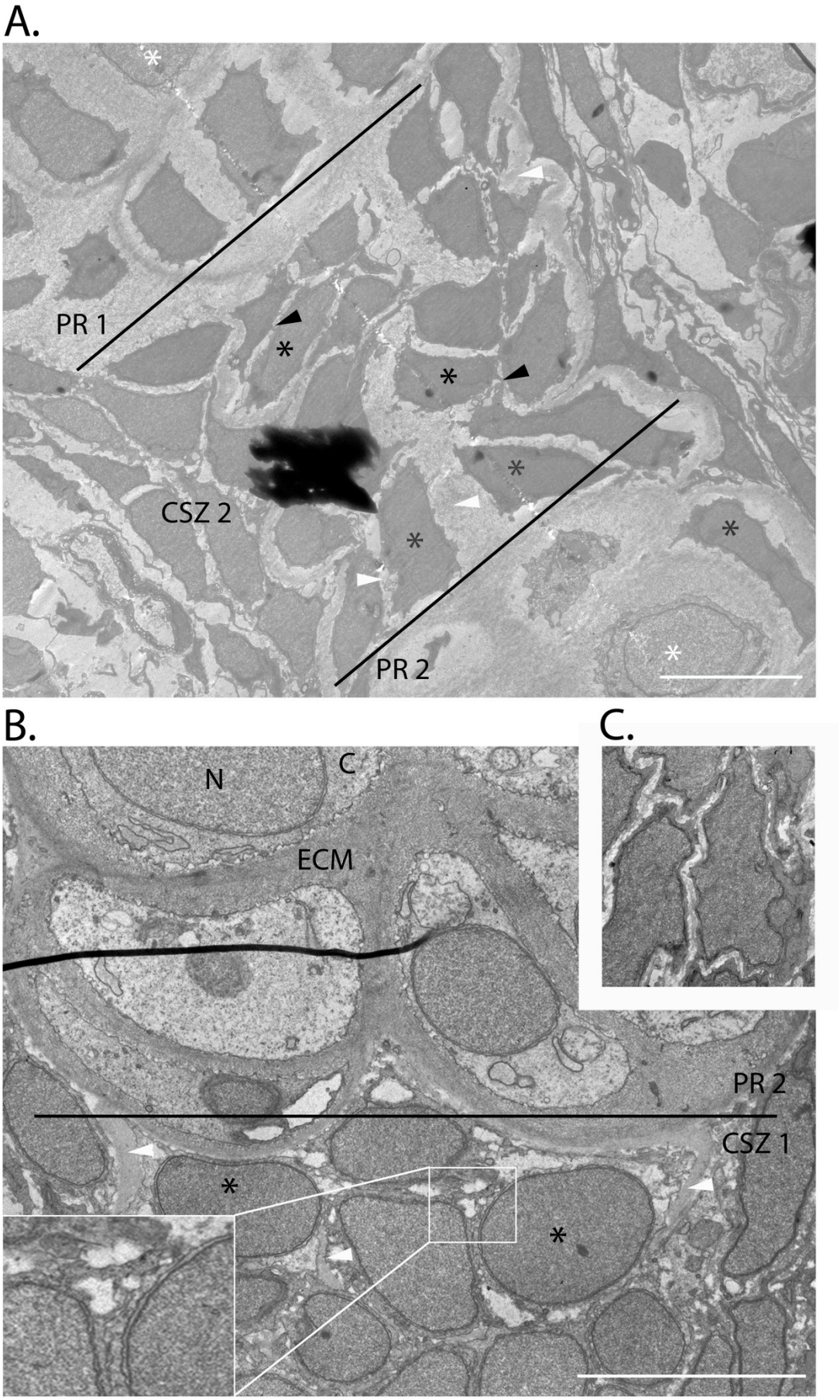


Figure 7
Detection of apoptosis in control areas (A and B) and FD (C). All images (except inset) are composed of several optical sections recorded with the confocal laser-scanning microscope and merged into a single image using Helicon Focus software. (A) Specimen at 36 hpf showing the developing olfactory pit, excited with the 488-nm laser and recorded with the green and red detector opened. Acridine orange shows all vital cell nuclei in the imaged area in green and acidic organelles in red. A yellow signal results from superimposed green and red signal and indicates the apoptotic nature of the cell. (B) shows the same optical sections as in (A) but excited by the 543-nm laser and thus showing propidium iodide staining only. This image shows the same apoptotic cells as the acridine orange staining. Note grouping of apoptotic cells in the future olfactory opening. (C) Pectoral fin of a 5.7-mm larva, recorded with both the 488- and 543-nm laser activated. This recording shows no signal of apoptosis in CSZ 0 nor in CSZ 1. Inset: alcian blue preparation of the same fin with CSZ clearly visible in the center of the FD. Dorsal to the top and rostral to the left in all figures.
FD: fin disk, CSZ: cartilage subdivision zone. Scale bars = 20 μm.

and in the cytoplasm of some epithelial cells, presumably corresponding to lysosomes (Figure 7A). When the same optical sections were visualized using the 543 nm laser, only the nuclei of apoptotic cells showed a red signal, representing propidium iodide staining. The olfactory region showed several isolated apoptotic cells and furthermore a well-defined group of apoptotic cells where the olfactory opening is formed (Figure 7B). During the development of the CSZs in the FD, neither acridine orange nor propidium iodide showed red nuclei in the remaining cartilage of the FD or in the CSZs (Figure 7C). Occasionally, apoptotic cells were detected in the pectoral fin epithelium.

Third, ultrastructural observations confirmed that the cells in the four regions of cartilage subdivision are viable and without signs of apoptosis, such as condensed chromatin or apoptotic cell bodies (Kouri-Flores et al., 2002). TEM observations allowed us also to distinguish three stages in the subdivision process. (Figure 8) First, the matrix inside the CSZs loses its fibrillar structure and becomes more homogeneous. Matrix can nevertheless be observed well beyond the point at which collagen type II (Dewit et al., 2010) and alcian blue staining (this study) disappear. The chondrocytes

Figure 8
TEM micrographs of early (A), intermediate (B), and late (C) stage of cartilage subdivision. (A) TEM image of the upper boxed region indicated in Figure 4B, showing an early stage of CSZ 2 of an 8.0-mm larva. The white asterisks label unchanged chondrocytes in the remaining proximal radials 1 and 2 (PR 1 and PR 2). Gray asterisks label former chondrocytes that are partially detached but still completely surrounded by degrading matrix (white arrowheads). The cells display an irregularly folded cell membrane; they are less voluminous and have a more electron dense cytoplasm. Black asterisks point to former chondrocytes that have broken through their former lacunae and have established cell-to-cell contacts with each other (black arrowheads). (B) TEM image of the lower boxed region indicated in Figure 4B, showing an intermediate stage of cartilage subdivision in CSZ 1 of an 8.0-mm larva. The black asterisks indicate vital former chondrocytes in the CSZ still partially surrounded by degrading ECM (white arrowheads), but with established cell-to-cell contacts (inset). (C) TEM image of the lower boxed region indicated in Figure 4C, showing a late stage of CSZ 1 in a 10-mm larva, when all cartilage matrix remnants have disappeared and resident cells show a mesenchymal cell phenotype. Dorsal to the top in all figures. ECM: extracellular matrix, C: cytoplasm, N: nucleus, CSZ: cartilage subdivision zone. Scale bars in A and B = 5 μm, scale bar in C = 1 μm.


A.
PR 1
CSZ 2
PR 2
B.
N
C
ECM
C.
PR 2
CSZ 1

surrounded by this degrading matrix are smaller, have more electron-dense cytoplasm, a higher nucleo-cytoplasmic ratio and an irregular cell shape; they are partially detached from their lacunae. In contrast, the chondrocytes in the remaining cartilage completely fill their lacunae and retain an electron-lucent cytoplasm that contains many small granules. These cells have a low nucleo-cytoplasmic ratio (Figure 8A). In a second stage of subdivision, the matrix between neighboring chondrocytes fragments and gradually disappears (Figure 8B). However, remnants of cartilage matrix facing the perichondrium remain visible for several more days. The cells inside the fragmented matrix now display an even higher nucleo-cytoplasmic ratio and show elaborate cell-to-cell contacts (Figure 8B), which are never observed in the chondrocytes of the cartilage

persisting elsewhere in the FD. In the third and final stage of subdivision, no more matrix remnants can be found (Figure 8C). The former subdivision zones, which can now be called interradial areas, show connective tissue with fibroblasts and a perichondrium has formed around each proximal radial. The changes that we observed in the matrix and chondrocytes of the subdivision zones are represented schematically in Figure 9.

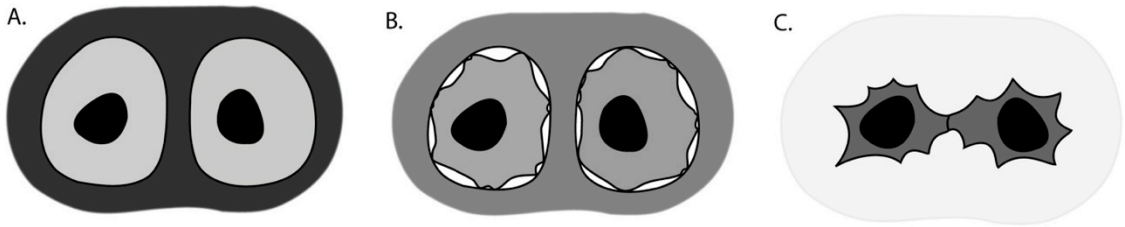


Figure 9
Schematic representation of dedifferentiation in chondrocytes in the CSZs. In (A), two chondrocytes are shown before subdivision. Their nucleus is presented in black and their cytoplasm is light grey. These cells completely fill their lacunae in the cartilage matrix, in dark grey. In (B), the earliest signs of subdivision are presented, degrading matrix is indicated by a lighter grey, the cell cytoplasm becomes more electron-dense and the cells do not fill their lacunae since they detach from the matrix. In (C), subdivision is completed, the cartilage matrix has disappeared and the cells resemble fibroblasts, with high nucleo-cytoplasmic ratio, electron-dense cytoplasm, and cell-to-cell contacts. CSZ: cartilage subdivision

Discussion

This article characterizes the cartilage subdivisions in zebrafish that transform the larval pectoral fin endoskeleton into its adult morphology. Although cartilage subdivisions are common in vertebrate skeletal development, the subdivisions in the teleost pectoral fin endoskeleton are unique because they occur in differentiated cartilage tissue. In contrast, the interradials of the pectoral fin in basal actinopterygians (Kälin, 1938; Cohn et al., 2002; Davis et al., 2004) and the interphalangeal joints in the tetrapod autopodium (Casanova and Sanz-Ezquerro, 2007; Montero et al., 2007) form by subdivision prior to cartilage differentiation.

The development of the zebrafish fin skeleton was previously described by Grandel and Schulte-Merker (1998). Our observations confirm that the reorganization of the pectoral fin endoskeleton starts with a round of proliferation in the FD cartilage near the end of the second wpf, at approximately 5.0mm NL. This is followed by the separation of the FD from the coracoid cartilage (CSZ 0) and the formation of the CSZ 1 in the center of the FD. We equally found that, at approximately 6.5mm NL (appr. 22dpf), the second phase of the reorganization includes the elaboration of the distal rim mesenchyme and the growth of the FD by another proliferation round and by appositional growth at the outer rim. These events are followed by the anlage of lepidotrichia and distal radials and by the formation of CSZ 2 and 3. This mode of pectoral fin formation is also observed in a wide variety of teleost species (Harrison, 1895; Derjugin, 1910; Goodrich, 1919; Kälin, 1938; Bouvet, 1974). Therefore, it is plausible that our observations in zebrafish are representative for teleosts.

In our specimens, we found that especially the second phase of the pectoral reorganization, in which the lepidotrichia, distal radials, and CSZ 2 and 3 develop, shows considerable developmental variation. This variation appeared to be linked to the individual growth rate of the specimens. In this study, specimens that grew faster than the average growth rate exhibited a developmental pattern similar to that described by Grandel and Schulte-Merker (1998). This is not surprising, since the specimens used by these

authors had a faster average growth rate than the specimens of our study (the growth curve calculated by these authors was Y=0.259 X +1.774 (R^2=0.927); compare to Y=0.237 X + 1.6165 (R^2=0.9654) in this study), most likely because their larvae were fed three times a day, while our larvae were fed only twice. In our fast-growing specimens and in the specimens studied by Grandel and Schulte-Merker (1998), the anlage of CSZs 2 and 3 appear in the centre of each FD half, expanding proximally and distally to individualize the four proximal radials (8.0-8.5 mm NL / 25–30 dpf). Simultaneously, the anlage of lepidotrichia and distal radials appear quickly over the entire fin fold.

In specimens with a growth rate lower than average, the second phase of the reorganization equally starts by an increase in cell numbers in the distal rim mesenchyme and the FD. However, in these specimens this increase appears to spread more slowly, resulting in a much slower appearance of new distal radials and lepidotrichia. Such slow-growing larvae also show a big time gap between the formation of CSZs 2 and 3. Interestingly, CSZ 2 never appears before the anlage of lepidotrichium 4, while CSZ 3 only appears when lepidotrichium 7 is present. Finally, the FD of these larvae shows far less appositional growth at its outer rim. This lower amount of new chondrocytes along the outer rim could explain why CSZ 2 and 3 do not form in the center of the FD halves, as in fast growing specimens, but rather at the distal edge. In both cases these CSZs first appear at the edge of the double-layered FD; only in fast-growing fish, this area of the FD no longer constitutes the distal border due to the addition of new chondrocytes. Furthermore, insufficient addition of new chondrocytes at the ventral rim of the FD could contribute to the size reduction, and even loss, of the fourth proximal radial, as we observed in some slow-growing specimens. Various studies have shown different effects of reduced growth rate on variation in morphological outcome. In the evolution of miniaturized species, which show a drastically reduced growth rate compared with their ancestral species, various organ systems and skeletal elements are lost (Hanken and Wake, 1993). In contrast, a reduced growth rate per se can also lead to an increase in number of meristic

characters such as vertebrae, scales, or tooth rows (Levin, 2010; Shkil et al., 2010).

Together, our observations suggest that the formation of CSZs 2 and 3 does not depend on the size of the FD cartilage but is rather linked to the formation of lepidotrichia in the fin fold. This is interesting because the appearance of the lepidotrichia has already been linked to the subdivision of the larval pectoral musculature into the adult muscle groups (Thorsen and Hale, 2005). This would imply that the reorganization of the fin musculature and the fin endoskeleton are synchronized, perhaps linked to a single developmental event, the formation of the dermal skeleton.

Since musculature, dermal skeleton, and endoskeleton are differently regulated throughout their development (Eames et al., 2004; Abzhanov et al., 2007), they can be seen as separate developmental modules (Schlosser, 2004). Variation in the modular design of the pectoral appendages provides an elegant perspective on both fin and limb evolution (Shubin and Davis, 2004). Nevertheless, there are currently very few studies attempting to identify developmental links between these developmental modules. In teleosts, the dermal skeleton is the major skeletal structure of the adult fin, yet the endoskeleton retains an important function during early development. Our research shows that during the transition from the larval to the adult fin skeleton these two different skeletal modules develop in a coordinated fashion. Understanding the mechanisms behind this coordination will certainly be required to fully understand how the biphasic nature of pectoral fin development could have originated.

Having established the timing of the FD cartilage subdivisions, and the developmental events associated with it, we further evaluated several cellular mechanisms that could lead to cartilage loss. Based on (1) the lack of evidence for apoptosis, both in acridine orange / propidium iodide staining and from ultrastructural observations, (2) the lack of chondroclast or macrophage digestive activity, and (3) the ultrastructural evidence for chondrocyte dedifferentiation, we propose that dedifferentiation of the resident chondrocytes accomplishes the subdivisions. In a dedifferentiation process, a

differentiated cell loses its specific phenotypical characteristics, transforming into an undifferentiated mesenchymal cell. Dedifferentiation can be part, however, of a transdifferentiation process. Transdifferentiation is a transformation of one differentiated cell type into another cell type (Okada, 1991). In some cases, this can be accompanied by cell division, whereas in others it is not (Beresford, 1990). Transdifferentiation can occur either directly or indirectly with respect to the timeframe of the phenotypic transformation. In direct transdifferentiation, the cells possess characteristics of both cell types simultaneously during the transition period, whereas indirect transdifferentiation implies a dedifferentiation phase in which the phenotypic characteristics of the cell first disappear before a new phenotype is established (Slack and Tosh, 2001).

The slow degradation of the cartilage ECM offers a sufficiently large time window in which dedifferentiation events can be observed. Before subdivision, the chondrocytes of the FD are completely embedded in cartilage matrix, which itself is an easily recognizable form of ECM. Consequently, these chondrocytes do not show cell-to-cell contacts. Furthermore, these chondrocytes have an electron-lucent cytoplasm, containing large numbers of small granules, large mitochondria, several Golgi-complexes, an elaborate endoplasmic reticulum, and a low nucleo-cytoplasmic ratio. All these traits correspond to the typical chondrocytic ultrastructure previously described by Holtrop (1971; 1972a; 1972b) and Huntington (1983). Undifferentiated mesenchyme and fibroblastic cells, on the other hand, are not completely embedded by their ECM and have plenty of cell-to-cell contacts. These cells are irregularly branched, their nucleo-cytoplasmic ratio is high and their cytoplasm is very electron dense (Knese, 1979).

Our TEM observations show that degrading cartilage ECM persists inside the subdivision zones well beyond the point where the matrix looses its alcian blue signal. First, the ECM looses its fibrillar ultrastructure, while still completely surrounding the resident cells. These cells can thus only be former FD chondrocytes, although their ultrastructure is now clearly different. Although chondrocytes normally completely fill their lacunae, these cells are

highly branched and detached from the ECM in several areas. Their nucleo-cytoplasmic ratio has strongly increased and their cytoplasm has become more electron-dense. In a later stage of subdivision, the ECM becomes fragmented when the resident cells break through and reestablish cell-to-cell contacts. These ECM fragments gradually become smaller and disappear, at which point it is no longer possible to distinguish former chondrocytes from the surrounding perichondral connective tissue.

