Collagen duplicate genes of bone and cartilage participate during regeneration of zebrafish fin skeleton

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ARTICLE INFO

ABSTRACT

The zebrafish fin is widely used as a model for skeleton regeneration. For years, the nature of the fin skeleton has been controversial as its extracellular matrix shows hybrid characteristics of both bone and cartilage. The presence of co-orthologs genes also increases the complexity of these tissues. In this article, we have identified and described the expression of fibrillar collagens in zebrafish fin skeleton. We found that genes coding for types I, II, V, XI and XXVII collagens are duplicated, showing in several cases, different expression domains. We also identified specific genomic features, such as the presence of type XXIV collagen and the absence of type III collagen in the zebrafish genome. Our study showed that actinotrichia-forming cells and osteoblasts synthesize a wide variety of these fibrillar collagens during fin regeneration. An intertrichial domain expressing most of the collagens was located in the transition between the mesenchyme condensations of actinotrichia and lepidotrichia and may determine an important niche associated with fin skeleton morphogenesis. We also confirmed the hybrid nature of the fin exoskeleton and provided a complete description of those fibrillar collagens expressed during the formation of the fin skeleton.

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1. Introduction

The zebrafish fin is an important model for skeletal regeneration. The dermoskeleton of the fin comprises rays with a proximal lepidotrichium and a distal bundle of actinotrichia (Duran et al., 2011a; Becerra et al., 1983). The lepidotrichia are formed by two symmetrical parenthesis-shape bone-like structures that are segmented and branched. Actinotrichia are rods of hyper-polymerized collagen that rise from the most distal segment of lepidotrichia (Akimenko et al., 2003).

Following amputation, fin regeneration takes place as a sequence of events, which includes wound healing, blastema formation and outgrowth of new actinotrichia and lepidotrichia. Distal proliferation and proximal differentiation control actino-trichia and lepidotrichia formation (Nechiporuk and Keating, 2002; Johnson and Bennett, 1999). In distal positions, skeletal forming cells called Actinotrichia-Forming Cells (AFC) first appear under the epidermis to initiate actinotrichia synthesis (Tu and Johnson, 2011; Santos-Ruiz et al., 2002). More proximally, skeletal-forming cells of osteoblast lineage (Tu and Johnson, 2011) forms lepidotrichia between the epidermis and the actinotrichia. In more proximal regions, a group of these osteoblasts surrounds each newly-formed lepidotrichia and continues its synthesis from the external surface (Santamaría and Becerra, 1991). During the synthesis of actinotrichia and lepidotrichia, a number of collagens are exported to the extracellular matrix (ECM) where fibrillo-genesis takes place.

Fibrillar collagens are the most abundant proteins in the extracellular matrix (van der Rest and Garrone, 1991). Each collagen

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molecule is a protein complex formed by three polypeptide chains called alpha-chains. These chains form a trimer that may be coded by the same chain/gene, giving rise to a homotrimer (e.g. type II collagen comprises three identical chains coded by Col2a1), or different gene/chains, giving rise to a heterotrimer (e.g. type I collagen is formed by two Col1a1 chains and one Col1a2 chain). Each alpha-chain is specific to a particular type of collagen.

Biosynthesis of a fibrillar collagen molecule starts with the translation of the alpha-chains and their post-translational modifications by hydroxylases, chaperones and glycosyltransferases. These modifications allow proper folding, trimerization and future crosslinking. The procollagen is further secreted to the ECM where proteinases cleave the propeptides of the chain ends to produce the mature collagen molecule (Canty, 2005). These fibrillar collagen molecules are then ready to initiate fibrillogenesis.

Seven different types of fibrillar collagens are known: I, II, III, V, XI, XXIV and XXVII. Types I and II are highly abundant in connective tissues (major collagens) while the others, sometimes called minor collagens (Types III, V, XI, XXIV and XXVII), are usually involved in fibrillogenesis of types I and II (Gordon and Hahn, 2009).

The skeleton of mammals comprises bone and cartilage, which differ in fibrillar collagen composition. Collagen type I and its fiber-associated types V and XXIV are more typically involved in bone formation while collagen type II and its related types XI and XXVII are more typical of cartilage differentiation (Birk, 2001; Fichard et al., 1997). Collagen types V and XI show an incomplete excision of their non-collagenic domains, giving rise to mature molecules with globular domains at their ends (Birk et al., 1990; Kadler et al., 2008). These domains exert steric impediments during fibrillogenesis, altering the accretion of molecules and thus controlling the diameter of fibrils. Although collagen types XXIV and XXVII have also been suggested to regulate fibrillogenesis, their role is still unclear.

