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Evolution of Developmental Control Mechanisms

Actinotrichia collagens and their role in fin formation

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ABSTRACT

The skeleton of zebrafish fins consists of lepidotrichia and actinotrichia. Actinotrichia are fibrils located at the tip of each lepidotrichia and play a morphogenetic role in fin formation. Actinotrichia are formed by collagens associated with non-collagen components. The non-collagen components of actinotrichia (actinodins) have been shown to play a critical role in fin to limb transition. The present study has focused on the collagens that form actinotrichia and their role in fin formation. We have found actinotrichia are formed by Collagen I plus a novel form of Collagen II, encoded by the *col2a1b* gene. This second copy of the collagen II gene is only found in fishes and is the only Collagen type II expressed in fins. Both *col1a1a* and *col2a1b* were found in actinotrichia forming cells. Significantly, they also expressed the *lysyl hydroxylase 1* (*lh1*) gene, which encodes an enzyme involved in the post-translational processing of collagens. Morpholino knockdown in zebrafish embryos demonstrated that the two collagens and *lh1* are essential for actinotrichia and fin fold morphogenesis. The *col1a1* dominant mutant *chihuahua* showed aberrant phenotypes in both actinotrichia and lepidotrichia during fin development and regeneration. These pieces of evidences support that actinotrichia are composed of Collagens I and II, which are post-translationally processed by Lh1, and that the correct expression and assembling of these collagens is essential for fin formation. The unique collagen composition of actinotrichia may play a role in fin skeleton morphogenesis.

Introduction

The teleostean fin is supported by two types of skeletal elements named lepidotrichia and actinotrichia. Lepidotrichia are calcified, segmented and branched bone-like rays that extend along the whole length of the fin, shaping it. Actinotrichia are non-calcified fusiform spicules found in brush-fashioned groups at the distal tip of each ray, where they provide a flexible support to the fin edge (Becerra et al., 1996; Montes et al., 1982).

Actinotrichia are the first fin skeleton formed during development. They appear inside the fin fold, serving as a scaffold for the migration into the fin fold of the mesenchymal cells that will form the future fin connective tissue (Wood, 1982; Wood and Thorogood, 1984). They are synthesized by the so-called actinotrichia forming cells (AFC), whose exact identity and origin (epidermal or mesenchymal) is still unknown. Later in development, lepidotrichia start growing from the fin fold base by sequential addition of segments to its distal end. At this stage actinotrichia are located at the distal tip of each lepidotrichia, where new segments are being synthesized. Interestingly, when fins are amputated at the lepidotrichia level, proximal to actinotrichia, actinotrichia appear anew at the amputation site prior to lepidotrichia regrowth. Lepidotrichia regenerate, as during development, by addition of new segments to their distal tip, where the actinotrichia are. Thus, both during development and regeneration, lepidotrichia seem to grow "towards the actinotrichia". This apparent interrelation has driven some authors to suggest a morphogenetic role for actinotrichia in lepidotrichia morphogenesis, possibly by inducing differentiation of scleroblasts or lepidotrichia forming cells (LFC) (Santamaría and Becerra, 1991).

Other authors like Dane and Tucker suggested actinotrichia might be responsible for maintaining the structural integrity of the early fin fold (Dane and Tucker, 1985). This role seems to be supported by a recent study also suggesting that loss of actinotrichia during evolution may have led to loss of lepidotrichia and contributed to the fin-to-limb transition (Zhang et al., 2010).

In spite of their possible morphogenetic role and evolutive significance, little is known about the ontogeny, function and molecular nature of actinotrichia.

Actinotrichia are found in actinopterygian (ray-finned) fishes. Homologous structures named ceratotrichia are present in chondrichthyes (Geraudie and Meunier, 1980). Ceratotrichia received some attention in the past century, when several studies tried to establish their biochemical composition. Ceratotrichia showed features similar to both elastin and collagen. These features led Krükenberg to name "elastoidin" to the constitutive protein of ceratotrichia (Krukenberg, 1885). Elastoidin was proposed to be responsible for the abnormal size of ceratotrichia fibrils (Damodaran et al., 1956; Kimura and Kubota, 1966; Sastry and Ramachandran,

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1965). Later studies showed that elastoidin was a mix of collagenous and non-collagenous proteins, but neither the particular collagen types nor the identity of the non-collagenous proteins could be determined (Ramachandran, 1962; Sastry and Ramachandran, 1965). As to the teleostean actinotrichia, the presence of, at least, a collagen fraction on them was confirmed by electron microscopy studies that showed the typical banding pattern of collagen in actinotrichia longitudinal sections (Montes et al., 1982). On the other hand, the non-collagen fraction of actinotrichia has very recently been identified as Actinodins. Actinodin genes (*and*) are only present in fish lineages and, significantly, they seem to be implicated in fin/limb evolution (Zhang et al., 2010).