Despite the fact that it was not possible to determine the final fate of these cells, the evidence for dedifferentiation and the lack of apoptosis suggests that former FD chondrocytes can contribute to inter-radial connective tissue or even perichondral bone formation. These scenarios would then represent cases of direct or indirect transdifferentiation, respectively. We hope to answer these questions in the future by means of lineage mapping of the FD chondrocytes. The lack of accurate lineage data is the main reason why *in vivo* loss of cartilage by transdifferentiation is still controversial. This is because specific lineage mapping is the only way to provide irrefutable proof of the transformation from one differentiated cell type to another (Eguchi and Kodama, 1993). Nevertheless, there is ample evidence that chondrocytes and fibroblasts undergo transdifferentiation under culture conditions, when provided with the specific transcription factors or a different adhesion substrate (Holtzer et al., 1960; Mackie et al., 1987; Sato et al., 1988; Yin et al., 2010). Other reports on cartilage transdifferentiation *in vivo* are however quite rare. It has been described in the middle section of the Meckelian cartilage in mice, which turns into fibroblastic tissue forming the sphenomandibular ligament (Harada and Ishizeki, 1998). The symphysis area of the Meckelian cartilages in the cichlid *Hemichromis bimaculatus* (an advanced teleost species) displays changes in cell shape that are very reminiscent of the features described here in the CSZs (Huysseune and Sire 1992). Hypertrophic chondrocytes very near the replacement front during mammalian and avian long bone development have been suggested to transdifferentiate (Roach et al., 1995; Roach, 1997; Bianco et al., 1998; Blumer et al., 2005).

Although transdifferentiation of cartilage into fibrous tissue may be rare, many other types of transdifferentiation in connective tissues have been described. The occurrence and importance of transdifferentiation in connective tissue remodeling has already been addressed by Beresford (1981) and has received new evidence over the last decades. Examples are cartilage and chondroid bone into bone (Gillis et al., 2006; Hammond and Schulte-Merker, 2009), bone into chondroid bone (Witten and Hall, 2002; Witten and Hall, 2003), bone into cartilage (Witten et al., 2005), fibroblastic tissue into bone or cartilage (Badi, 1972; Choi et al., 2010), hyaline- into fibro-cartilage (Kim et al., 2005), periosteal chondrogenesis (Li et al., 2004), perichondral osteogenesis (Bast, 1944; Vilmann, 1977; Blumer et al., 2005), fat into bone (de la Fuente and Helms, 2005), and muscle into bone (Gersbach et al., 2004). All these findings support the idea that the different phenotypes of connective tissues are neither fixed nor terminal, but rather form a continuous spectrum in which differentiation can be modulated by a variety of factors. This phenotypic continuity is also illustrated by the numerous types of connective tissue with intermediate phenotypical characteristics, like bone fracture callus, chondroid bone, and fibrocartilage (Beresford, 1981; Hall and Witten, 2007).

Our observations thus add to the growing consensus that the phenotypes of connective tissues are not terminal and that phenotypic plasticity can play an important role in various developmental and homeostatic processes, in this case the transformation of the zebrafish pectoral fin endoskeleton. In addition, our data allow future comparisons between these naturally occurring cartilage subdivisions and cartilage loss in cartilage degenerative disease, such as non-inflammatory osteoathritis, where chondrocytes themselves induce ECM degradation and loose their phenotypic characteristics (Loeser, 2006).

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3.3. MMP14 - mediated remodeling during zebrafish pectoral cartilage transformation

by

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MMP14 - mediated remodeling during zebrafish pectoral cartilage transformation

Abstract

Matrix metalloproteinases (MMPs) play an important role in both pathological and normal events of cartilage degradation in tetrapod species. Nevertheless many questions about the regulation and the physiological effects of MMP activity remain unanswered. Here we present the first investigation on the involvement of several MMPs during cartilage degradation in the zebrafish endoskeleton. In zebrafish and other teleost species, cartilage degradation occurs in several localized regions of the pectoral fin endoskeleton. These regions are called cartilage subdivision zones (CSZ) and they transform a disk-shaped cartilaginous element, the fin disk cartilage, into four rod-like proximal radials. Previous investigation showed that cartilage degradation in the CSZ was not associated with apoptosis, nor with macrophage- or chondroclast- mediated resorption, but rather with chondrocyte dedifferentiation. We selected specific MMPs, i.e. MMP2, 9, 13 and 14, in order to evaluate whether this cartilage degradation in the zebrafish pectoral cartilage resembles any pathological or normal cartilage degradation event, known from tetrapod models. We found that all MMPs under investigation can be found in zebrafish chondrocytes, but only MMP14 was involved in the formation of CSZs. MMP14-mediated degradation of uncalcified cartilage is known to be of vital importance for normal skeletal development in tetrapods. Our data indicate that the degradation of embryonic cartilages by MMP14 is not limited to tetrapod species, but is rather a highly conserved feature of osteichthyan chondrogenesis, which makes zebrafish a highly useful model organism to investigate more detailed aspects of MMP14-mediated cartilage remodeling in the future.

Introduction

The capacity to remodel extracellular matrix (ECM) is crucial for the development, growth and homeostasis of almost all types of tissues, especially cartilage, where cells are completely embedded in their ECM. Collectively, matrix metalloproteinases (MMPs) are capable of degrading all the structural protein components of the ECM (Massova et al., 1998; Vu and Werb, 2000; Brinckerhoff and Matrisian, 2002; Murphy et al., 2002). Therefore, these proteinases have been the subject of intensive study in a wide range of developmental and pathological conditions (Cawston and Wilson, 2006; Amalinei et al., 2010; Hua et al., 2011). In many cases, however, the physiological effects of MMP-mediated degradation remain poorly understood. MMPs can cause a variety of complex physiological effects on cells and tissue, since their degradation can release signaling molecules or activate other proteinases, present in latent form at the cell surface or in the ECM. However, the most profound effects of MMP-mediated degradation are associated with alterations in ECM composition and the availability of different binding sites for cell-matrix receptors, such as integrins. These translate changes in the extracellular environment into a variety of cellular responses such as proliferation, survival, migration and invasion (Geiger et al., 2001; DeMali et al., 2003; Humphries et al., 2004; Larsen et al., 2006).

In cartilage biology, various MMPs have been shown to play an important part in many aspects of chondrogenesis and cartilage pathogenesis (Imai et al., 1997; Holmbeck et al., 1999; Ishiguro et al., 2002; Murphy et al., 2002; Holmbeck et al., 2003; Kuroki et al., 2005; Malemud, 2006). However, many questions remain and detailed examinations of cartilage tissue in tetrapod models pose significant practical challenges. Teleost species, such as zebrafish, have many practical advantages over conventional model organisms and the field of cartilage biology has already benefited greatly from the contribution of studies that used zebrafish as a model organism (Kimmel et al., 2001; Crump et al., 2004; Clement et al., 2008; Flanagan-Steet et al., 2009; Carney et al., 2010; Eames et al., 2011). However, the relatively large

phylogenetic distance between zebrafish and humans requires detailed comparative study between zebrafish and tetrapod chondrogenesis, before zebrafish can be used as a reliable model organism. In the most recent decade, many studies have shown the highly conserved nature of cartilage development (Bauer et al., 1998; Crotwell et al., 2001; Yan et al., 2005; Avaron et al., 2006; Crotwell and Mabee, 2007; Hammond and Schulte-Merker, 2009), but the role of MMPs during zebrafish chondrogenesis has not yet been investigated. Here we present the first data on the involvement of MMPs in zebrafish cartilage remodeling.

One of the most characteristic remodeling events in the zebrafish endoskeleton is the transformation of the pectoral fin disk cartilage. In this event, common to all teleosts, a disk-shaped cartilage that supports the larval pectoral fin, subdivides into four rod-like cartilage radials (Grandel and Schulte-Merker, 1998; Dewit et al., 2011). These subdivisions are accomplished by the formation of four distinct cartilage subdivision zones (CSZs), in which cartilage tissue is lost. Our previous report indicates that this loss of cartilage is not due to chondroclast-mediated resorption or chondrocyte apoptosis, but likely due to the dedifferentiation of the chondrocytes in the CSZs (Dewit et al., 2011).

Here we investigate the involvement of several MMPs, in particular MMP2, 9, 13 and 14, in this remodeling process. MMP2 and 9 are also called gelatinase A and B, respectively, and are capable of degrading many cartilage ECM components, such as fibronectin, aggrecan and proteoglycan link protein (Murphy et al., 2002). In mature human articular cartilage, MMP2 is present in all chondrocytes (Duerr et al., 2004), while MMP9 can only be detected in some superficial chondrocytes (Söder et al., 2006). In pathological conditions, such as osteoarthritis (OA), these two MMPs are up-regulated and expressed in all chondrocytes near the OA lesion (Agarwal et al., 2003; Duerr et al., 2004; Davidson et al., 2006). MMP13 or collagenase 3 degrades both collagen type II and aggrecan, the two major components of cartilage ECM (Murphy et al., 2002). This proteinase is expressed in the long bone metaphysis, from proliferative to hypertrophic chondrocytes (Ohkubo et al.,

2003; Behonick et al., 2007). Furthermore, this collagenase is strongly up-regulated in OA cartilage (Davidson et al., 2006).

MMP14 is also called MT1-MMP and is the only membrane-bound MMP under investigation. Unlike MMP2, 9 and 13, which are secreted in inactive form, the pro-domain of MMP14 is cleaved intracellularly. As a result, this proteinase is always present in active form on the cell membrane, however, its activity remains regulated by other proteins, for example by interactions with MMP inhibitors (Itoh and Seiki, 2006). Apart from the degradation of many cartilage matrix components, such as collagen type II, aggrecan, fibronectin and dermatan sulfate proteoglycan (Murphy et al., 2002), this proteinase can also activate MMP2 (Zhou et al., 2000) and 13 (Knauper et al., 2002). In tetrapod long bones, MMP14 is mainly expressed in the perichondrium and the superficial cartilage bordering the metaphysis (Holmbeck et al., 2003; Yang et al., 2008). There, it allows the transition from the superficial chondrocytes in the uncalcified ECM and perichondrium to the bony tissue of the periosteum, a process proven to be essential for proper long bone growth (Holmbeck et al., 2003). Unlike MMP2, 9 and 13, this proteinase is not significantly up-regulated in OA cartilage (Davidson et al., 2006). However, this does not exclude the possibility that this proteinase is involved in OA, since it can activate proteinases, which do show up-regulation (Imai et al., 1997).

Here, we examine the distribution of MMP2, 9, 13 and 14 during the period of intense remodeling of the pectoral fin endoskeleton by means of immunohistochemistry. As a result of a genome duplication common to all teleosts MMP14 is represented by two paralogs MMP14α and β in zebrafish (Hurley et al., 2007). Both paralogs are examined by paralog specific antibodies. We also study the role of these MMPs by using both a broad spectrum and a more specific MMP inhibitor. This analysis sheds light on how the remodeling of the pectoral fin endoskeleton is accomplished. It also allows us to assess whether the pectoral fin disk cartilage transformation in zebrafish corresponds with the observations in either normal or pathological degradation of cartilage tissue in tetrapods.

Material and methods

Wild type zebrafish, *Danio rerio*, embryos were raised in E3 medium (Brand et al., 2002) at a standard temperature of 28.5˚C. After 6 days post-fertilization (dpf), the larvae were transferred to previously aerated tap water at 26˚C. From 5 dpf until 14 dpf, the larvae were fed with dry food (ZM 000, ZMsystems, Winchester, UK) twice a day. At 12 dpf, freshly hatched *Artemia salina* nauplii were introduced, in order to gradually replace the dry food. These were then given twice a day for the remainder of the rearing time.

Prior to fixation all larvae, ranging from 10 to 40 dpf, were euthanized with ethyl 3-aminobenzoate methanesulfonate salt (MS222, Sigma-Aldrich, St. Louis, USA) and their notochordal length (NL) was measured (Cubbage and Mabee, 1996). These measurements were taken with an ocular micrometer on a MZ APO stereomicroscope (Leica, Heerbrugg, Switzerland). Since larvae of equal age can show considerable developmental variation, especially in relatively late developmental stages, these NL measurements provided a more accurate developmental staging system (Grandel and Schulte-Merker, 1998; Parichy et al., 2009).

Specimens used for whole-mount immunohistochemistry were fixed overnight in 4% PFA (pH 7.5) at 4°C, dehydrated and stored in methanol at -20°C. Prior to staining, larvae were rehydrated and pectoral fins were dissected to allow for optimal tissue orientation prior to visualization. The pectoral fins were permeabilized for 20 min. in 0.1% proteinase K and for 20 min. in 2% hyaluronidase, both in PBS, pH 7.5 at 25°C. After rinsing and blocking (1% BSA and 1% DMSO in PBS, pH 7.5), they were incubated overnight at 4°C with the primary antibodies (Collagen II: II-II6B3 (Hybridoma Bank, Iowa City, U.S.A.), MMP2: 55111, MMP9: 55345, MMP13: 55114, MMP14α: 55115, MMP14β: 55116 (Anaspec Inc., Fremont, U.S.A.)), diluted 1⁄300 in blocking solution. All anti-MMP antibodies used in this study are raised against the zebrafish proteins. After rinsing, the samples were incubated at 25°C for 2 h with the secondary antibody (Alexa anti-rabbit 488, Alexa anti-mouse 595 (Invitrogen, Carlsbad, U.S.A.)) diluted 1⁄300 in blocking solution. After rinsing, specimens were transferred to 30% glycerin, finally

mounted in vectashield (Vector Laboratories Inc., Burlingame, U.S.A.) and observed using a confocal laser scanning microscope (CLSM) (D-eclipse-C1, Nikon, Melville N.Y., U.S.A.). For staining controls, the same protocol was applied, without the use of a primary antibody. The double staining protocol was controlled by using each antibody separately on two specimens and comparing these signals with a double-stained specimen.

In order to obtain positive control tissue for MMP9, the caudal fin of larvae at 5 dpf was amputated just caudally to the tail blood vessels. Larvae were allowed to recover for 5 hours to allow macrophages to respond to the injury (Yoong et al., 2007). Subsequently, larvae were fixed and processed for immunohistochemistry as described above. Positive control tissue for MMP2, 14 α and β was obtained by amputating the distal portion of the caudal fin, at the level of the third lepidotrichia joint. Larvae were allowed 5 days of regeneration (Bai et al., 2005) then larvae were fixed and processed as described above. All amputations were performed under appropriate anesthesia with ethyl 3-aminobenzoate methanesulfonate salt (MS222, Sigma-Aldrich, St. Louis, USA).

Specimens used for immunohistochemistry on paraffin sections were fixed overnight in 4% PFA (pH 7.5) at 4°C and processed for paraffin embedding. Transverse and sagittal serial sections of 5 µm were made, mounted on uncoated slides and dried at 40˚C. After deparaffination, sections were rehydrated and washed with PBS. Endogenous peroxidase was blocked by 1% H_2O_2 in 100% methanol for 20 min. After washing, sections underwent antigen retrieval in a preheated citrate buffer (1.8ml 1M citric acid and 8.2ml 1M Na-citrate in 1l distilled water) at 95°C for 20 min. Sections were allowed to cool down inside the retrieval buffer, washed with PBS and submerged in block solution (3% BSA, 1% milk powder in PBS, pH 7.5) for 2h. The slides were then covered with the primary antibodies (see above), diluted 1/500 in block solution and left overnight at 4˚C. After washing, sections were impregnated for 2 h at 25°C with the secondary antibody, a biotin-coupled polyclonal goat anti-rabbit immunoglobulin (E0432, Dako), diluted 1/500 in blocking solution. Subsequently, sections were rinsed and impregnated with

ABC-kit and DAB-kit, according to manufacturers' instructions (K0492 and K3468, resp., Dako). Sections received final rinsing before being coverslipped with DEPEC and photographed using a light microscope (AXIO Imager Z1, Zeiss, Göttingen, Germany) fitted with an Axiocam camera.

In order to assess the role of the MMPs under investigation during zebrafish cartilage remodeling, we treated larvae with two MMP-inhibitory compounds, one broad spectrum inhibitor, GM6001 (Millipore, Billerica, USA) and one specific MMP inhibitor, ND322 that only inhibits MMP2, 9 and 14.

These compounds were dissolved in DMSO as stock solutions and stored at -20°C. Stock solutions were concentrated, in order to prepare treatment solutions of 20 ml E3 medium containing 0.1% DMSO supplemented with either 0.25 mM 6001, 5 μM, 10 μM or 20 μM ND322. The concentration used for GM6001 was adapted from Bai et al. (2005), while the concentrations of ND322 were chosen in accordance to a preliminary study performed at the University of Notre Dame. Each treatment started with 10 larvae at 18 dpf and was maintained for 8 days. The overall experimental design is presented in table 1, which also lists the number of surviving larvae in each treatment. Medium was refreshed every 48h. Control groups of 10 siblings were raised over the same timespan in 20 ml E3 medium, containing only 0.1% DMSO. All groups were fed small amounts of *Artemia salina* nauplii three times a day.

At 26 dpf, all larvae were measured for NL, fixed and stained whole mount with alcian-blue/alizarin-red (Walker and Kimmel, 2007). Specimens were cleared until all background staining had dissipated, pectoral fins were dissected and mounted, according to Dewit et al. (2011). Photographs were taken using a light microscope (AXIO Imager Z1; Zeiss, Göttingen, Germany)

sibling	CONTROL	GM6001	ND322	
A	6	8	6	5µM
B	6	7	9	5µM
	4	5	5	10µM
	7	4	0	20µM

fitted with an Axiocam camera. Series of partially focused images were merged using Helicon Focus software (version 4.6). The degree of cartilage subdivision was estimated using the following equation:

$$\%\ \mathrm{CS} = \frac{\text{total surface CS} - \left(\frac{\text{progression surface CS}}{2}\right)}{\text{total surface FD}} \times 100$$

The '% CS' is the percentage of cartilage subdivision. The 'total surface CS' is the surface of all the regions of the fin disk cartilage showing signs of cartilage degradation. The 'progression surface CS' consists of the surface where cartilage degradation is evident, but remnant matrix is still visible, as opposed to regions that have become completely free of alcian blue staining. The 'total surface FD' is the surface of the entire fin disk cartilage. All surface measurements were taken using ImageJ software, version 1.44o (Abramoff et al., 2004).

The average '% CS' of the left and right pectoral fins was used for further statistical analysis. To test whether the treatment and length had an effect on the average '% CS', we fitted a linear mixed model with treatment and notochordal length as explanatory variables. Afterwards, we also tested whether changes in length were affected by the treatment by including the interaction between treatment and length in the previous model. Sibling information was always included as a random factor to avoid pseudoreplication. The average '% CS' was logarithmically transformed, assumptions of normality and homoscedasticity were met and no outliers with high influence (by using Cook's distance) were present. In addition, a

Table 1

This table gives the number of surviving larvae for each subgroup (n=10) at the end of the MMP-inhibition experiment at 26 dpf. Each treatment, i.e. control, GM6001 and ND322, was performed in four independent groups (subgroups) that contained 10 larvae of equal age (18 dpf). Two different sibling groups (A and B) were used that originated from different parent groups (each parent group contained 2 males and 3 females). The first column of this table shows how these sibling-groups were distributed over the experiment. The last column indicates which of the three tested concentrations of ND322 was given to each subgroup.

generalized linear mixed model was fitted to verify whether mortality was affected by the treatment (sibling as random effect). The model showed that mortality did not differ significantly between treatment groups ($F_{2,8}$=0.17; P=0.85). All tests were considered significant if the P-value was less than 0.05 and the statistical analyses were performed in SAS 9.2 (SAS Institute Inc. 2002-2006, Cary, NC, USA).