The nature of the fin skeletal elements is controversial as they present features of both bone and cartilage. We recently showed that actinotrichia-forming cells and lepidotrichia osteoblasts synthesize collagen types I and II (Duran et al., 2011a). These results are further supported by the presence of other bone and cartilage genes in the fin skeleton, such as *type X collagen* (Avaron et al., 2006; Eames et al., 2012), *sparc* (*osteonectin*) (Rotllant et al., 2008), *sox9* (Smith et al., 2006) and *sp7* (Green et al., 2009). Although several studies have reported the presence of both cartilage and bone markers during fin morphogenesis (Marí-Beffa et al., 2007), little is known about how these molecules interact to give rise to this special extracellular matrix.

Genetic studies in zebrafish are always a challenge due to the redundancy produced by the numerous co-ortholog or paralog genes in its genome: most of the genes described in mammals are duplicated in zebrafish. Nevertheless, novel genes may be discovered which are specific to fish. Some of these genes have been recently identified as "ohnologs" paralog genes, which have been lost in other vertebrates (Postlethwait, 2007). To date, many co-ortholog genes have been identified in zebrafish, but their functions remain unclear as they have been poorly studied. For example, most vertebrates form type I collagen with two different alpha chains: Col1a1 and Col1a2. Nevertheless, a third alpha chain, Col1a3, has been found in several teleost fish. Currently, this chain has been re-classified as Col1a1b and is considered to be the product of a gene duplication of *col1a1* (Le Guellec et al., 2003). Another example is type II collagen, which is coded by a single gene in most animals, *col2a1*. Recently, two alternative genes, *col2a1a* and *col2a1b*, have been discovered in zebrafish (Duran et al., 2011a). These two genes show differential expression, with Col2a1b protein expressed in the fin. Fang et al. (2010) have further described the expression of several minor collagen genes (*col5a1*, *col5a3*,

col11a1 and *col11a2*) during zebrafish development (Fang et al., 2010). The expression domains of these genes suggest they are involved in skeleton development, as widely described in mammals (van der Rest and Garrone, 1991; Eyre et al., 2002; Kahai et al., 2004; Wu et al., 2010). However, a complete understanding of the expression and function of all fibrillar collagen and their co-orthologs during zebrafish skeletogenesis is still missing.

In the present study, we identify all fibrillar collagen genes in zebrafish, uncovering several duplicated genes, which are essential for the understanding of ECM composition in zebrafish skeleton. We also studied the expression pattern of those collagens that participate during development and regeneration of the fin skeleton. In general, our results confirm the unique nature of the zebrafish fin skeleton, which shows a characteristic molecular composition, hybrid between bone and cartilage. Interestingly, most of these collagen genes are expressed in a transition region between those of active synthesis of lepidotrichia and actinotrichia. We have named this expression domain the "intertrichial region" and discuss its potential function during zebrafish skeletogenesis below.

2. Material and methods

2.1. Animals and fin amputations

All zebrafish used in this study were maintained at 28.5 °C under standard conditions (Westerfield, 2000). AB wild type strain of zebrafish was used for regeneration studies. Fish were anaesthetized in 0.02 mg/mL tricaine and fins were cut two-ray segment proximal to the first branch.

2.2. Identification of fibrillar collagen in zebrafish

Ensembl and BLAST (Basic Local Alignment Search Tool, BLASTP and BLASTX) applications were used to identify collagen genes in the zebrafish genome (Zv9) by comparison with human (GRCh38.p2), mouse (GRCm38.p3), chicken (Galgal4), *Xenopus* (JGI 4.2), gar (LepOcu1), medaka (HdrR) and *Fugu* (FUGU 4.0). Accession numbers are described in Table 1. Dendrograms to identify different collagen groups were constructed through CLUSTALW alignment using Njplot software (http://doua.prabi.fr/software/njplot). Bootstrap values were obtained after 1000 iterations.

2.3. Transcriptional analysis

Total RNA from control and regenerating fins at 1, 2, 3, 4 and 7 days post-amputation (dpa) was extracted using TRI-Reagent (Sigma) and DNAse treated according to manufacturer's instructions. cDNA was obtained from 1 μg total RNA using the i-Script kit (Bio-Rad) according to manufacturer's instructions.

Gene expression analysis was performed by qRT-PCR using SYBR Green in an iCycler detection system (Bio-Rad). Reactions were performed in triplicate and the absence of primer dimers was confirmed by examining dissociation curves. The expression of Beta-Actin and Gapdh genes was used as the normalizer. Relative quantification of expression level was performed using the comparative Ct method (Pfaffl, 2001). All gene expression analyses were performed from pools of fin tissue from at least 10 individuals. qRT-PCR was performed at least in two experimental replicate and one extra independent biological replicate to confirm the pattern. Primers used are shown in Supplementary Table S1. Statistical analyses (t student and Mann–Whitney tests were performed with Prism v 5.0 (GraphPad).