Though recent immunohistochemical studies have suggested actinotrichia are composed of Collagen type II, which would interact with other collagens, such as Collagen IX (Huang et al., 2009), the type of collagen that forms actinotrichia has not been identified yet. Moreover, there is very little information about the genetic mechanisms that control actinotrichia synthesis.

Actinotrichia are composed not by a bundle of discretely separated collagen fibrils, but rather of hyperpolymerized collagen molecules (Montes et al., 1982). Given the size of the actinotrichia, such a degree of polymerization would require a careful post-translational processing during collagen biosynthesis and the collaboration of other molecules to reach such a large size (Gross and Dumsha, 1958; Ramachandran, 1962; Sastry and Ramachandran, 1965). One good candidate for this task is Lysyl hydroxylase 1 (*lh1* or *plod1*), which has been described in cells surrounding the actinotrichia during fin development (Schneider and Granato, 2007). Lysyl hydroxylases are essential for collagen biosynthesis, catalyzing the addition of hydroxyl groups to lysine residues. These hydroxylysine residues serve as attachment sites for carbohydrate chains and participate in the formation of intermolecular cross-links (Myllyharju and Kivirikko, 2004; Risteli et al., 2004).

This article deals with the collagenous nature of actinotrichia and describes the expression of *col1a1a*, *col2a1b*, and *lh1* during fin development and regeneration. *col2a1b* is a new collagen which might have evolutive significance. During zebrafish development, morpholino knockdown of *col2a1b*, *col1a1a* and *lh1* genes show fin fold phenotypes. On the other hand, *col1a1a chihuahua* mutation also shows aberrant skeletal phenotype during fin formation. These evidences suggest that actinotrichia and lepidotrichia are composed of a combination of Collagen I alpha 1a and Collagen II alpha 1b chains. In actinotrichia, specifically, these collagens are probably post-translationally modified by Lysyl hydroxylase 1.

Materials and methods

Animals

Zebrafish (*Danio rerio*) wild type and the mutant *col1a1a*^{dcl24} specimens were maintained in standard conditions (Westerfield, 2000). The *col1a1a*^{dcl24} mutant (*chihuahua* or *chi*; (Fisher et al., 2003) was a kind donation of Shannon Fisher. We used heterozygotes since homozygotes are not viable. Except for heterozygous mutants, all experiments on zebrafish were performed on wild type AB line at 28.5 °C. Goldfish (*Carassius auratus*, Linnaeus, 1758) were maintained at 25 °C in system of tanks of 300 l. Animals were grown up to about 15–20 cm body size before experiments. In regeneration experiments, the caudal fin of anesthetised specimens was amputated and let grow back. The amputation plane was in all cases located between the second and third segments proximal to the first ray branching.

Histochemistry, immunohistochemistry and in situ hybridization

Collagen fibrils were revealed in whole fins with the picrosirius-polarization method (Becerra et al., 1983). Briefly, fins were fixed with 4% paraformaldehyde (PFA) in PBS, pH 7.4, and stained for one hour in Picrosirius solution. After staining, fins were washed to remove excess staining, dehydrated and mounted. Images were captured using a Nikon Optiphot microscope with polarization optics. The length and number of actinotrichia was measured with ImageJ software in picrosirius-stained, whole-mount fins from ten wild-type AB and six *chihuahua* heterozygote fishes. Statistical significance was assessed using Mann–Whitney *u*-test (Prism program).

Immunostaining was performed in tissue sections from fins and embryos preserved with the CryoWax method described by Durán et al. (2011). Briefly, fins and embryos were preserved by sequential immersion in isopentane and methanol at −80 °C and then embedded in polyester wax and sectioned with a microtome. Embedding, sectioning, mounting and storage were carried out at 16 °C.