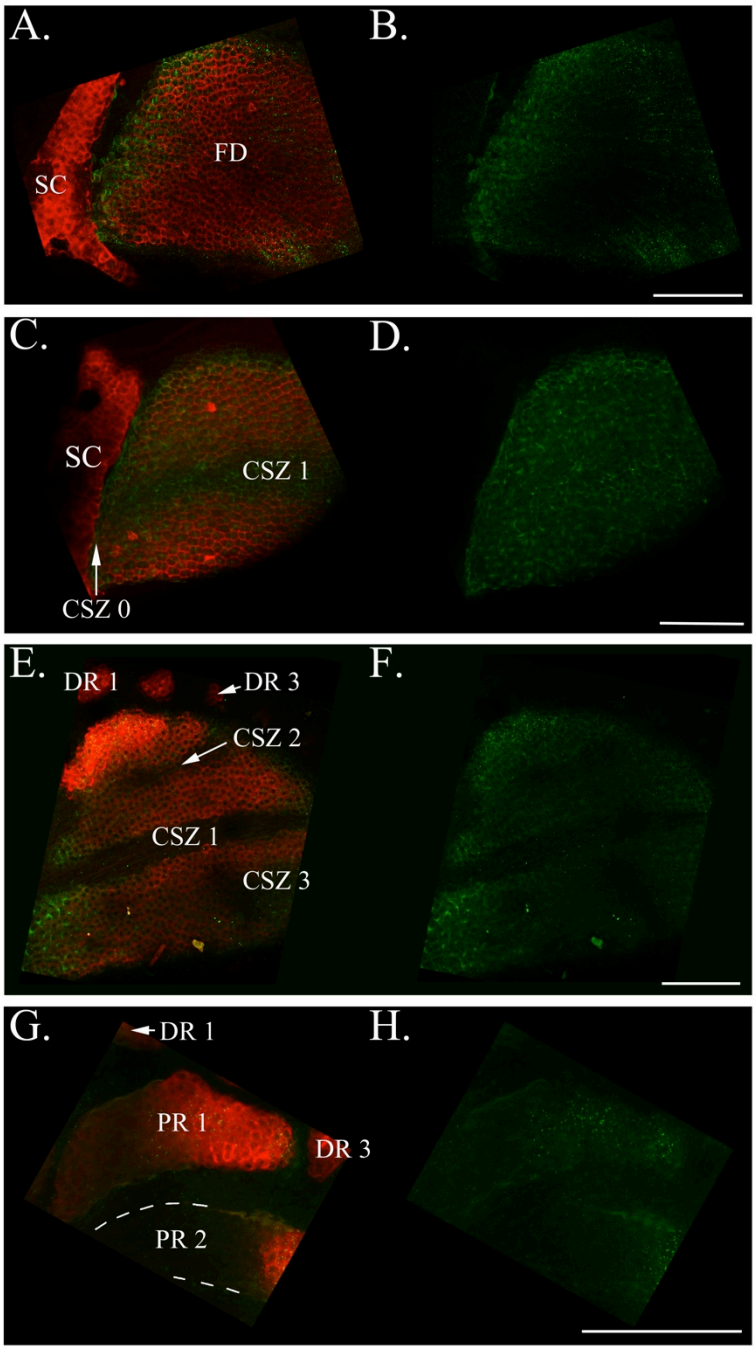

Figure 2
Z-stack of CLSM recording of the pectoral fin after whole mount immunostaining for collagen type II (red) and MMP14α (green). In (A, C, E, G) the merged image of both red and green channels is shown, while (B, D, F, H) show only the green, MMP14α signal. (A, B) 5 mm larva (18 dpf), (C, D) 6.2 mm larva (21 dpf), (E, F) 7.6 mm larva (25 dpf) and (G, H) portion of the pectoral fin of a 8.3 mm larva (29 dpf). Dashed line in (G) indicates the boundaries of the 2nd proximal radial (PR 2) which can no longer be stained due to the presence of perichondral bone.
Further abbreviations: CSZ 0-3: cartilage subdivision zone 0-3, DR 1-3: distal radial 1-3, FD: fin disk cartilage, PR1-2: proximal radial 1-2, SC: scapulocoracoid cartilage. Scale bars = 100 μM.

Results

MMP14α is dynamically distributed during the remodeling of the fin endoskeleton

The developmental sequence of the pectoral cartilage subdivisions was previously described (Dewit et al., 2011). Here, we evaluate the presence of MMP2, 9, 13, MMP14α and β during these events.

MMP14α first appears in the most proximal region of the fin disk cartilage in 5.2 - 5.4 mm larvae (16-20 dpf) (Figure 2 A-B). This timing corresponds perfectly with the onset of CSZ 0 formation between the fin disk cartilage and the scapulocoracoid cartilage of the shoulder girdle. MMP14α is, however, not limited to CSZ 0 and is also observed in the most proximal chondrocytes of the disk cartilage, while the chondrocytes of the scapulocoracoid remain negative. Between 5.6 and 6.2 mm NL, both CSZ 0 and CSZ 1 have formed and MMP14α can be seen in the former chondrocytes of the subdivision zones and in the chondrocytes of the remaining halves of the fin disk cartilage (Figure 2 C-D). Later, MMP14α gradually disappears, first inside CSZ 0 and 1, but soon also in the chondrocytes of the remaining fin disk cartilage. Between 7.5 and 8 mm NL, CSZ 2 and 3 form, but, unlike CSZ 0 and 1, they do not show MMP14α positive cells. At this point, only the proximal areas of proximal radial 3 and 4 still show MMP14α (Figure 2 E-F). When the proximal radials are finally individualized MMP14α can no longer be detected in the cartilage or the interradial tissue. However, at this time the proximal radials will undergo perichondral ossification and MMP14α can be detected in the transition region between perichondrium and periosteum (Figure 2 G-H). Regions covered with perichondral bone are impenetrable to antibodies in whole mount staining and therefore appear negative for collagen type II and MMPs.

Although an MMP14α signal can clearly be observed in regions of both cartilage loss and cartilage persistence, there is a clear difference in the distribution of MMP14α around the cells. In persistent cartilage MMP14α is mostly concentrated at a single lacunar wall, more specifically the youngest matrix wall between two daughter chondrocytes (white arrowheads in Figure

3). In addition to this clearly concentrated MMP14α, the entire periphery of the chondrocytes shows faint MMP14α signal (Figure 3 A-B). In contrast, regions of cartilage loss, such as CSZ 0 and 1, show cells with strong MMP14α signal along the entire cell periphery (asterisk in Fig. 3 C-D). Older regions of these subdivision zones are, however, the first areas to lose MMP14α signal completely. At this point some cells at the boundaries of the CSZs show chondrocytes with strong MMP14α signal along their entire periphery (asterisk in Figure 3 E-F).

MMP14β, MMP2, 9 and 13, on the other hand, are not detected during fin disk subdivision, despite verification of the antibodies on various control tissues. MMP13 could be detected in zebrafish chondrocytes, even in the proximal radials of the pectoral fin, yet only after subdivisions had occurred (Figure 4 A-B). In tetrapods, MMP13 is found to be strongest in early hypertrophic chondrocytes (Ohkubo et al., 2003; Behonick et al., 2007). In the zebrafish pectoral fin endoskeleton, hypertrophic chondrocytes first appear around the dorsal and ventral cartilage canals of the girdle, where the scapular and coracoid ossifications will form, respectively (Grandel and Schulte-Merker, 1998), and these cells are MMP13 positive. In zebrafish, MMP9 can be detected in several cells, probably macrophages, near a wound inflicted to the caudal fin in 5 dpf larvae (Figure 4 C-D) (Yoong et al., 2007). In mice, MMP9 is found in regions of known osteoclast activity (Vu et al., 1998; Ortega et al., 2004). In zebrafish, osteoclasts can be found in the tooth-bearing branchial arch (Figure 4 E) (Witten et al., 2001). MMP2 and MMP14α are located in 5-day-old regeneration blastema of the caudal fin in 3 weeks old zebrafish (Bai et al., 2005) (Figure 4 F-G). In contrast to MMP14α, MMP14β could not be detected in the regeneration blastema.

Immunohistochemistry on paraffin sections generally confirms our whole mounts observations, although this approach generated considerably more background signal, especially in dermal and epidermal tissues (Figure 5). Although it is possible that the dermal and epidermal signal corresponds to some extend to the presence of MMPs, neither our whole mount observations

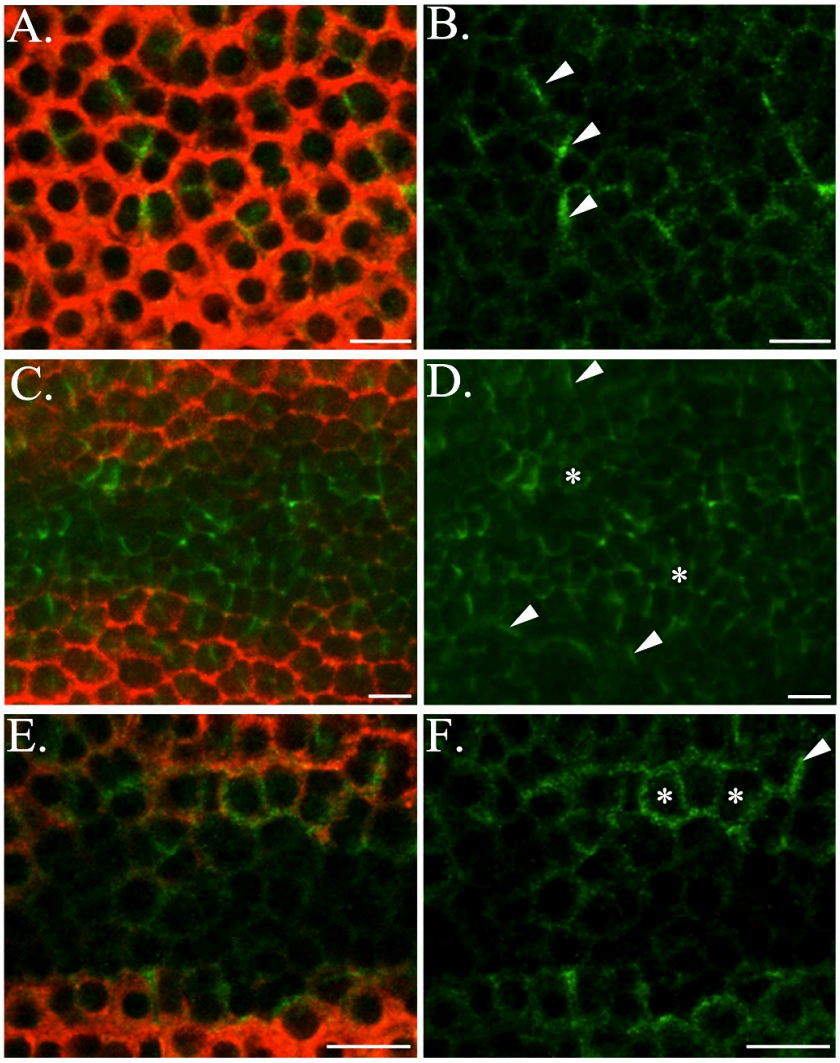

Figure 3
Z-stack of detailed CLSM recording of the pectoral fin endoskeleton after whole mount immunostaining for collagen type II (red) and MMP14α (green). In (A, C, E) the merged image of both red and green channels is shown, while (B, D, F) show only green, MMP14α signal. (A, B) show detailed images of persistent fin disk cartilage in a 6.2 mm larva, with faint MMP14α along the entire cell and concentrated MMP14α at the youngest matrix wall separating chondrocyte daughter cells (indicated by white arrowheads). (C, D) show detailed images of cartilage subdivision zone 1 (CSZ 1), which no longer shows collagen type II, in a 6.2 mm larva. MMP14α in the CSZ 1 is strong along the entire cell periphery (white asterisks), while only a single matrix wall stains strongly for MMP14α in the persistent cartilages (white arrowheads). (E, F) show detailed images of a mature CSZ 1, in a 7 mm larva. MMP14α has mostly disappeared in the center of CSZ 1, while at the borders some chondrocytes still show MMP14α around their entire periphery (white asterisk). Chondrocytes located just a little deeper in the persistent cartilage show localized MMP14α (white arrowhead).
Scale bars = 10 μM.

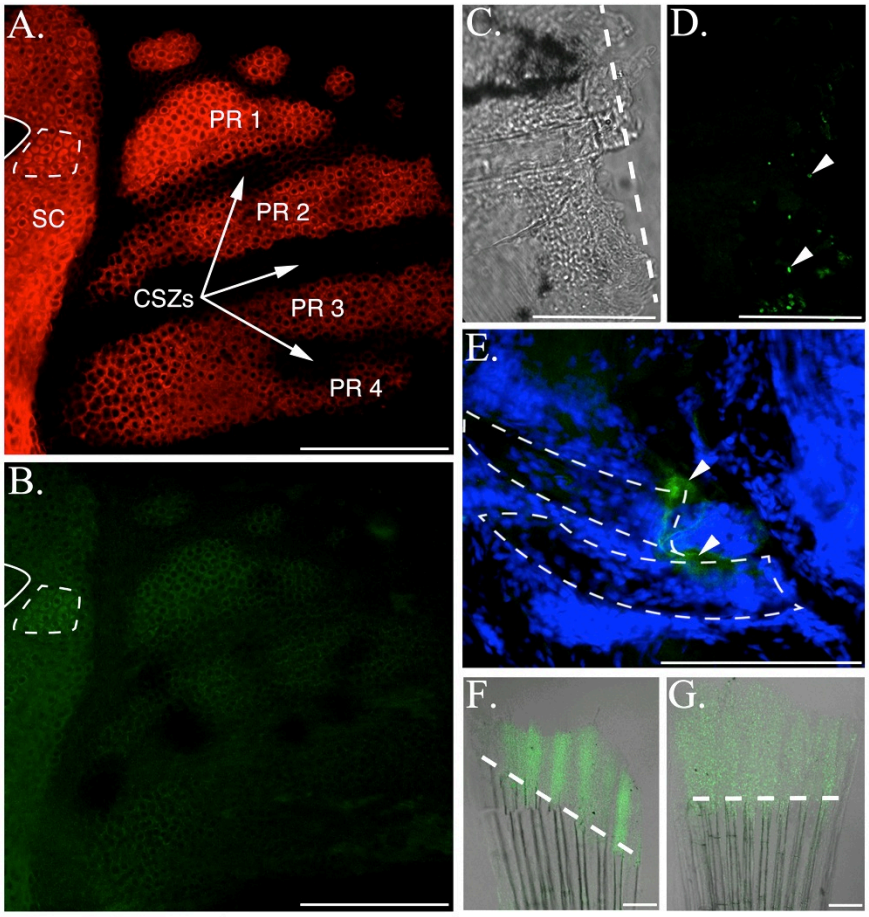

Figure 4
(A, B) show Z-stack of CLSM recording of whole mount immunostaining for collagen type II (red) in (A) and MMP13 (green) in (B). (B) shows the faint presence of MMP13 in the scapulocoracoid cartilage (SC) and in some areas of the proximal radials (PR 1-4), but not associated with the cartilage subdivision zones (CSZs). Dashed lines in (A, B) indicate hypertrophic chondrocytes located around the scapular cartilage canal (full lines), which show slightly stronger MMP13 signal. (C, D) show Z-stack of CLSM recording of an amputated caudal fin after whole mount immunostaining for MMP9, performed 6 hours after amputation. (C) shows bright field image of the amputation wound (dashed line), while (D) shows the corresponding fluorescent image with MMP9 positive cells (presumably macrophages) that are recruited to the wound (white arrowheads). (E) shows Z-stack of CLSM recording of tooth region after whole mount immunostaining for MMP9, in a 21 dpf larva. MMP9 positive cells (likely osteoclasts), marked by arrowheads, can be found at the base of a tooth in resorption (teeth are marked by the dashed lines), blue signal is DAPI nuclear counterstain. (F, G) show Z-stack of CLSM recording of a 5-day-old regeneration blastema (dashed line marks amputation site) of the caudal fin after whole mount immunostaining for MMP2 in (F) and MMP14α in (G). The fluorescent images were merged with their corresponding bright field image. All scale bars = 100 μM.

nor the available literature can confirm this observation. Of all MMPs investigated in this paper, MMP14α is the only MMP that is distinctively present in the fin disk cartilage during subdivision (Figure 5 A-E). However, unlike in whole mount staining, MMP2 and 9 appear faintly, and MMP13 more distinctively, present in the chondrocytes of the scapulocoracoid. We also analyzed the presence of these MMPs in the cartilaginous radials of the median fins, which, at 1 month post-fertilization, possess regions of appositional growth, mature and hypertrophic chondrocytes (Figure 5 F-M). MMP2 is detected in all these chondrocytes, but appears strongest in the recently added chondrocytes, which still show a flattened cell shape. MMP9 and 13 are also present throughout the chondrocytes of these radials, although a stronger signal can be found in mature and hypertrophic chondrocytes. MMP14α, on the other hand, cannot be detected in the cartilage of the median fin radials, just as it was absent in the pectoral girdle.

Pectoral fin disk cartilage subdivision is significantly delayed by MMP-inhibitors

We inhibited MMP-mediated ECM degradation during the period of pectoral fin disk cartilage subdivision (from 18 to 26 dpf), with a broad-spectrum (GM6001) and a MMP2, 9 and 14 specific (ND322) inhibitory compound (Figure 6). Larval survival was approximately 60% and did not differ significantly between treatment groups ($F_{2,8}$=0.17;P=0.85). Only the 20 μM ND322 treatment showed obviously higher mortality, with 100% mortality within 48 hours. Overall, the linear mixed model showed that the average percentage of cartilage subdivision differed significantly between treatments ($F_{2,62}$ = 38.84; P < 0.0001) (Figure 6). The highest values of cartilage subdivision were observed in the control treatment and these were significantly different from the GM6001 (t_{62} = 3.70; P = 0.0005) and the ND322 treatment (t_{62} = 8.77; P < 0.0001). Values of cartilage subdivision in the GM6001 group were also significantly higher than those in the ND322 group (t_{62} = 5.58; P < 0.0001).

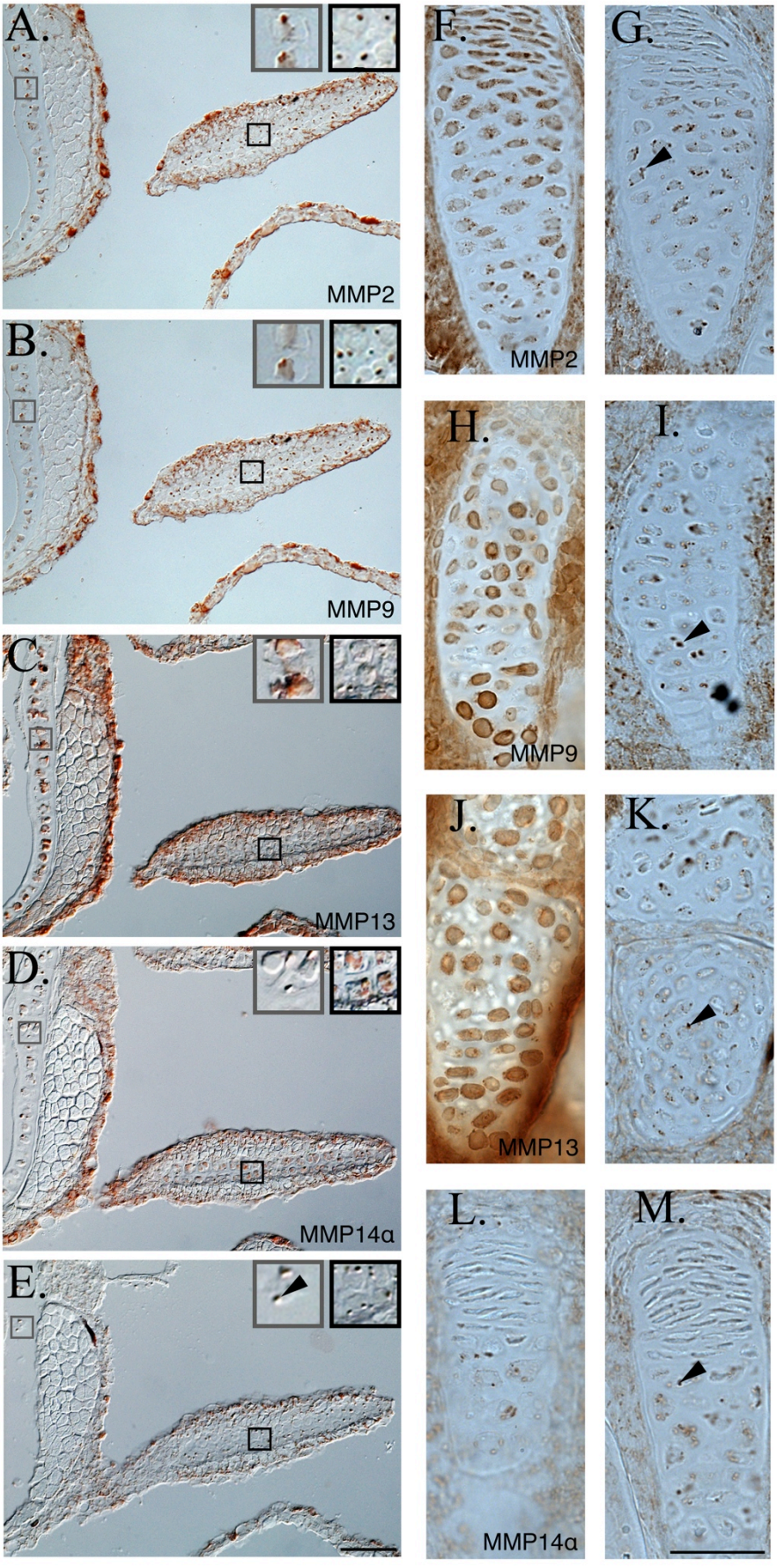
A.
MMP2
B.
MMP9
C.
MMP13
D.
MMP14α
E.
F.
MMP2
G.
H.
MMP9
I.
J.
MMP13
K.
L.
MMP14α
M.

Figure 5
Immunological staining on paraffin sections of the pectoral region in 6.5 mm larvae (A-E) and the median fin radials of 1-month-old larvae (F-M), with caudal fin radials in (F, G, L, M) and anal fin radials in (H-K). MMP2 is stained in (A, F), MMP9 in (B, H), MMP13 in (C, J) and MMP14α in (D, L). Negative controls are given in (E, G, I, K, M). These negative controls show globular structures inside chondrocyte lacunae (black arrowheads), presumably artefacts of the tissue fixation and embedding, and a general background signal in epidermal and mesenchymal tissues. (A) and (B) show weak staining in the scapulocoracoid (grey inset) for MMP2 and MMP9 respectively, but no staining in the fin disk cartilage (black inset). (C) shows clear MMP13 staining in the scapulocoracoid (grey inset), while the fin disk cartilage shows no signal (black insert). Only (D) shows staining in the fin disk cartilage (black insert) indicating MMP14α, but this MMP is not detected in the scapulocoracoid (grey insert). In (F), (H) and (J) the chondrocytes of the median radials show positive MMP2, 9 and 13 staining, respectively, while MMP14α is not detected in median fin cartilage (L).
Scale bar for (A-E) is given in (E) and represents 50 μM. Scale bar for (F-M) is given in (M) and represents 50 μM.

Despite the significant effect of both inhibitors on pectoral fin disk cartilage subdivision, the subdivision process was not stopped by these treatments. The average percentage of CSZ significantly increased with length ($F_{1,62}$ = 327.57; $P < 0.0001$) and the extent of this increase, i.e. the slopes of the fitted curves in Figure 6, was significantly different between treatments ($F_{2,60}$ = 7.55; P = 0.0012). More specifically, the slope or the progression of the average percentage of cartilage subdivision with length was highest in the ND322 treatment and was significantly different from the progression in both the control (t_{60} = 3.00; P = 0.0039) and the GM6001 treatment (t_{60} = 3.77; P = 0.0004). There was no difference between the control and the GM treatment (t_{60} = -0.83; P = 0.41).

Discussion

Zebrafish pectoral cartilage degradation compared to cartilage loss in tetrapods

We find that the transformation of the pectoral cartilage is at least partially accomplished by MMP14α. The first appearance of this proteinase coincides perfectly with the onset of pectoral fin disk cartilage subdivision.

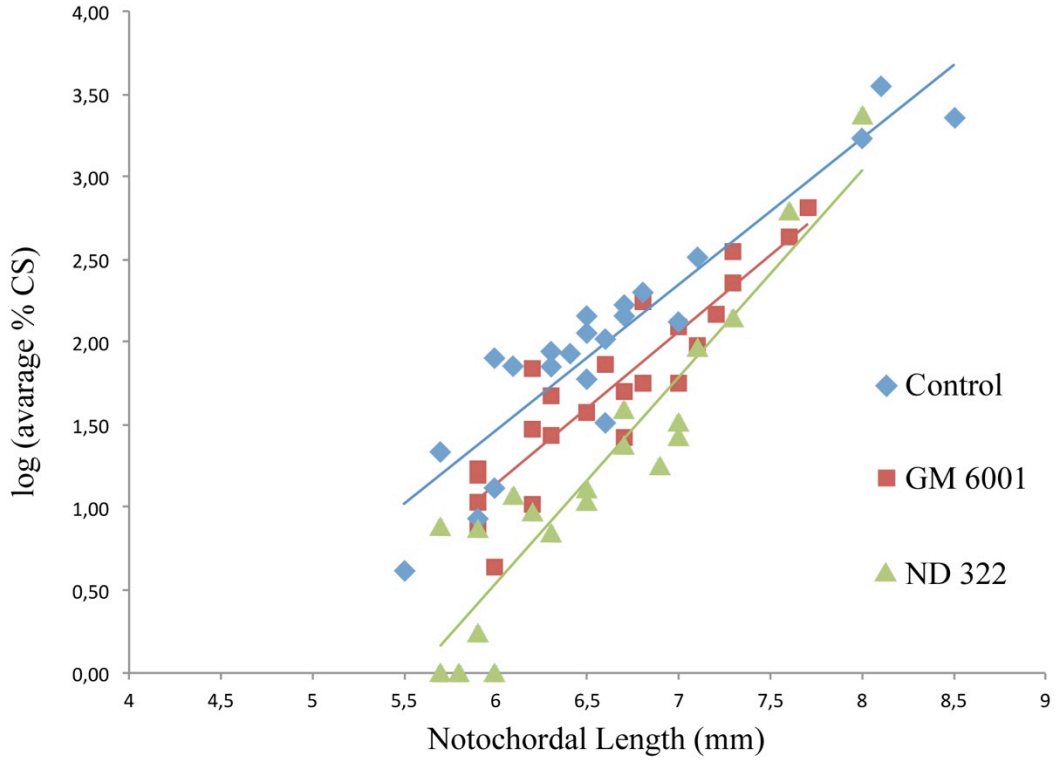

Figure 6
This graph shows the extent of cartilage subdivision, expressed as average percentage of cartilage subdivision (%CS) (see Material and Methods). The average %CS was logarithmically transformed and plotted on the Y-axis, while the notochordal length of each larva was plotted on the X-axis. Blue diamonds, red squares and green triangles represent control, broad spectrum inhibition by GM6001 and specific inhibition of MMP2, 9 and 14 by ND322, respectively. Different concentrations ND322, i.e. 5 and 10 μM, are merged in a single treatment group in order to maintain statistical power.

Furthermore, the MMP-inhibition experiment indicated that larvae raised under MMP-inhibitory conditions had significantly less surface area of the fin disk cartilage subdivided than their siblings, which were raised under control conditions. This delay in cartilage subdivision progression was found to be significant for both the broad spectrum (GM6001) and the selective (ND322) inhibitory compound. Since the latter compound only inhibits MMP2, 9 and 14 (Grobelny et al., 1992; Gooyit et al., 2011) and neither MMP2 nor MMP9 could be detected by immunohistochemistry in the fin disk cartilage, we can conclude that the inhibitory effect of ND322 specifically on MMP14α resulted in the significant retardation of pectoral cartilage subdivision. Interestingly, ND322 had a significantly stronger retardation effect on cartilage subdivision

than GM6001, although GM6001 is a more potent inhibitor of MMP14 than ND-322 (Ki values of 13.4 nM and 210 nM, respectively). GM6001 is, however, poorly water soluble and this may account for the observed differences in cartilage subdivision (Gooyit et al., 2011).

MMP14-dependent remodeling of cartilage tissue was previously described by Holmbeck et al. (2003). These authors identified numerous sites in the mouse skeleton, i.e. the calvarial cartilages, the posterior portion of the meckelian cartilage and the groove of Ranvier in long bone metaphysis, which all showed highly localized MMP14 expression in non-hypertrophic cartilage. This *Mmp14* expression was found to be closely associated with the degradation of both collagen type II and proteoglycans and with a high incidence of apoptosis. In *Mmp14* null-mutant mice all these skeletal sites were characterized by the presence of 'ghost cartilage' in regions where cartilage tissue could no longer be detected in wild type mice. This 'ghost cartilage' appeared to be void of proteoglycans, contained apoptotic chondrocytes and many empty chondrocyte lacunae. However, some lacunae of the 'ghost cartilage' showed entrapped proliferative cells, expressing collagen type I and osteocalcin. This suggests that some former chondrocytes had escaped apoptotic cell death and had switched to an osteoblast-like cell phenotype.

Our previous observations on the cellular events in the CSZs of the zebrafish pectoral fin endoskeleton showed that neither chondrocyte hypertrophy and chondroclast-mediated resorption, nor chondrocyte apoptosis are involved in these events. However, we did find clear signs of chondrocyte dedifferentiation in concomitance with the degradation of the ECM (Dewit et al., 2011). The only major difference between these findings and the observations by Holmbeck et al. (2003) on mouse cartilage is the absence of apoptosis. This can, however, be explained by an important difference between zebrafish and murine hyaline cartilage. Hyaline cartilage in zebrafish and especially the fin disk cartilage is far more cell-rich with much smaller quantities of ECM between neighboring chondrocytes than murine hyaline cartilage or tetrapod hyaline cartilage in general (Witten et al., 2010).

We observed that dedifferentiating chondrocytes of zebrafish CSZs reestablish cell-to-cell contacts with neighboring chondrocytes well before all ECM is degraded (Dewit et al., 2011). Although cell-to-cell contacts are not required for chondrocyte survival, they are known to be essential for the survival of other types of connective tissue, including fibroblastic and osteogenic cells, both of which are found in the interradial area that is formed out of the CSZs (Hall and Miyake, 1995; Hall and Miyake, 2000).

Subdivision was also found to occur in the absence of MMP14α and even following MMP inhibition. Indeed, despite the clear presence of MMP14α in CSZ 0 and 1, we found no MMP14α associated with the formation of CSZ 2 and 3. In addition, we found that fast growing juveniles, larger than 8 mm, showed complete cartilage subdivision even under MMP inhibition, while their moderately and slow growing siblings showed significantly slower progression of cartilage subdivision. This difference of treatment effectiveness for siblings with different growth rates resulted in an overall steeper progression curve of the ND322 treatment. The progression curve of the GM6001 treatment was, however, not significantly steeper than the control group, but most likely this is caused by the absence of fast growing siblings reaching over 8 mm in this group.

One likely cause for the apparent lack of delayed cartilage subdivision in fast growing larvae is that MMP14α may have been present before the onset of treatment at 18 dpf. However, a pilot study revealed that an earlier onset of treatment would require feeding with dry food, since not all larvae had switched to live food at this time. Yet, even minimal amounts of dry food resulted in rapid deterioration of the water quality of the small treatment volumes, which was followed by high mortality rates. Therefore treatment was only started when all siblings could survive on live food.

An alternative explanation for the incomplete effect of MMP inhibition and for the absence of MMP14α in CSZ 2 and 3 is inspired by Holmbeck et al. (2003). These authors still observe some degree of cartilage degradation even in *Mmp14* null-mutant mice. The cartilage regions normally characterized by MMP14 degradation in wild type mouse still underwent

apoptosis in the mutant mice and the few surviving cells in these regions no longer produced cartilaginous ECM. In addition, some ECM components, like proteoglycans, disappeared from the ECM, suggesting that some redundancy may exist with other MMPs or that other catabolic enzymes are involved in these remodeling events in an MMP14-independent manner. Other studies also report that the loss of cartilage matrix, whether under normal conditions or associated with pathophysiological processes, is always dependent on both the cessation of new matrix synthesis and *Mmp* expression (Szabova et al., 2009), but also on the presence of natural MMP inhibitors (Oh et al., 2001; Sahebjam et al., 2007).

In conclusion, our observations agree with these previous observations on tetrapod cartilage remodeling that MMP14α can mediate the degradation of cartilaginous ECM, but MMP14-mediated ECM degradation does not appear to be the driving factor behind chondrocyte dedifferentiation in these regions, just as the occurrence of apoptosis is not affected in remodeling murine cartilages. Additionally, we find that, although MMP14α has a clear functional role in cartilage ECM degradation, it is likely that other currently unknown factors are equally involved in this process.

It is, however, unlikely that MMP2, 9 and 13, have a significant role in pectoral cartilage subdivision, since these MMPs could not be detected during this event. Therefore, the loss of cartilage tissue in the fin disk transformation shows little resemblance to OA lesions in tetrapod articular cartilage, since *MMP2*, *9* and *13* are all up-regulated in OA cartilage degradation (Murphy et al., 2002; Davidson et al., 2006). However, the fact that all these MMPs were found in other cartilaginous elements of the zebrafish skeleton, suggests that these MMPs are nevertheless part of the catabolic arsenal of the zebrafish chondrocyte, just as in their tetrapod counterparts (Cawston and Wilson, 2006; Sakakura et al., 2007).

The role of MMP14 in persistent cartilage

The presence of MMP14α in persistent regions of the fin disk cartilage adds to the growing body of data that the functions of MMP14 far exceed

mere ECM degradation. In tetrapods, MMP14 is known to modulate various signaling pathways by creating functionally active protein fragments and by shedding other proteins, such as syndecan and CD44, from the cell membrane (Itoh and Seiki, 2006). In cartilaginous tissue specifically, MMP14 is found to be crucial for proper chondrocyte proliferation in the growth plate of long bones, although it is only moderately expressed in these proliferative chondrocytes (Zhou et al., 2000; Szabova et al., 2009). In addition, MMP14 rather counter-intuitively allows cultured bovine chondrocytes to respond to cyclic compressions with an increase in ECM production. More specifically, cyclic compression induces chondrocyte spreading and subsequent retraction and together these cell shape changes result in an increased production of ECM components. Integrins were found to be essential for the initial spreading, but MMP14 is required for the subsequent cell retraction, suggesting that the role of this proteinase in chondrogenesis is far more complex than mere ECM degradation (Spiteri et al., 2010). Interestingly, the fin disk cartilage does show chondrocyte proliferation in concomitance with the presence of MMP14α (Grandel and Schulte-Merker, 1998; Dewit et al., 2011). However, this does not explain the concentrated presence of MMP14α at the youngest matrix wall, between two daughter cells. In this case MMP14α may contribute to the production of new matrix at this thin matrix wall.

In conclusion, it appears that MMP14α performs multiple functions during the transformation of the zebrafish fin disk cartilage. Many of these functions are also known from tetrapod chondrogenesis, but remain far from completely understood. We found that the zebrafish pectoral cartilage can serve as a valuable and easily accessible model system to study both the up-stream regulation and the down-stream physiological effects of MMP14α-mediated degradation during ECM degradation, chondrocyte proliferation and matrix production.

Acknowledgments

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3.4. Expression of Cathepsin-K in the pectoral and median fins of medaka, *Oryzias latipes*

by

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Expression of cathepsin K in the pectoral and median fins of medaka, *Oryzias latipes*

Abstract

Cathepsin K (CTSK) is a cysteine proteinase that is associated with endochondral bone formation and bone remodeling since it is strongly expressed in osteoclast cells. We evaluated the expression of ctsk during skeletal development in the pectoral and median fins of medaka, *Oryzias latipes* through observations of a *ctsk*:mEGFP reporter line and *in situ* hybridization for *ctsk*. Both the reporter line and *in situ* hybridization clearly identify regions known for their osteoclast activity. However, in addition to this osteoclast-related signal, the reporter line revealed additional distinctly positive signals during pectoral and median fin development. Using detailed confocal imaging, mEGFP was found in the mesenchymal tissue surrounding the developing cartilaginous radials and the bony fin rays of all observed fins. In addition to this mesenchymal source of mEGFP signal, a strong mEGFP signal was found in many isolated cells in the developing fins. Both the mesenchymal cells and the strong mEGFP-positive cells, however, preceded the formation of calcified matrix in both dermal and cartilaginous structures of the fins. This suggests that an osteoclast-independent source of *ctsk* contributes to the intense tissue remodeling associated with the formation of both bony rays and cartilaginous radials. However, these observations were only partially confirmed by *in situ* hybridization. Possible reasons for this discrepancy are discussed. Interestingly, both the transgenic line and the *in situ* analysis failed to detect *ctsk* expression in the subdivision zones of the pectoral fins, while all other cartilaginous fin radials formed in association with *ctsk* expression at least according to the transgenic reporter line. These results support our previous findings on a chondro/osteoclast independent mechanism for the unique type of remodeling occurring in the pectoral fin endoskeleton.