The antibody JAS'96 against selachian ceratotrichia was used to recognize both actinotrichia and actinotrichia forming cells (AFC). This antibody binds specifically to epitopes in actinotrichia and AFC of teleost fishes, recognizing the unique combination and conformation of proteins that constitute the actinotrichia and that are not found anywhere else in the fish body (Santamaria et al., 1996). Goat anti-Collagen I α1 (CN AB758, 1:10 dilution, Chemicon; (Zhao et al., 2006) and mouse anti-Collagen II (II-II6B3, 1:50 dilution, Developmental Studies Hybrydoma Bank; (Huang et al., 2009) antibodies were used for Collagen I and II recognition, respectively. Goat anti-rat IgG-Alexa Fluor 488 conjugated, donkey anti-mouse IgG-Alexa Fluor 488 conjugated and donkey anti-goat IgG conjugated with Alexa Fluor 568 (Molecular Probes) were used as secondary antibodies. In some cases, 1 μg/mL Hoechst 33258 was added to the secondary antibodies to counterstain nuclei. With a Leica SP5 II laser broadband confocal microscope, fin sections were optically dissected by sequential acquisition of confocal sections. Projections of the Z-stack and 0.7 μm individual confocal planes were obtained with the Leica LAS AS software.

In situ hybridization with digoxigenin-labeled RNA-probes for *col1a1a*, *col2a1b* and *lh1* was performed as previously described (Akimenko et al., 1994). Collagen probes were designed to include gene regions with the lowest possible similarity to other collagen genes. This involved the inclusion of 3′UTR in many cases. Primers for *col1a1a* were: Fw 5′-AACACGTCTGGTTCGGAGAG-3′; Rv 5′-AAATCCTGACCTGGTGTTGG-3′; Primers for *col2a1b* were: Fw 5′-AACAGAAGTGCTTCCGAACG-3′; Rv 5′-TTGTCCTGATTCGAGGCTCT-3′. *lh1* probe *(plod1)* was a kind gift from Dr. Granato (Schneider and Granato, 2007).

Proteomics

Goldfish actinotrichia were cleaned from surrounding tissues by 1 h trypsin digestion, followed by manual isolation with forceps. Actinotrichia were then washed 3 times with ethanol. The fibrils were cryopowdered and solubilized in Laemmli buffer (Laemmli, 1970) under reducing conditions with beta-mercaptoethanol, for 10 min at 100 °C. Proteins were separated by SDS-PAGE electrophoresis. Gels were stained with Coomassie blue and the bands were cut and sent to UCO proteomics service for protein identification. The proteomic "Peptide Mass Fingerprinting LC-MS/MS by MALDI TOF/TOF" analysis was carried out in University of Cordoba (UCO), a member of the ProteoRed network. The obtained peptides were identified using the Uniprot database.

Cloning of col2a1b and semi-quantitative RT-PCR

Total RNA was extracted from both regenerating and non-regenerating (control) fins using TRI Reagent (Sigma), according to the manufacturer's protocol. DNA was removed using DNase I (Sigma). 1 μg of total RNA was reverse-transcribed using the i-Scrip kit (Bio-Rad) according to the manufacturer's instructions. A full-length cDNA of *col2a1b* was obtained and cloned into the pGEM-T vector. As *col2a1a* and *col2a1b* sequences share a 74% identity, the best

discrimination was obtained with primers designed in the 3′UTR. The primers used for *col2a1b* cloning were Fw 5′-AGCGGCTCACGGATGAT-′3 and Rv 5′-TCACCATCATCTCCTGGTTTC-′3. The primers used for Semi-quantitative RT-PCR were: *col2a1a*, Fw 5′-CCTCTGAAATCCAGC-CATGT-′3; Rv 5′-GACTGCTGTGGTTCCAGTCA-′3 and for *col2a1b*, Fw 5′-AACAGAAGTGCTTCCGAACG-′3; Rv 5′-TGCTCTGGTTTCTCCCTCAT-′3.

Real time quantitative RT-PCR

Gene expression analysis was performed by qRT-PCR using the fluorescent intercalating dye SYBR Green in an iCycler detection system (Bio-Rad). Reactions were performed in triplicate and the absence of primer dimmers was confirmed by examination of dissociation curves. The expression of Beta-Actin was used as the normalizer. Relative quantification of expression level was performed using the comparative Ct method (Pfaffl, 2001), were the value one was given to the sample with lower expression. All gene expression analysis was performed at least in two individual biological samples. Primers used for expression analysis were *col1a1a*, Fw 5′-GCTTTTGGCAAGAGGACAAG-′3; Rv 5′-TGTCTTCGCA-GATCACTTCG-′3, for *col2a1b*, Fw 5′-AACAGAAGTGCTTCCGAACG-′3; Rv 5′-TGCTCTGGTTTCTCCCTCAT-′3 and for Beta-actin, Fw 5′-TACAAT-GAGCTCCGTGTTGC-′3; Rv 5′-CACAATACCAGTAGTACGACCAGA-′3. Statistical analysis was performed using StatGraphic Centurion program. Significant differences were determined by ANOVA (for 3 or more samples).