Introduction

In medaka (*Oryzias latipes*), like in zebrafish and in other teleost species (Grandel and Schulte-Merker, 1998; Schreiber, 2006), the early pectoral fin endoskeleton forms as a continuous cartilaginous structure composed of both girdle and fin disk cartilage (Grandel and Schulte-Merker, 1998; Dewit et al., 2011). While the fin disk cartilage supports the muscular portion of the larval pectoral fin, collagenous fin rays or actinotrichia support the epithelial fin fold. The early pectoral fins of teleosts are fully functional and retain their special morphology for some time (Grandel and Schulte-Merker, 1998). However, they undergo a unique transformation once the median fins and the vertebral bodies start to form. In the pectoral fin, bony fin rays or lepidotrichia replace the actinotrichia of the larval fin fold, while the fin disk cartilage is subdivided into four proximal radials (Grandel and Schulte-Merker, 1998). For zebrafish, we established that these cartilage subdivisions are accomplished by chondrocyte dedifferentiation without the involvement of apoptosis or resorption (Dewit et al., 2011), i.e. without the involvement of clast cells. Since the transformation of the pectoral skeleton can found in a similar developmental setting in all teleost species, including medaka (own observations), it is likely that the underlying mechanisms behind this skeletal transformation are equally similar in all teleosts. The use of medaka, a teleost species rather distantly related to zebrafish, allows us to asses whether or not some of our previous findings on the pectoral cartilage transformation in zebrafish are indeed common in teleost species.

Cathepsin K (CTSK) is a cysteine proteinase, capable of degrading elastin, gelatin and collagen at neutral and acidic pH (Bromme et al., 1996; Kafienah et al., 1998). This proteinase is most abundantly expressed in chondro/osteoclast cells (Haeckel et al., 1999; Boyle et al., 2003; Chatani et al., 2011; To et al., 2012), which are responsible for the replacement of calcified cartilage during endochondral bone formation and for the remodeling of bony tissue throughout life in both tetrapods (Currey, 2003) and teleosts (Witten et al., 2001; Witten and Huysseune, 2009). The strong expression of CTSK in osteoclasts makes this proteinase suitable as an osteoclast marker.

It is, however, important to note that *ctsk* expression is found in many different types of mammalian tissues. Apart from osteoclast, other specifically differentiated macrophages, such as epithelioid cells and multinucleated giant cells, also express *CTSK* in various soft tissues (Bühling et al., 2001). In addition, epithelial cells of the respiratory, digestive and renal system have been reported to express *CTSK* (Haeckel et al., 1999). Smooth muscle cells and macrophages are dependent on CTSK during vascular remodeling (Sukhova et al., 1998). In thyroid epithelial cells, CTSK-mediated degradation could aid in the release of thyroid hormone (Tepel et al., 2000). Cathepsin-K is even involved in the Toll-like receptor (TLR) 9-mediated activation of dendritic cells (Takayanagi, 2010). This wide expression of *ctsk* suggests that this proteinase has a wider range of functions than solely in osteoclast-mediated resorption.

Recently, To et al. (2012) generated a transgenic *ctsk* reporter line, which allows the *in vivo* visualization of *ctsk* expressing cells, such as osteoclasts. This particular transgenic line contains the coding sequence of membrane-bound EGFP (*mEGFP*) under control of the *ctsk* promoter (To et al., 2012). The analysis of this *ctsk* reporter line serves a double purpose in this doctoral research. On one hand, it allow us to assess whether clast-like cells are absent during the medaka's pectoral transformation just as in zebrafish. On the other hand, CTSK is capable of degrading many ECM components, such as elastin, gelatin and collagen, at neutral pH (Bromme et al., 1996; Kafienah et al., 1998) and this proteinase is expressed in many types of tissues. Combining these factors makes this proteinase a suitable candidate to be expressed by the dedifferentiated chondrocytes in the pectoral subdivision zones, accomplishing matrix decomposition in these regions in a macrophage- or chondroclast-independent manner.

Material and Methods

The transgenic *ctsk* reporter line, used in this analysis, was previously described in To et al. (2012). All embryos provided were raised in E3 medium (Brand et al., 2002) at a standard temperature of 28.5°C. After hatching, at 6 days post-fertilization (dpf), the larvae were transferred to aerated tap water at 26°C. At this point, juveniles were fed with dry food (ZM 000) (ZMsystems) three times a day. At 14 dpf, larger-grained dry food was given (ZM 100) (ZMsystems), while at 30 dpf regular fish food flakes were used for the remainder of the rearing time.

From 14 dpf onwards, ten randomly picked juveniles were selected every two days. These fish were anaesthetized with 0.01% ethyl 3-aminobenzoate methanesulfonate salt (MS222, Sigma-Aldrich), their notochordal length (NL) (Cubbage and Mabee, 1996) was measured and the mEGFP signal was observed in live animals using a stereomicroscope (SZX12, Olympus) fitted with a fluorescent lamp (U-RFL-T, Olympus). At 21 dpf, larvae of specific NL, i.e. 4 (n=5), 5 (n=5) and 6.5 mm (n=5), were selected for detailed live imaging with the confocal laser-scanning microscope (D-eclipse-C1, Nikon). Prior to imaging larvae were anaesthetized with 0.1% MS222. After imaging, larvae were euthanized with 0.5% MS222, fixed in 4% buffered paraformaldehyde (PFA) for 24 hours at 4°C, stained with alcian blue/alizarin red according to Walker and Kimmel (2007), and visualized according to the procedure outlined in Dewit et al. (2011). The remainder of the transgenic fish was raised to adulthood in order to form a brood stock.

In order to confirm that the observed mEGFP signal represented true *ctsk* expression, *in situ* hybridizations were performed on 4 (n=4), 5 (n=3) and 6.5 mm (n=3) fish, according to the protocol described in (To et al., 2012). The specimens were first observed under a stereomicroscope (MZ APO, Leica), followed by embedding of the specimens in epon for serial sectioning, according to the procedure outlined in Verstraeten et al. (*in press*). All sections were mounted in Depex and examined using the microscope (AXIO Imager Z1, Zeiss) equipped with DIC and fitted with an Axiocam camera.

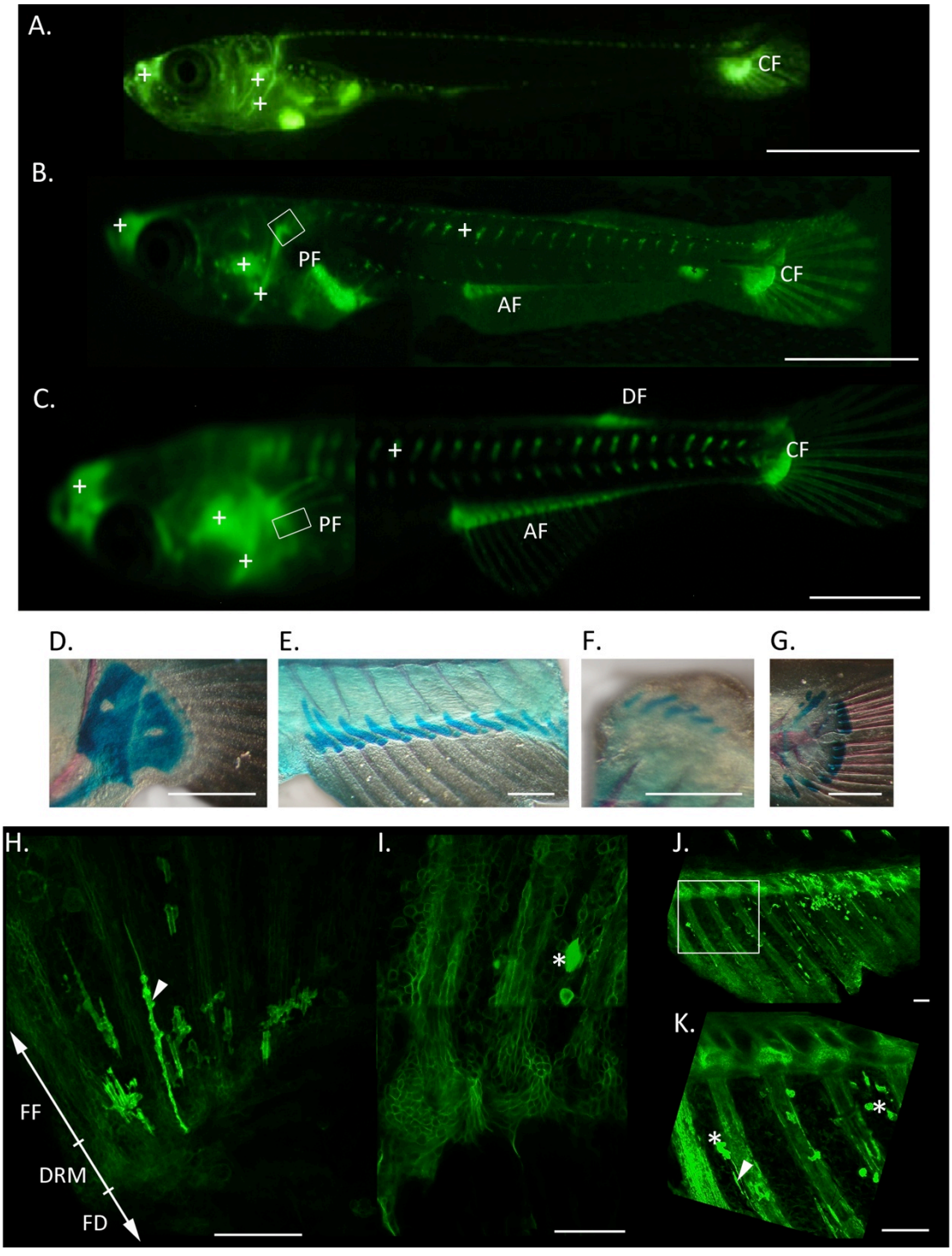
Results

At 4 mm NL, prior to the pectoral fin transformation and prior to the formation of the dorsal and anal fin, ossified regions, such as (1) the haemal and neural arches, (2) the tooth-bearing mandibular and seventh branchial arches and (3) the cleithrum, are distinctly positive for mEGFP (Figure 1 A). In addition to this osteoclast-related mEGFP signal (marked by + in figure 1), the region of the developing hypurals in the caudal fin is equally positive (Figure 1 A). However, the hypurals of the caudal fin do not show any sign of bone formation at this point (data not shown).

At approximately 5 mm NL (between 17 and 21 dpf), positive signals are visible in the pectoral fins, more specifically in the dorsal region of the distal rim mesenchyme (DRM) and in the proximo-dorsal region of the fin fold mesenchyme (FFM) (Figure 1 B). This first appearance of mEGFP signal in the pectoral fins coincides with the earliest steps in the formation of both distal radials in the DRM and lepidotrichia in the FFM, respectively. The earliest step in lepidotrichia formation is the local disappearance of collagenous fin rays or actinotrichia, which is easily observable under a stereomicroscope. Concomitant with the appearance of mEGFP in the pectoral fins, the median fin fold and underlying mesenchyme of the median septum become mEGFP-positive, just caudally to the anal opening (Figure 1 B). This mEGFP signal coincides both in timing and location with the formation of the cartilaginous fin radials and the bony fin rays of the developing anal fin.

Figure 1
Lateral view of *ctsk*:mEGFP expression in (A) 4 mm, (B) 5 mm and (C) 6.5 mm NL larvae and (+) indicates regions of known osteoclast-related *ctsk*:mEGFP expression. (D - G) Alcian blue/alizarin red skeletal staining of the specimen depicted in (C), with in (D) the pectoral fin, in (E) the anal fin, in (F) the dorsal fin, and in (G) the caudal fin. (H) Confocal stack of the boxed region in the pectoral fin of (B). (I) Confocal stack of the boxed region in the pectoral fin of (C). (J) Confocal stack of the anal fin of (C). (K) Higher magnification of the box in (J). The white arrowheads and asterisks in (H-K) indicate giant mEGFP-positive cells that are either very elongated or rounded, respectively.
Abbreviations: AF: anal fin, CF; caudal fin, DF: dorsal fin, DRM: distal rim mesenchyme, FD: fin disk cartilage, FFM: fin fold mesenchyme, PF: pectoral fin.
Scale bars in (A-C)= 1mm, in (D-G)= 500μm, in (H-K)= 50μm

At 6.5 mm NL (between 24 and 28 dpf), the mEGFP signal in the pectoral and anal fins has clearly expanded in accordance with the formation of new fin radials and lepidotrichia (Figure 1 C). In the pectoral fin, the entire DRM and fin fold have become mEGFP-positive. At this point, the majority of the distal radials and lepidotrichia are forming and the subdivision of the



pectoral cartilage is clearly visible on alcian blue/alizarin red stained specimens (Figure 1 D). However, no mEGFP-positive cells could be detected in the subdivision zones of the proximal pectoral cartilage (Figure 1 C). In the anal fin (Figure 1 E), the mEGFP signal has spread caudally, corresponding to the rostro-caudal addition of new radials to the anal fin (Figure 1C). At this time the dorsal fin starts to develop (Figure 1 F) and, similar to the earliest anlage of the anal fin in 5 mm larvae, this dorsal fin also forms in mEGFP-positive tissue (Figure 1 C). Note that all cartilaginous fin radials, with the exception of the hypurals, remain free of perichondral bone at this point and even the lepidotrichia of the pectoral and anal fin only show the earliest signs of ossification (Figure 1 D-G).

Detailed analysis through laser scanning confocal microscopy of these fin-associated regions revealed that the mEGFP-signal in all the fins originated from similar tissues. In the pectoral fins, the distal radials develop at the distal border of the fin disk cartilage, which is occupied by mesenchymal tissue, called the DRM. This mesenchymal tissue continues into the fin fold where it is called FFM. Similar pre-skeletal mesenchyme can be found in the median septum and median fin fold where caudal, anal and dorsal fins develop. While cartilaginous radials form in the mesenchymal tissue of the median septum, the lepidotrichia develop in the fin fold. In the dorsal and anal fins, distal radials develop in between the internal cartilaginous radials and the external lepidotrichia. In the caudal fin, no distal radials are formed and the lepidotrichia articulate directly with the hypurals. The DRM and FFM of the pectoral fin and the pre-skeletal mesenchyme of the median fins clearly exhibit mEGFP-signal, just prior to the appearance of cartilaginous radials and lepidotrichia. However, once differentiated cartilage tissue appears, the mEGFP-signal is lost. Only the interradial tissue and especially the perichondrium remain mEGFP-positive (Figure 1 H-K). In the FFM of all observed fins, an extensive mEGFP-signal is found when actinotrichia are still present. Once lepidotrichia form and mature, the FFM in between lepidotrichia shows a reduced mEGFP signal, while the mEGFP

signal of the mesenchymal tissue between the two half rays of a single lepidotrichium retains a stronger signal.

However, inside the generally positive fin mesenchyme additional large and isolated cells can clearly be distinguished. Unlike the mesenchymal tissue, which show only mEGFP signal at their cell periphery, these giant cells appear completely filled with mEGFP (white arrowheads and asterisks in Figure 1 H-K). In earlier stages of fin development, these giant mEGFP-positive cells are found in more proximal regions, i.e. the DRM and the median septum mesenchyme for the pectoral and median fins respectively (Figure 1 H-K). In more advanced stages of fin development, these giant cells can no longer be found in the proximal regions where cartilaginous radials form. Instead, they appear to have spread more distally into the fin fold associated with the developing lepidotrichia. This trend was identified in all fins observed and is clearly shown in Figure 1 J., which represents the same anal fin as shown in Figure 1 E. In the anterior and developmentally more advanced region of the anal fin giant mEGFP-labeled cells can only be found distally in the fin fold, while in less advanced more caudal regions a high concentration of these large cells is found in the proximal part of the septum and fin fold mesenchyme. If Figure 1 J is compared to the skeletal staining in Figure 1 E it becomes clear that these giant mEGFP-labeled cells appear concomitant with the earliest signs of lepidotrichia formation, spreading more distally as lepidotrichia mature and grow outward. In addition, it is important to note that two kinds of these giant mEGFP cells can be distinguished. First, very long and slender cells that extend in parallel to the forming lepidotrichia can be identified just next to the lepidotrichia (white arrowheads in Figure 1 H and K). Second, more rounded cells, often with extensive filopodia can be seen, mostly in between lepidotrichia, although some are clearly associated with the bony fin rays (white asterisks in Figure 1 I and K).

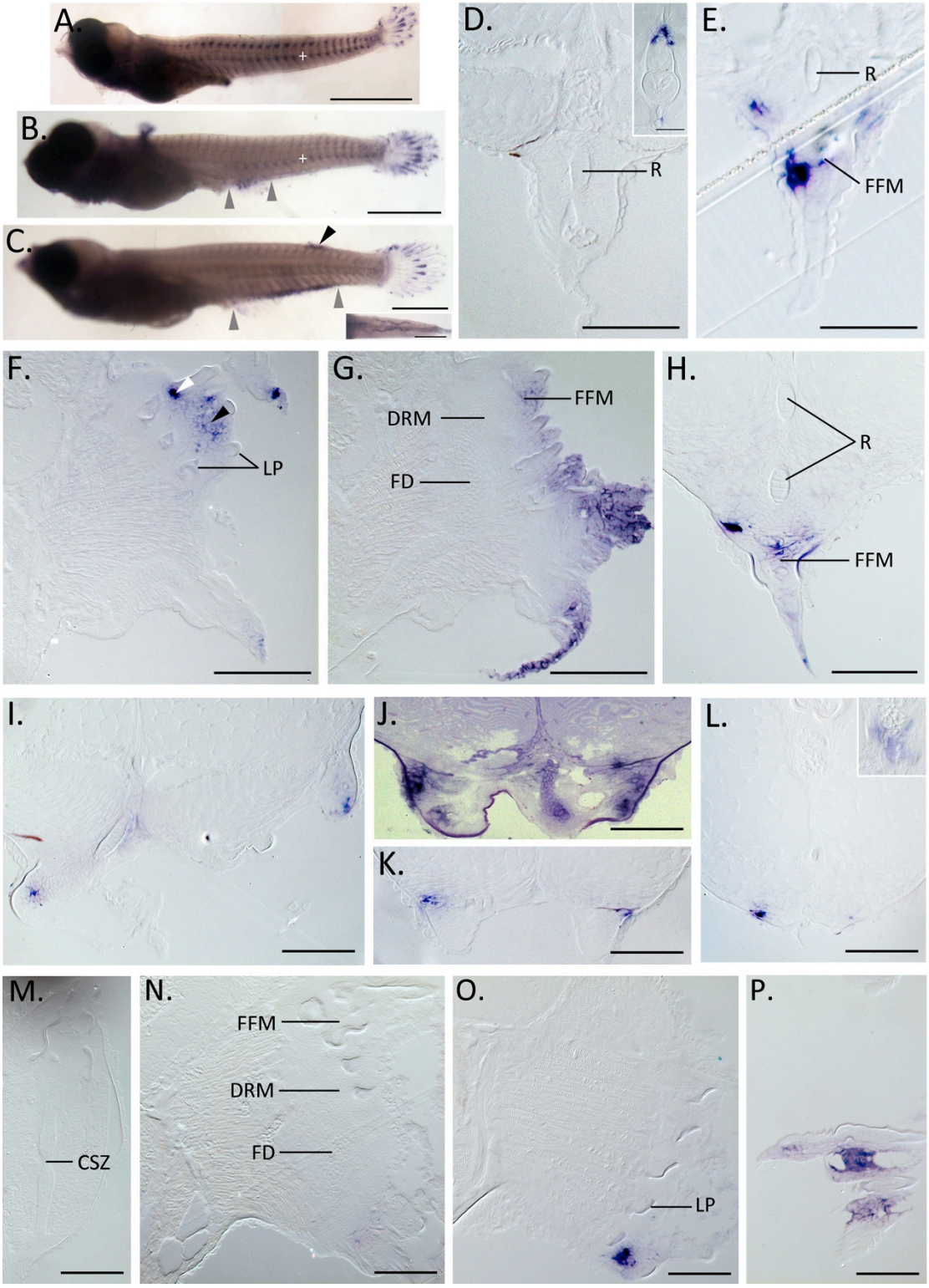
In order to confirm that the observed mEGFP signal represents true *ctsk* expression, whole mount *in situ* hybridization for *ctsk* in 4, 5 and 6.5 mm wild type medaka were performed by Dr. T.T. To from the laboratory of Dr. Winkler. Observations on whole mounts showed that all specimens exhibited

osteoclast-related *ctsk* expression in the haemal and neural arches and tooth-bearing regions, although this signal was harder to identify or absent in larger specimens of 5 and 6.5 mm (Figure 2 A-C). In 4 mm larvae, fin-related *ctsk* expression could only be identified in the caudal fin, while the pectoral and anal fins showed *ctsk* expression only in 5 mm larvae and the dorsal fin only in the 6.5mm larvae (Figure 2 A-C, pectoral fin not shown). However, more detailed observations of the *ctsk* expression in the developing fins revealed that the *in situ* hybridization signal is clearly less abundant than the mEGFP signal of the transgenic reporter line and even completely absent in the regions where the cartilaginous fin radials are forming.

Ctsk expression was therefore studied in more detail by embedding and sectioning these specimens. In 4 mm specimens, *ctsk* expression was found in the haemal and neural arches, tooth-bearing regions and the FFM of the caudal fin. No *ctsk* expression was found surrounding the developing hypurals (data not shown). In 5 mm larvae, *ctsk* expression could be detected in the FFM of both the anal and the pectoral fin (Figure 2 D-G). However, expression associated with the developing cartilaginous radials, i.e. the distal radials in pectoral fins and both the distal and the proximal radials of the anal fin, could not be detected (Figure 2 D-G). In the pectoral fins, the most proximal *ctsk* expression was found in the FFM surrounding the developing

Figure 2
Lateral view of whole mount *ctsk in situ* hybridization in (A) 4 mm, (B) 5 mm and (C) 6.5 mm NL larvae and (+) indicates regions of known osteoclast-related *ctsk* expression. Grey arrowheads in (B) and (C) mark the anal fin, while the black arrowhead in (D) marks the dorsal fin. Insert in (C) shows ventral view of the anal fin in (C). (D-G) DIC micrograph of 5 mm NL larvae, with in (D-E) the anal fin and in (F-G) the pectoral fin. Insert in (D) shows the neural and haemal arches found in section (D). White and black arrowheads in (F) indicate strong *ctsk* expression in a single large cell, and diffuse *ctsk* expression in multiple mesenchymal cells, respectively. (H-P) DIC micrograph of 6.5 mm NL larvae, with in (H-L) the anal fin and in (M-P) the pectoral fin. (J) Was counterstained with toluidine blue. Insert in (L) shows the haemal arch found in section (L).
Abbreviations: CSZ: cartilage subdivision zone, DRM: distal rim mesenchyme, FFM: fin fold mesenchyme, FD; fin disk cartilage, LP: lepidotrichium, R: anal fin radial.
Scale bars in (A-C) = 1mm, in (D-P) = 50 μM

lepidotrichia, while the DRM was negative. In the anal fin, the most proximal *ctsk* signal was found just underneath the epidermis in the border region between median septum and fin fold mesenchyme. The fin fold of the anal fin did not show expression in every section (Figure 2 D-E), while the pectoral fin fold was found to be overall positive for *ctsk* expression (Figure 2 F-G).



In the 6.5 mm larvae, only the anterior portions of the anal fins were preserved intact, while more caudal regions showed severe rupturing and deformation of the tissue (Figure 2 H-L). Similar damage was found in the dorsal fins (not shown). In the most anterior and best-preserved region of the anal fin (Figure 2 H), *ctsk* signal was found only in a subset of the sections and always located in the FFM. In all sections, *ctsk* expression was absent in the tissues surrounding the cartilaginous radials. Just as in 5 mm larvae, the most proximal *ctsk* expression was found in superficial mesenchyme at the body-to-fin fold border. In more caudal sections, the anal fin was severely ruptured, compromising our interpretation of the exact location of the *ctsk* expression. In many sections the cathepsin signal appeared to be located in FFM, which was split in two halves along its median plane, creating an artificial double ridge of *ctsk*-expressing mesenchyme (Figure 2 I). However, some sections in the most caudal regions of the anal fin show less severely distorted tissue presenting *ctsk* expression in two separate ventral ridges, one on each side of the median plane, apparently lined with an intact epidermis (Figure 2 J-K). The exact extent of the damage was, however, difficult to asses even if sections were counterstained with toluidine blue (Figure 2 J). The, currently unidentified, double ridge of *ctsk*-expressing tissue can also be seen in the insert of figure 2 C. In the pectoral fins of the 6.5 mm larvae, the cartilage subdivision zones of the pectoral fin disk cartilage are clearly visible, but do not show any signs of *ctsk* expression (Figure 2 N). The more proximal and dorsal regions of the FFM, which showed *ctsk* expression in 5 mm larvae, did not retain *ctsk* expression (Figure 2 O). More ventrally (Figure 2 O) and distally (Figure 2 P), the pectoral FFM did, however, retain its *ctsk* expression. The *ctsk* expression in all examined fins consisted of diffusely positive mesenchymal cells, as well as some isolated large and strongly positive cells (arrowheads Figure 2 F). Finally, it should be noted that the osteoclast-related *ctsk* signal of the haemal and neural arches was either absent or poorly visible in most of the 6.5 mm larvae, while this signal served as a positive control in virtually all sections of the 4 and 5 mm larvae (compare inserts in Figure 2 D and Figure 2 L).

Discussion

Detailed observation of the mEGFP from the *ctsk* reporter line showed that *ctsk* is expressed in all developing fins. More specifically, fin mesenchyme showed expression of *ctsk* just prior to and during the formation of both cartilaginous and dermal fin radials. However, these observations could only partially be confirmed by whole mount *in situ* hybridizations for *ctsk* on representative stages of medaka development.

The most obvious reason for these inconsistencies would be an incomplete detection of *ctsk* by the *in situ* hybridization. Evidence for this hypothesis can be seen in the incomplete staining of osteoclasts in the haemal and neural arches of larger specimens, while these cells were adequately stained in the smaller specimens. All specimens were treated for 1 h in 20ug/ml proteinase K. This digestive treatment allows for probe penetration, but may have been insufficient to reach the internal tissues of the larger specimens. However, a further increase in digestive treatment would not be advisable, since many external tissues already sustained considerable damage. In particular, the bad condition of the thin external fin folds resulted in the loss of precious data. If this *in situ* analysis is repeated in the future, it would be advisable to use paraffin or cryo-sections for the analysis, which would allow tissues to remain intact, while both internal and external tissues are equally exposed to the probe. Such analysis could, in addition, reveal whether the bilaterally paired ridges of *ctsk*-positive tissue in 6.5 mm larvae represent true morphological structures or an artifact caused by tissue rupturing and deformation during the staining protocol.

These technical difficulties considered, the differences between the data obtained by *in situ* hybridization and the transgenic reporter line remain substantial. The most striking difference can be found prior to and during the formation of the cartilaginous fin radials. The reporter line clearly shows mEGFP signal in the fin mesenchyme prior to the formation of cartilaginous radials, which disappears inside the differentiating cartilages, but remain in the perichondral and interradial regions. None of this cartilage-associated

mesenchyme shows expression by *in situ* hybridization, despite positive staining in the nearby FFM. The lack of staining associated with the cartilaginous radials is therefore not likely to be caused by insufficient probe penetration, although further analysis is required to rule out this possibility.

Another difference between transgenic line and *in situ* signals can be found in the FFM. The transgenic line shows that the entire FFM becomes mEGFP positive just prior to lepidotrichia development. This signal coincides with the disappearance of collagenous fin rays or actinotrichia and was confirmed by *in situ* hybridization, at least for the pectoral FFM. Due to the bad conditions of most fin folds in the *in situ* hybridizations, this observation could not be confirmed for the median fins. However, once lepidotrichia have formed, the transgenic line shows a strong reduction of mEGFP signal between the lepidotrichia, while the mEGFP signal remains relatively strong in the mesenchyme between two lepidotrichium half rays. In contrast, in hybridized specimens, the FFM completely loses its *ctsk* expression once lepidotrichia are well established. In fact, the only mesenchymal tissue that sometimes retains expression is located between the lepidotrichia.

These inconsistencies between the transgenic reporter line and the *in situ* hybridizations may have been caused by a number of reasons. While *in situ* hybridization detects *ctsk* messenger RNA (mRNA), the reporter line shows the translated protein product of the construct mRNA, whose transcription is controlled by the *ctsk* promoter. If mRNA levels are low, they could be missed by *in situ* hybridization. However, similarly low concentrations of construct mRNA, could still result in detectable amounts of mEGFP if translation occurs fast or over an extensive period of time. This could make the transgenic line more sensitive to low levels of *ctsk* expression.

Alternatively, the mEGFP produced by translation of the construct mRNA could have a significantly longer lifetime than the mRNA strands of both the construct and the endogenous *ctsk* gene. Since EGFP is stable in physiological conditions, the lifetime of the mEGFP signal depends on how fast cells will target the membrane-bound EGFP for degradation. Therefore mEGFP can potentially be present in cells that no longer express *ctsk*. This

could explain why the entire proximal region of the pectoral FFM remained mEGFP-positive in 6.5mm larvae, while only the ventral region, which develops last, showed *ctsk* expression with *in situ* hybridization. In addition, this scenario could explain the presence of mEGFP in FFM with well-established lepidotrichia, which appears negative in the *in situ* analysis.

A final reason for the discrepancies between observations made in the transgenic versus the hybridized specimens is the occurrence of ectopic construct expression. The location of the transgenic insertion in the genome occurs randomly. When a specific region of the genome is highly expressed during some developmental process, an inserted construct in that particular region can show expression by ectopic activation of its promoter sequence. In this case, the detected mEGFP signal does not represent true *ctsk* expression. Further analysis will be required to verify whether either of the processes, described above, are responsible for the observed discrepancies. Ectopic *mEGF* expression, caused by transcript insertion, has been reported in the zebrafish caudal fin (Hadzhiev et al., 2007).

There are, however, good indications that the mEGFP signal we observed truly corresponds to *ctsk* expression, despite the inability of our *in situ* analysis to confirm many of these observations. Published images of another medaka *ctsk* reporter line show EGFP signals in both the anal and dorsal fin identical to the ones described in this report (Chatani et al., 2011). It is rather unlikely that the same transcript insertion causes similar artifacts in two independent lines. In addition, these authors show a *ctsk* whole mount *in situ* with clear *ctsk* expression surrounding the cartilaginous radials of the anal fin. This specimen likely received more extensive digestive treatment than our specimens, judging by the extensive damage to the specimen. This suggests that technical imperfections in our *in situ* analysis are the most likely cause for the discrepancies we observed. While future research certainly requires a more elaborate *in situ* analysis, other techniques could also aid in solving the discrepancies between the reporter line and the ISH results, such as a qPCR.

Some of our observations, however, did appear consistent in both the transgenic line and the *in situ* hybridization analysis. The transgenic line

revealed giant cells that were first found once actinotrichia started to disappear in the most proximal region of the fin fold. These cells were found more distally in the FFM when lepidotrichia had developed further. Detailed observations on the cell morphology of these cells suggested that two distinct types could be distinguished: one appeared strongly elongated and situated alongside the lepidotrichia, while the other appeared rounded with extensive filopodia situated both between and next to the lepidotrichia. Both types of giant cells appeared completely filled with mEGFP, which suggests high transcription rates of the construct in these cells. In the *in situ* hybridizations, similar intensely stained regions could be identified in the FFM indicating large amounts of *ctsk* mRNA (white versus black arrowhead in Figure 2 F). However, the bad overall condition of the fin folds in the *in situ* hybridizations made detailed observation on these cells unreliable making it hard to confirm with certainty that these regions correspond to the giant cells observed in the transgenic line.

At this point, we can only speculate on the identity of these cells, although it is highly unlikely that they are osteoclasts, since their first appearance clearly precedes bone formation and acid phosphatase activity assays fail to reveal these cells, which would be expected if these cells were osteoclasts (own observations; (Chatani et al., 2011; To et al., 2012). It seems likely that these *ctsk*-positive cells contribute to the resorption of the actinotrichia and may even contribute to the development of new nerves and blood vessels, which form concomitantly with each lepidotrichium (Carmeliet and Tessier-Lavigne, 2005; Thorsen and Hale, 2007).

Another consistent observation in this analysis was the complete absence of *ctsk* during the subdivision of the pectoral fin disk cartilage. This confirms previous observation in zebrafish showing that chondro/osteoclast cells are not involved in the subdivision process (Dewit et al., 2011). Furthermore, this data shows that, although CSTK is capable of degrading cartilaginous ECM components at neutral pH, it is not involved in the decomposition of the cartilage extracellular matrix during the remodeling of the pectoral endoskeleton.

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4. General discussion

In this doctoral thesis, I used zebrafish and medaka to investigate some of the cellular mechanisms that underlie the skeletal transformations in teleost pectoral fins. This is one of the most unusual skeletal transformations known in vertebrate species, which includes the loss of cartilage tissue in four regions of a previously continuous cartilaginous element. In this discussion, I will first address the unique aspects of teleost pectoral fin development. Second, I will elaborate on the mechanisms that drive the loss of cartilage tissue during the transformation of the pectoral endoskeleton and I will compare these findings with other known mechanisms of cartilage remodeling.

4.1. The biphasic nature of teleost pectoral fin development

In all teleosts, the pectoral fins have two distinct phases in their development, each characterized by a completely different skeletal morphology. Most teleost embryos hatch at an early stage of development. In many of these teleost hatchlings the dependence on the yolk sac quickly gives way to active feeding. At that point, their endoskeleton is limited to some cranial and splanchnocranial cartilages and the supportive elements of their premature pectoral fins (Grandel and Schulte-Merker, 1998; Kimmel et al., 2001). These early pectoral fins are supported by a single cartilage element that consists of both the scapulocoracoid of the girdle and an external disk-shaped cartilage. The fin disk cartilage separates a ventral and a dorsal muscle group, whose fibers run from the girdle to the actinotrichia of the epithelial fin fold. In zebrafish, this early skeleton forms as early as 5 days post-fertilization (dpf) and remains mostly unaltered for approximately two more weeks (Grandel and Schulte-Merker, 1998).

At approximately 20 dpf, when the zebrafish young reach a standard length of 5 mm, other elements of the skeleton, such as the vertebral column and the median fins, start to form (Bird and Mabee, 2003). This is followed by the formation of scales and the pelvic fins, which complete this second period of skeletal development roughly 10 days later, when the larvae's standard length reaches approximately 10 mm (Sire et al., 1997; Grandel and Schulte-Merker, 1998). Simultaneously with the formation of the adult skeleton, the premature pectoral fins transform to their adult morphology. Lepidotrichia form in the fin fold and the fin disk cartilage subdivides in order to form four individual proximal radials. Interestingly, all these skeletal changes appear to be regulated by thyroid hormone (Brown, 1997; Shkil et al. unpublished data). In truly metamorphic teleosts, mostly flatfish, the characteristic eye migration equally coincides with the formation of vertebral bodies, median fins and the transformation of the larval pectoral cartilage. Furthermore, all these changes in the flatfish skeleton have been shown to be thyroid hormone-dependent

(Schreiber, 2006). Together, these data suggest that, even in the non-metamorphic zebrafish, this second period of skeletal development could be seen as a period of larval-to-juvenile transition (Liu and Chan, 2002).

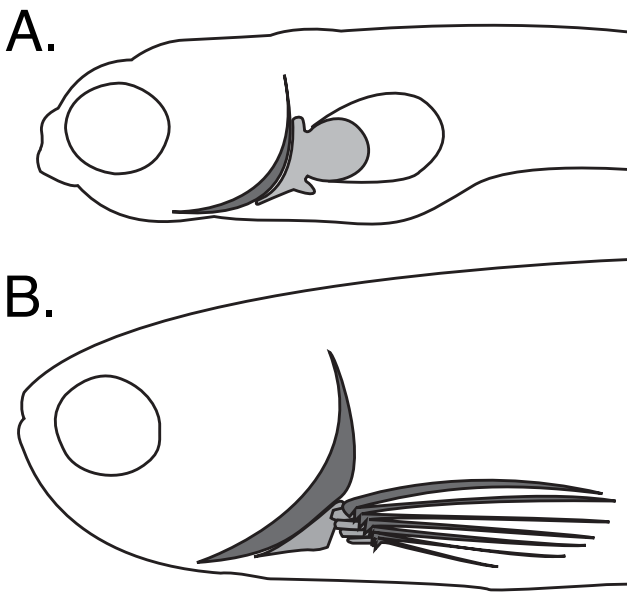
The biphasic development of the teleost pectoral fins possibly evolved in the wake of a genome duplication event that occurred in the actinopterygian lineage between 226-316 MYA and is common to teleost species only (Hoegg et al., 2004; Hurley et al., 2007). This genome duplication event is responsible for the occurrence of many paired paralog genes in teleosts that are only represented by a single ortholog in other gnathostomes.

Of relevance to the cartilaginous skeleton, one example of such duplicated genes is the transcription factor *Sox9* (Chiang et al., 2001b; Cresko et al., 2003; Koopman et al., 2004). This transcription factor promotes crest-like behaviors in neural plate cells (Cheung and Briscoe, 2003) and helps to determine crest-derived chondrogenic lineages (Spokony et al., 2002; Yan et al., 2002; Mori-Akiyama et al., 2003). In addition, *Sox9* is an upstream factor in the morphogenesis and differentiation of cartilage and bone (Bi et al., 1999; Bi et al., 2001; Yan et al., 2002). The zebrafish ortholog genes *Sox9a* and *b* are expressed in separate sub-portions of the total ancestral *Sox9* expression domain. The diversification of paralog functions is clearly seen in *Sox9*-associated cartilage differentiation. At 5 dpf, *Sox9a* is expressed in the splanchnocranial condensations and in the pectoral girdle, but only to a minimal extent in the fin disk cartilage, while *Sox9b* is exclusively expressed in the fin disk condensation. Consistently with these differences in expression, loss of gene function by morpholino injection or mutation reveals that the pectoral fin disk is the only differentiated cartilage that remains when the *Sox9a* function is impaired, while the fin disk cartilage is the most severely affected element in larvae with impaired *Sox9b* function (Yan et al., 2005).

These observations offer some insight into the unique developmental regulation of the teleost fin disk cartilage, compared to other cartilaginous elements of the teleost endoskeleton, but also compared to the pectoral endoskeleton in other gnathostome taxa. The contribution of the endoskeleton to the adult pectoral fins is in no other gnathostome taxon as small as in

teleosts. It is well established that the precursors for the pectoral endoskeleton are formed by proliferation of undifferentiated limb bud mesenchyme. The relatively early differentiation of these precursors into the fin disk cartilage inevitably coincided with a relatively early stop in fin bud outgrowth, which resulted in the relatively small contribution of the endoskeleton to the adult pectoral fins of teleosts. The exact cause of the endoskeletal reduction in teleosts is currently unknown, but is highly likely that both the teleost genome duplication and heterochronic shifts in gene expression, similar to the one described in Sakamoto et al. (2009), could have facilitated this evolutionary trend. In any case the reduction of the endoskeleton would never have compromised the functionality of teleost pectoral fins, since the lepidotrichia-supported fin fold has been the main locomotory actor in neopterygian species long before teleost developed their early-phase, we could say larval-phase, pectoral morphology.

Furthermore, there are good indications that the establishment of a larval-phase pectoral fin has resulted in the evolution of some completely new locomotory strategies in more derived teleost species. The larval pectoral fins of teleosts possess a rather specific orientation with respect to the major body axis. The anterior edge of the fin points dorsally and the ventral and dorsal muscle groups respectively face towards the rostral and caudal ends of the larvae. During the formation of the adult pectoral fins in zebrafish, cartilage subdivisions and allometric growth of the body change the positioning of the pectoral fins to a more ventral position, with the previously dorsal edge of the fin now facing rostrally (Grandel and Schulte-Merker, 1998) (See Figure 1). The latter adult position of the pectoral fins is



also found in basal actinopterygians and chondrichthyans and allows for considerable maneuverability and optimum performance in breaking, but only limited forward propulsion (Drucker and Lauder, 2003).

However, many derived taxa of teleosts, such as perciforms, appear to have retained the positioning of the larval pectoral fins. Lepidotrichia and cartilage subdivision zones form normally, but the overall outline and position of the former fin disk cartilage remains practically unaltered (Thorsen and Westneat, 2005; Schreiber, 2006) (personal observations). Interestingly, many of these taxa have developed locomotory strategies that depend heavily on strong and frequent undulations of the pectoral fins, which give them unique maneuverability and even an energy-efficient method of slow-speed forward propulsion, called labriform locomotion (Westneat, 1996; Thorsen and Westneat, 2005; Jones et al., 2007). I propose that the position and articulation of the larval pectoral endoskeleton had some preadaptive value, which enabled the majority of advanced teleost taxa to develop these new locomotory strategies.

Additional indications for the unique nature of the teleost pectoral fins, comes from the evidence we collected on the composition of the pectoral fin disk cartilage. We examined the distribution of fibronectin during zebrafish chondrogenesis and found that this glycoprotein is strongly present in the fin disk cartilage prior to its subdivision (Dewit et al., 2010). In tetrapods, different splice-isoforms of fibronectin perform different roles during chondrogenesis. Fibronectin is essential for the establishment of chondrogenic condensations (Kulyk et al., 1989; Gehris et al., 1996; Gehris et al., 1997). Once these condensations differentiate into cartilaginous elements, fibronectin is down-regulated and only expressed in a cartilage-specific isoform, whose function is still poorly understood (Zanetti et al., 1990; White et al., 1996). We found that, unlike in tetrapods, zebrafish cartilage

Figure 1
Schematic representation of the position and orientation of the pectoral fin, prior to (A) and after (B) the second stage in pectoral development. The endoskeleton and dermal bones are shown in light and dark grey, respectively.

condensations of the splanchnocranium, pectoral and median fins formed without detectable amounts of fibronectin in the ECM, although we did find a short period immediately after initial differentiation in which fibronectin could be detected in all the cartilages we observed. In the fin disk cartilage, however, fibronectin could be detected very clearly and over an extended period of time (Dewit et al., 2010).

At this point, I can only speculate on the role of fibronectin in zebrafish cartilage. Histological analysis nevertheless suggests that the presence of fibronectin coincides with an early phase in zebrafish chondrogenesis marked by the presence of stacked chondrocytes. In many teleost species, cartilage condensations form by intercalating precursor cells, which organize themselves in rows or plates of just one cell wide (Huysseune and Sire, 1992; Kimmel et al., 1998). These stacked precursors are characterized by cell-to-cell contacts and N-cadherin expression prior to their differentiation into chondrocytes (Liu et al., 2003). At that point, they gradually lose cell-to-cell contacts and start the production of cartilage ECM (Huysseune and Sire, 1992). I hypothesize that this mode of chondrogenesis does not require a temporary condensation-specific ECM as in tetrapods, whose chondrogenic condensations are many cells wide in cross section. The stacked organization of chondrocytes remains visible for some time after elements have differentiated, but is eventually lost by interstitial and appositional growth (Kimmel et al., 1998). We found that fibronectin could transiently be detected in various newly differentiated zebrafish cartilages prior to the disruption of the stacked chondrocyte organization. In the fin disk cartilage, however, both the stacked chondrocyte organization and fibronectin are retained significantly longer than in other cartilaginous elements, which suggests that this element is retained in an early differentiation phase until the fin endoskeleton is remodeled into its adult morphology (Dewit et al., 2010).

In the introduction of this thesis, I summarized our current understanding of endoskeletal pattern formation in the pectoral appendages. In tetrapods, the endoskeletal pattern is formed during the cartilage condensation stage prior to the appearance of differentiated cartilage. Despite

our considerable understanding of the many processes that are involved in cartilage development, no morphogens are currently known to be directly accountable for the specific endoskeletal pattern in tetrapod limbs. In stead, reaction-diffusion based computer models suggest that the skeletal patterns in tetrapod limbs could arise from relatively simple molecular feedback loops if these involve an activator that directly or in-directly stimulates the production of a faster spreading inhibitor. Interestingly, many of such activating and inhibitory molecules are known to operate during chondrogenesis, but until this day it remains unclear how their regulation is linked during the different stages of chondrogenesis. If such information were to be discovered, it would undoubtedly improve our understanding of endoskeletal pattern formation in tetrapod limbs. However, such information could equally provide a potential explanation for the significant difference between pattern formation in tetrapod limbs and actinopterygian fins. In actinopterygians and more specifically in polypterids, *Lepidosteus* species and teleosts, the adult pattern of the pectoral endoskeleton forms very differently, i.e. by the subdivision of an apparently differentiated cartilaginous disk. In stead of attempting to uncover the developmental regulation behind this unusual method of endoskeletal pattern formation, this doctoral research focused on the mechanisms that are responsible for the skeletal reorganization that makes this method of pattern formation possible, i.e. the mechanism of cartilage loss inside the subdivision zones.

4.2. The mechanism of fin disk cartilage subdivision

In the present work, we have evaluated all known ways in which cartilaginous tissue can be degraded. According to the literature, the disappearance of chondrocytes and their elaborate ECM can only be accomplished by a limited number of cellular mechanisms. Specialized resorption cells, such as macrophages (Blumer et al., 2008) or chondroclasts (Vu et al., 1998), can resorb cartilaginous tissue. The resident chondrocytes can undergo programmed cell death, apoptosis, and be resorbed secondarily by the surrounding tissue or the previously mentioned specialized resorption cells (Bronckers et al., 2000). Finally, chondrocytes could stop the production of new ECM components and resorb their previously secreted ECM (Yocum et al., 1995; Holmbeck et al., 2003).

I found that no apoptosis or resorption by either macrophages or chondroclasts could be identified in the cartilage subdivision zones of the zebrafish pectoral endoskeleton (Dewit et al., 2011). In addition, I confirmed that chondroclasts and macrophages were equally absent during fin disk subdivision in medaka (Chapter 4). I did find, however, that major ECM components, such as proteoglycans, collagen type II and fibronectin, disappear relatively early in the subdivision process (Dewit et al., 2010), at a point when the resident chondrocytes are still recognizable. The ultrastructure of this early-phase cartilage subdivision zones shows mesenchymal-like cells completely enclosed by amorphous ECM. In adjacent regions of persistent cartilage, on the other hand, the resident cells show the typical ultrastructure of chondrocytes and the ECM shows the distinctive fibrillar ultrastructure, as expected from cartilaginous matrix. These observations indicate that the chondrocytes in the cartilage subdivision zone dedifferentiate, suggesting that they are themselves responsible for the loss of cartilage matrix in these regions (Dewit et al., 2011).

Ultrastructural analysis further showed that the degrading ECM subsequently fragments and that the now mesenchymal cells (former chondrocytes), re-establish cell-cell contacts amongst each other and with perichondral mesenchyme. Once the former cartilaginous ECM has

disappeared completely, it becomes impossible to distinguish the former chondrocytes from the perichondral tissue (Dewit et al., 2011). Although the absence of apoptosis suggests that the former chondrocytes survive the cartilage-to-interradial transition, further investigation is required in order to assess to which extent former chondrocytes actually contribute to the connective tissue of the interradial regions, or even to the perichondral bone that forms around proximal radials.

If former chondrocytes do in fact contribute to differentiated interradial tissues, the dedifferentiation of the fin disk chondrocytes only represents the first phase of a transdifferentiation event, as defined by Thowfeequ et al. (2007), i.e. the transition of one differentiated cell-type into another in which both cell-phenotypes can be distinguished by molecular and biochemical markers. Testing this hypothesis requires long-term lineage mapping of the fin disk chondrocytes. This was attempted with the micro-injection of DiI cell-tracker, but the impractical location and small dimensions of the fin disk cartilage, together with the high mortality of successfully injected larvae, made this approach practically unfeasible. For future attempts, I propose that transgenic lines with, for example, a photo-convertible fluorescent construct could be used in combination with dual-photon microscope photo-conversion in order to trace the fin disk chondrocytes.

Many different vertebrate cell types, including chondrocytes, have shown the ability to transdifferentiate *in vitro* (Thowfeequ et al., 2007; Schlegel et al., 2009). As early as 1981, Beresford reviewed the literature in support of the concept that the phenotypes of connective tissues are not terminal and that phenotypic plasticity can play an important role in various developmental and homeostatic processes. Despite numerous recent studies showing different types of phenotype transitions in connective tissue, transdifferentiation remains a controversial subject, mainly because few studies have provided the detailed fate-mapping needed in order to follow cells during their phenotypic transformation (Witten and Hall, 2002; Witten and Hall, 2003; Li et al., 2004; Blumer et al., 2005; de la Fuente and Helms, 2005; Kim et al., 2005; Gillis et al., 2006; Choi et al., 2010). Such crucial information

has currently been provided for the contribution of differentiated cells in tissue regeneration and repair in several vertebrate species (Tanaka, 2003; Knopf et al., 2011; Tanaka and Reddien, 2011). Evidence for the plasticity of the cartilage phenotype specifically can be found in the existence of intermediate tissue types, such as chondroid bone and fibrocartilage (Hall and Witten, 2007). Furthermore, various studies have reported that cells entrapped in chondrocyte lacunae could show molecular and cellular characteristics of the osteoblastic or fibroblastic phenotype, which suggests that the resident chondrocytes have switched to another phenotype (Roach et al., 1995; Harada and Ishizeki, 1998; Holmbeck et al., 2003; Hammond and Schulte-Merker, 2009).

While the observed dedifferentiation of chondrocytes in the zebrafish fin disk cartilage could explain the arrest of cartilaginous matrix production in the cartilage subdivision zones, it remained unclear how these dedifferentiating cells could resorb their ECM. Therefore, I evaluated the presence of a subset of proteinases, which are capable of degrading cartilage ECM components at neutral pH. I found that the presence of MMP14 (synonym MT1-MMP) coincides in both timing and location with the reorganization of the fin disk cartilage in zebrafish. In addition, I found that inhibition of MMP14-mediated degradation did significantly retard fin disk cartilage subdivision. However, our data equally suggested that other currently unknown catabolic enzymes are also involved in fin disk cartilage subdivision (Chapter 3).

Holmbeck et al. (2003) described the occurrence of MMP14-dependent cartilage remodeling of three separate regions of uncalcified cartilage in the mouse endoskeleton; (1) the posterior region of the Meckelian cartilage, which transforms into the sphenomandibular ligament, (2) the calvarial cartilage of the skull, and (3) the groove of Ranvier, which is the superficial region of uncalcified cartilage bordering the proliferative and hypertrophic chondrocytes of the long bone metaphysis. Interestingly, the findings of these authors and our observations bear strong resemblance. In mouse, the absence of MMP14-mediated degradation results in remnant ‘ghost cartilage’ in regions where

cartilage tissue would normally have disappeared (Holmbeck et al., 2003). This 'ghost cartilage' is remnant cartilaginous matrix devoid of proteoglycans and containing empty chondrocyte lacunae, suggesting that even without MMP14 activity these cartilages still degrade to some extent. In zebrafish, impaired MMP14-function results in the retardation of fin disk cartilage subdivision, but not in the complete stop of cartilage loss. Both studies suggest that even without MMP14-function the loss of the chondrocyte phenotype and even some ECM degradation still proceed in these specific cartilaginous regions.

Different from our observations in zebrafish, murine MMP14 remodeling involves chondrocyte apoptosis. Yet, in the mouse, some chondrocytes appear to escape cell death by switching their cell phenotype, as suggested by the presence of osteocalcin expressing cells in some lacunae of the 'ghost cartilage' (Holmbeck et al., 2003). It is, however, important to note that in most cases the proportion cell-to-matrix is much smaller in tetrapods than in zebrafish cartilage. Thicker ECM walls could make it impossible for dedifferentiated mouse chondrocytes to re-establish cell-cell contacts, which is required for both fibroblast and osteoblast survival, and thus illicit apoptosis. Ultrastructural observations that would support such an interpretation are only available for the posterior portion of the murine Meckelian cartilage, which is an embryonic cartilage with relatively thin ECM walls. Here, fibroblastic cells occupying former chondrocyte lacunae are described, many of which show cell-cell contacts and no signs of apoptosis (Harada and Ishizeki, 1998). This, together with our data, suggests that the re-establishment of cell-to-cell contacts may be required for the dedifferentiated chondrocytes to escape apoptosis during MMP14-associated remodeling.

Although the molecular signals that induce the loss of uncalcified cartilage in specific regions of the zebrafish pectoral fins and in several sites of the murine endoskeleton are currently unknown, many aspects of MMP14-associated cartilage loss in zebrafish and mouse are highly similar. Therefore, I propose that chondrocyte dedifferentiation, whether or not followed by

apoptosis, and MMP14-mediated ECM degradation could represent a conserved pathway of cartilage catabolism common to all Osteichthyes. In order to test this hypothesis, the presence of MMP14 should be examined during cartilage loss in more basal actinopterygian and tetrapod species. Polypterids could represent valuable test subjects in this regard because of their basal phylogenetic position, but also because of the development of their pectoral endoskeleton. All basal actinopterygians form a multitude of proximal radials out of a continuous disk-shaped condensation (Grandel and Schulte-Merker, 1998). However, in polypterids this condensational disk also develops into a cartilaginous disk before subdivision begins (Budgett, 1902). This implies that ECM is secreted before the interradials lose their skeletogenic nature, suggesting that ECM degradation is required for polypterid pectoral development just as in teleosts. However, amphibian species could also present the opportunity to test our hypothesis on the conserved nature of MMP14-associated remodeling of uncalcified cartilages. It was recently described that during amphibian metamorphosis some cartilages of the splanchnocranium are drastically reduced in size (Rose, 2009). However, the developmental mechanism behind this loss of uncalcified cartilage was not identified.

It will, however, be equally important to discover more up-stream factors, responsible for inducing the loss of the chondrocyte phenotype and regulating MMP14-mediated ECM degradation. Wnt14 could represent a possible candidate for such an up-stream factor, since it inhibits chondrogenesis during joint formation and is known to reverse the chondrocytes' phenotype *in vitro*. Another possible factor may be thyroid hormone, which is known to regulate many skeletal changes in teleost larvae, including the pectoral cartilage subdivision (Shkil et al. unpublished data; Schreiber, 2006). In frog metamorphosis, thyroid hormone signaling has been linked directly to MMP14 expression (Shi and Ishizuya-Oka, 2001; Fu et al., 2007). Also in mouse, indications exist for the involvement of thyroid hormone in MMP14 regulation (Holmbeck et al., 2003). Some phenotypic traits of MMP14–deficient mice (Holmbeck et al., 1999) resemble both cretinism and

hypothyroidism symptoms in humans (McLean and Podell, 1995) and traits of thyroid hormone receptor knockout models (Göthe et al., 1999).

In conclusion, this research revealed that the zebrafish fin disk cartilage is maintained in an early differentiation stage, characterized by stacked chondrocyte organization and fibronectin-rich ECM, until it is subdivided into the four proximal radials. This subdivision of the pectoral fin disk cartilage is accomplished by dedifferentiation of the resident chondrocytes and MMP14-mediated ECM degradation. Although the pectoral endo-skeleton transformation is specific to the teleost lineage, literature shows that various uncalcified cartilages in mouse disappear in a highly similar fashion. Therefore, I hypothesize that the remodeling of uncalcified cartilages may be accomplished by a conserved method of cartilage degradation, which involves chondrocyte dedifferentiation and MMP14-mediated ECM degradation. However, in order to consolidate this hypothesis, it will be essential to, first, identify the molecular signals that regulate the destruction of both mouse and zebrafish uncalcified cartilages and, second, discover similar events of cartilage degradation in other osteichthyan species.

5. Summary

English version

Teleost fish are the only gnathostomes that undergo a major architectural transformation in the skeleton of their pectoral appendages during their lifetime. Most teleosts resort to active feeding relatively early in their development. In this phase, their skeleton is limited to some cranial cartilages and the supportive cartilage of their premature, but functional pectoral fins (Kimmel et al., 1995; Schilling and Kimmel, 1997; Thorsen et al., 2004). These early pectoral fins are supported by a single cartilaginous element that comprises both the girdle, internal to the body wall, and a disk-shaped cartilage, called the fin disk cartilage, external to the body wall (Grandel and Schulte-Merker, 1998). The fin endoskeleton separates a dorsal and a ventral muscle group. These muscles span from the pectoral girdle to the collagenous fin rays or actinotrichia, which support the most distal portion of the fin, the epithelial fin fold.

Only when the remainder of the adult skeleton begins to form, will this early pectoral fin undergo one of the most unusual skeletal transformations known in vertebrate skeletogenesis (Grandel and Schulte-Merker, 1998; Bird and Mabee, 2003; Dewit et al., 2011). This second phase in pectoral development includes the formation of bony fin rays in the epithelial fin fold, *de novo* formation of distal cartilaginous radials at the base of each lepidotrichium and the complete transformation of the early pectoral endoskeleton. The previously continuous pectoral cartilage looses cartilage tissue in several well-defined regions, thereby separating the fin disk cartilage

from the girdle and subdividing the former into four rod-like proximal radials (Grandel and Schulte-Merker, 1998; Dewit et al., 2011).

In this doctoral thesis, I investigated which cellular mechanisms underlie the loss of cartilage tissue during the development of the pectoral fin endoskeleton in zebrafish (*Danio rerio*), a teleost species commonly used in developmental research.

First, I examined the composition of the extracellular matrix (ECM) during pectoral cartilage subdivision. The presence of certain ECM components, such as collagen type I and II, tenascin, elastin and fibronectin can reliably distinguish different types of connective tissues, as well as different developmental stages in cartilage development (Hall, 2005). Using immunohistochemical staining, I tested two hypotheses. First, that differences in ECM composition between presumptive interradial tissue and radial cartilages foreshadow the process of subdivision in the fin disk. Second, that the fin disk cartilage is not composed of mature cartilaginous tissue prior to subdivision. For the latter I compared the ECM composition with cartilages of the splanchnocranium and the median fins. Due to the limited availability of suitable antibodies for collagen type I and tenascin, I focused on collagen type II, elastin and fibronectin. Elastin was found to be absent from all the zebrafish cartilages tested, both cranial and post-cranial, but collagen type II and fibronectin could be detected.

The distribution of collagen type II was consistent with its expected distribution as a major structural component of cartilage tissue. This protein appears concomitant with sulphated proteoglycans once the cranial and post-cranial zebrafish cartilages differentiate. Likewise, during fin disk cartilage subdivision, this protein is lost simultaneously with the loss of proteoglycans. The distribution of fibronectin, however, showed remarkable differences with observations made on tetrapod cartilage. In tetrapods, fibronectin is strongly present in the condensation stage of cartilage development. This is the earliest step in chondrogenesis, in which mesenchymal precursor cells cluster together to form a cell condensation, which subsequently differentiates into a cartilaginous element when the secretion of cartilage ECM begins. In

differentiated tetrapod cartilage, fibronectin is still present, but to a lesser extent and in a differently spliced isoform. In the cranial and post-cranial cartilage of zebrafish, however, fibronectin could not be detected in the condensation stage, although it could transiently be detected shortly after differentiation. Interestingly, the fin disk cartilage showed considerably stronger fibronectin signal over an extended period of time, reaching a maximum just prior to the onset of cartilage subdivision. These findings suggest that the fin disk cartilage may be maintained in an early differentiation stage prior to its subdivision.

Second, I evaluated different ways in which cartilage tissue can be removed. According to the literature, the disappearance of chondrocytes and their elaborate ECM can only be accomplished by a limited number of cellular mechanisms. Specialized resorption cells, such as macrophages (Blumer et al., 2008) or chondroclasts (Vu et al., 1998), can resorb cartilaginous tissue. The resident chondrocytes can undergo programmed cell death, apoptosis, and be resorbed secondarily by the surrounding tissue or the previously mentioned specialized resorption cells (Bronckers et al., 2000). Finally, chondrocytes could arrest the production of new ECM components and resorb their previously secreted ECM (Yocum et al., 1995; Holmbeck et al., 2003).

Morphological observations and staining for (tartrate-resistant) acid phosphatase, could not reveal any resorption by either macrophages or chondroclasts in the cartilage subdivision zones of the zebrafish pectoral fin endoskeleton (Dewit et al., 2011). Using vital staining with acridin orange, I found no evidence for apoptosis either. Instead, ultrastructural analysis provided strong evidence for chondrocyte dedifferentiation. Cells entrapped in the partially degraded ECM of the subdivision zones showed no ultrastructural characteristics of chondrocytes, but rather resembled mesenchymal cells. When the degrading ECM subsequently fragmented, these former chondrocytes re-established cell-to-cell contacts amongst each other and with perichondral mesenchyme. Combined with the absence of apoptosis, these ultrastructural observations suggest that the former fin disk chondrocytes dedifferentiate and contribute to the interradial tissue that develops in the

wake of the cartilage subdivision zones. However, detailed lineage tracing of these dedifferentiating chondrocytes will be required to test this hypothesis.

Having discovered that cartilage subdivisions in the pectoral fin endoskeleton are likely accomplished by chondrocyte dedifferentiation, the focus of this doctoral research shifted to the identification of proteinases that could enable dedifferentiating chondrocytes to degrade their ECM. Thus, I used immunohistochemical staining to study the presence and distribution of several matrix metalloproteinases (MMPs), known to be essential in both normal and pathological cartilage loss in tetrapod models (Imai et al., 1997; Holmbeck et al., 1999; Ishiguro et al., 2002; Murphy et al., 2002; Holmbeck et al., 2003; Kuroki et al., 2005; Malemud, 2006). From all the MMPs studied, i.e. MMP2, 9, 13, 14α and 14β, only MMP14α coincided in both timing and location to fin disk cartilage subdivision. In addition, disruption of MMP14α-mediated degradation using both broad and narrow spectrum pharmacological inhibitors resulted in a significant retardation in the progression of the cartilage subdivision zones. However, cartilage subdivision was not stopped by MMP14α inhibition, indicating that chondrocyte dedifferentiation still proceeds even without MMP14α function. Interestingly, very similar observations have been made on three cartilages of the mouse endoskeleton that undergo resorption, i.e. (1) the calvarial cartilages of the cranium, (2) the posterior section of the Meckelian cartilage, and (3) the groove of Ranvier, which is situated alongside the metaphysis of long bones. All these cartilages are resorbed in an MMP14-dependent manner. In the absence of functional MMP14 degradation, the resorption of these cartilages is delayed, but the phenotype of the resident chondrocytes is lost nevertheless (Holmbeck et al., 1999; Holmbeck et al., 2003).

Finally, collaboration with the laboratory of Dr. Winkler (Singapore University) allowed us to study the involvement of another proteinase, cathepsin K, during the cartilage subdivision in the medaka (*Oryzias latipes*). Like the zebrafish, the medaka is a teleost species commonly used as a model in developmental research. By means of a transgenic reporter line and in situ hybridized specimens provided by our collaborators, I assessed

whether or not cathepsin K is expressed by the dedifferentiating chondrocytes of the subdivision zones. Moreover, the well-known expression of this proteinase in chondroclasts (Chatani et al., 2011; To et al., 2012) also offered the opportunity to confirm the lack of chondroclast involvement in pectoral fin cartilage subdivision. I found no cathepsin K-expression associated with pectoral cartilage subdivision. Instead, I observed cathepsin K expression associated with lepidotrichia development in the paired and the median fins, which was never previously described.

I conclude with a general discussion, emphasizing that the fin disk cartilage is the only element of the zebrafish endoskeleton that is maintained in an early-differentiated stage prior to subdivision. I further elaborate on the mechanism behind the loss of cartilage tissue during this subdivision process, which involves chondrocyte dedifferentiation and MMP14α-mediated ECM degradation and may represent a conserved mechanism of cartilage degradation amongst Osteichthyes.

Nederlandstalige versie

Teleostachtige vissen zijn de enige Gnathstomata die tijdens hun levensduur een grootschalige architecturale transformatie van het pectoraal skelet ondergaan. De meeste teleostsoorten beginnen zeer vroeg in hun ontwikkeling met actief foerageren. In deze vroege levensfase is het skelet beperkt tot enkele craniale kraakbeenelementen en het ondersteunend kraakbeen van de premature maar functionele pectorale vinnen (Kimmel et al., 1995; Schilling and Kimmel, 1997; Thorsen et al., 2004). Deze vroege pectorale vinnen worden ondersteund door één continu kraakbeenelement dat zowel de inwendig gesitueerde schoudergordel als een uitwendige schijfvormige structuur, ook wel het vinschijfkraakbeen genoemd, omvat (Grandel and Schulte-Merker, 1998). Het kraakbenig skelet of endoskelet van de vin vormt de scheiding tussen een ventrale en een dorsale spiergroep. Deze spieren reiken van de schoudergordel tot aan de collageneuze vinstralen, oftewel actinotrichia, die het meest distale gedeelte van de vin ondersteunen, nl. de epitheliale vinplooi.

Pas wanneer de rest van het adulte skelet zich begint te ontwikkelen, ondergaat deze vroege pectorale vin een voor vertebraten erg uitzonderlijke skeletale verandering (Grandel and Schulte-Merker, 1998; Bird and Mabee, 2003; Dewit et al., 2011). Deze tweede fase in de pectorale ontwikkeling omvat de ontwikkeling van benige vinstralen, die lepidotrichia worden genoemd, in de epitheliale vinplooi, de vorming van distale kraakbeenradialia aan de basis van elk lepidotrichium en de volledige transformatie van het vroege pectorale endoskelet. Het voorheen ononderbroken pectoraal kraakbeen verliest kraakbeenweefsel in verschillende duidelijk afgelijnde zones, waardoor het vinschijfkraakbeen van de schoudergordel loskomt en onderverdeeld wordt in vier staafvormige proximale radialia (Grandel and Schulte-Merker, 1998; Dewit et al., 2011).

In deze doctoraatsthesis onderzocht ik welke cellulaire mechanismen aan de basis liggen van het verdwijnen van kraakbeenweefsel tijdens de ontwikkeling van het pectorale endoskelet in zebravissen (*Danio rerio*), een teleostsoort die veelvuldig gebruikt wordt in ontwikkelingsonderzoek.

Eerst en vooral heb ik de samenstelling van de extracellulaire matrix (ECM) onderzocht tijdens de opdeling van het pectorale kraakbeen. De aanwezigheid van bepaalde ECM-componenten, zoals collageen type I en II, tenascine, elastine en fibronectine, kan namelijk op een betrouwbare manier worden gebruikt om verschillende bindweefseltypes te onderscheiden. Bovendien kan hiermee een onderscheid gemaakt worden tussen verschillende stadia in de kraakbeenontwikkeling (Hall, 2005).

Ik heb enkele hypotheses getest met behulp van immunohistochemische kleuringen. Ten eerste wilde Ik erachter komen of er voor het proces van kraakbeenopdeling al verschillen bestaan in de ECM-samenstelling van het interradiaal weefsel en de kraakbenige radialia. Ten tweede ging ik na of het vinschijfkraakbeen wel degelijk uit matuur kraakbeenweefsel bestaat voordat het wordt opgedeeld. Voor deze laatste hypothese heb ik de ECM-samenstelling van het vinschijfkraakbeen vergeleken met de kraakbenige elementen van het splanchnocranium en de mediane vinnen. Ons onderzoek spitste zich voornamelijk toe op collageen type II, elastine en fibronectine, omdat voor collageen type I en tenascine geen geschikte antilichamen beschikbaar waren. Elastine werd in geen enkel van de craniale en post-craniale kraakbeenderen teruggevonden, maar collageen type II en fibronectine konden wel aangekleurd worden.

De verdeling van collageen type II in the ECM voldeed aan de verwachtingen voor de belangrijkste structurele component van kraakbeenmatrix. Dit eiwit verschijnt gelijktijdig met de opkomst van gesulfateerde proteoglycanen, wanneer de craniale en post-craniale kraakbeenderen van de zebravis differentiëren. Tijdens het opdelen van het pectorale kraakbeen verdwijnt dit proteïne eveneens gelijktijdig met het verlies van gesulfateerde proteoglycanen uit de ECM. De verdeling van fibronectine vertoont daarentegen belangrijke verschillen met de bevindingen bij de kraakbeenontwikkeling van Tetrapoda. Bij Tetrapoda is fibronectine sterk aanwezig in de condensatiefase van de kraakbeenontwikkeling. Dit is de eerste stap in de chondrogenese, waarbij mesenchymale precursoren samenclusteren tot een celcondensatie. Deze condensatiecellen zullen dan

verder differentiëren tot kraakbeenweefsel en kraakbeenspecifieke ECM gaan produceren. In gedifferentieerde kraakbeenmatrix is fibronectine nog steeds aanwezig, maar wel in mindere mate en in een andere isovorm. In het craniale en post-craniaal kraakbeen van zebravis kan fibronectine slechts tijdelijk opgespoord worden, kort nadat de kraakbeenelementen differentieerden. Het was dan ook erg opmerkelijk dat fibronectine veel sterker en over een beduidend langere periode aanwezig was in de matrix van het vinschijfkraakbeen net voor dit werd opgedeeld. Deze vaststelling doet vermoeden dat het vinschijfkraakbeen in een vroeg differentiatiestadium wordt gehouden tot het zich opdeelt.

Ten tweede onderzocht ik de verschillende manieren waarop kraakbeenweefsel kan verwijderd worden. Volgens de literatuur kunnen chondrocyten en hun uitgebreide ECM slechts verdwijnen door een beperkt aantal cellulaire mechanismen. Gespecialiseerde resorptiecellen zoals macrofagen (Blumer et al., 2008) en chondroclasten (Vu et al., 1998) kunnen kraakbeenweefsel resorberen. Residentiële chondrocyten kunnen apoptose ondergaan en secundair geresorbeerd worden door het omliggende weefsel of de hierboven genoemde resorptiecellen (Bronckers et al., 2000). Tot slot kunnen chondrocyten de- of trans-differentiëren en daarbij de secretie van nieuwe ECM stopzetten en hun oude ECM beginnen te resorberen (Yocum et al., 1995; Holmbeck et al., 2003).

Morfologische observaties en kleuring voor (tartraat-resistente) zure fosfatase konden niet aantonen dat de opdeling van het pectoraal kraakbeen wordt veroorzaakt door macrofagen of chondroclasten (Dewit et al., 2011). Ik vond eveneens geen bewijs voor apoptose bij een vitale kleuring met acridine oranje. Ultrastructurele observaties leverden daarentegen wel overtuigend bewijsmateriaal ten voordele van chondrocytdedifferentiatie. Cellen die nog steeds waren omsloten door restanten van kraakbenige matrix vertoonden geen ultrastructurele kenmerken van chondrocyten, maar leken meer op mesenchymale cellen. Deze voormalige chondrocytcellen maakten terug cel-cel contact met elkaar en met perichondrale cellen zodra de degraderende matrix fragmenteerde. De combinatie van deze bevindingen met de

afwezigheid van apoptose doet vermoeden dat de voormalige chondrocyten dedifferentiëren en vervolgens bijdragen tot de weefsels van de interradialia die zich in het kielzog van het opbrekend kraakbeen ontwikkelen. Om deze hypothese te bevestigen, zal de bestemming van de vinschijfchondrocyten in detail moeten worden gevolgd.

Nadat ik ontdekt had dat kraakbeenopdeling in de pectorale vin naar alle waarschijnlijkheid het gevolg is van chondrocytdedifferentiatie, richtte ik ons onderzoek op een ander aspect. Ik ging op zoek naar proteïnases waarmee chondrocyten hun eigen ECM kunnen resorberen. Hierbij gebruikte ik immunohistochemische kleuringen om de aanwezigheid en distributie van verschillende matrix metalloproteïnases (MMP) te onderzoeken. Deze MMP's zijn essentieel voor het degraderen van kraakbeen in Tetrapoda, zowel in normale als in pathologische omstandigheden (Imai et al., 1997; Holmbeck et al., 1999; Ishiguro et al., 2002; Murphy et al., 2002; Holmbeck et al., 2003; Kuroki et al., 2005; Malemud, 2006). Van alle MMP's die ik onderzocht, nl. MMP2, 9, 13, 14α en 14β, kon alleen MMP14α in tijdstip en locatie in verband gebracht worden met de opdeling van het vinschijfkraakbeen. Bovendien zorgde het onderdrukken van de functie van MMP14α door zowel farmacologische inhibitoren met een breed spectrum als door meer specifieke inhibitoren voor een aanzienlijke vertraging van het opdelingsproces. De kraakbeenopdeling werd echter niet stopgezet door de inhibitie van MMP14α, wat erop wijst dat chondrocytdedifferentiatie blijft doorgaan zonder de functie van MMP14α. Er werden eerder erg gelijkaardige vaststellingen gedaan op drie kraakbeenderen van het endoskelet bij muizen die resorptie ondergaan: (1) het kraakbeen in de calvaria van het cranium, (2) het achterste deel van het kraakbeen van Meckel en de (3) insnoeringen van Ranvier, die zich langs de metafyse van lange beenderen bevinden. Al deze kraakbeenderen worden geresorbeerd onder invloed van MMP14. Wanneer geen functionele MMP14-degredatie optreedt, loopt de resorptie van deze kraakbeenderen vertraging op, maar gaat het fenotype van de residentiële chondrocyten toch verloren (Holmbeck et al., 1999; Holmbeck et al., 2003).

Tot slot gaf een samenwerking met het laboratorium van Dr. Winkler (Singapore University) ons de kans om de rol van een ander proteïnase, cathepsine K, bij de kraakbeenopdeling in de Japanse rijstvis (*Oryzias latipes*) te bestuderen. De Japanse rijstvis is, net als de zebravis, een teleostsoort die vaak als model wordt gebruikt in ontwikkelingsonderzoek. Met behulp van een transgene reporterlijn en in situ gehybridiseerde specimens die ik via deze samenwerking ontvingen, was ik in staat om na te gaan of cathepsine K al dan niet tot expressie komt in de dedifferentiërende chondrocyten van de opdelingszones. Bovendien gaf de gekende expressie van dit proteïnase in chondroclasten (Chatani et al., 2011; To et al., 2012) ons ook de mogelijkheid om te controleren of chondroclasten effectief geen rol speelden in de opdeling van kraakbeen in de pectorale vinnen. Ik vond geen expressie van cathepsine K bij de opdeling van kraakbeen in de pectorale vinnen. Ik stelde echter wel expressie van cathepsine K vast bij de ontwikkeling van lepidotrichia in de gepaarde en mediane vinnen. Dit fenomeen was nog niet eerder beschreven.

Ik besluit met een algemene bespreking van de resultaten. Hierbij wordt de nadruk gelegd op het feit dat vinschijfkraakbeen het enige onderdeel van het endoskelet van zebravissen is dat behouden blijft in een vroeg gedifferentieerd stadium vóór de opdeling. Ik gaan verder in op het mechanisme dat kraakbeenverlies veroorzaakt tijdens dit opdelingsproces. Ik stel vast dat hierbij zowel chondrocytdedifferentiatie als de functie van MMP14α een rol spelen en dat dit heel waarschijnlijk een geconserveerd mechanisme voor kraakbeendegradatie is bij Osteichthyes.

6. References

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