Knockdown morpholino

Expression knockdown was performed by morpholino injection into embryos at 1–2 cell stage (Heasman, 2002). At least 50 morphants were obtained from 1 mM injections for each morpholino. Morpholino and 5′-mispair control sequences are shown in Table 1. Morphant descriptions were performed after polarization microscopy. Morphants were studied during 10 days, but because the activity of morpholinos lasts for about 3 days, morphants phenotypes were rescued after this time. Actinotrichia presence and measurement of the total area of the tail fin fold posterior to notochord (Fig. S1) were determined in 50 embryos at 72 hpf-embryos. Measurements were carried out with ImageJ. The statistical significance was also assessed by Mann–Whitney *u*-test (Prism Program).

Results

Morphogenesis of actinotrichia

The morphogenesis of actinotrichia has been studied during development and regeneration. Comparable morphogenetic events in both processes are summarized in Supplementary Table 1.

Actinotrichia and the AFC were located by immunofluorescence with the JAS'96 antibody, raised against dogfish (*Scyliorhinus canicula*; Linnaeus, 1758) ceratotrichia (Santamaria et al., 1996). During caudal fin development, labeling was first detected 30 h post-fertilization (hpf), at the distal ectoderm of the caudal fin fold (Fig. 1A). The first well-formed fiber-like structures were identified 36 hpf in the distal-most region of the fin fold (Fig. 1B). While still located next to the ectoderm, these fibers grew and extended throughout the whole length of the fin fold. At 72 hpf, the first labeled mesenchymal cells were observed inside the fin fold (Fig. 1C). The number of these cells increased and filled the whole fin fold, surrounding entirely the actinotrichia and separating them from the ectoderm. The size of the actinotrichia still increased after the mesenchymal cells had interposed themselves between the actinotrichia and the ectoderm. This growth could not be due to ectodermal synthesis any more, since the actinotrichia and the epidermis were physically separated. It can only be explained by a contribution of the mesenchymal cells to actinotrichia synthesis, a fact that is confirmed by the label observed in these cells.

During fin regeneration, JAS'96 immunoreactive label was first detected 2 days post-amputation (dpa). JAS'96 antibody recognized both AFCs and extracellular fibrils located beneath the basal epidermal layer at the distal ray blastema (Fig. 1D). At three dpa, actinotrichia had increased their diameter and had elongated proximally, (Fig. 1E). At five dpa (Fig. 1F), both actinotrichia and AFCs were surrounded by blastemal cells, and therefore separated from the epidermis. These results suggest that actinotrichia are synthesized by blastemal cells beneath the epidermis.

Table 1
Morpholinos and control sequences used in this study.

Gen name	Morpholino sequence	5-Mispair control
col1a1	GCAGAATATCCACAAAGCTGAACAT	GCAcAAaATgCACAAAcCTcAACAT
col2a1a	TCCGTGAATCCAGCAATCTGAACAT	TCCcTcAATgCAcCAATCTcAACAT
col2a1b	AGGTCTTCATCATCCGTGAGCCGCT	AGcTCTTgATCATCgGTcAcCCGCT
lh1	CGAGAAAAGCCAGAACTCCTCTCAT	CGtGtAAAGgCAcAACTgCTCTCAT

Composition of the actinotrichia

In order to clarify the biochemical nature of actinotrichia, we isolated and purified actinotrichia for a proteomic analysis. In this case, Goldfish actinotrichia were used. The goldfish (*Carassius auratus*) and the zebrafish belong to the same family (Cyprinidae) but the former can grow to a much bigger size than the latter, thus making it possible to isolate enough sample for this analysis. Proteins were separated by SDS-PAGE and a typical monomeric collagen-banding pattern was obtained, with only one monomeric band of about 140 kDa (Fig. S2). It is known that heterotrimeric collagens show a pattern composed of two bands of monomer chains. The heaviest one (around 140 kDa) corresponds to the alpha 1 chain, whereas the lightest (around 120 kDa) correspond to the alpha 2 chain. Homotrimeric collagens show only one monomer band of around 140 kDa. The only band obtained by SDS-PAGE presented around 140 kDa and should correspond to an alpha 1 chain. It was cut from the gel and subject to protein identification by LC-MALDI TOF/TOF. Results showed the presence of two proteins in the sample. The deduced proteins from Goldfish sequenced peptides were alpha 1 chains of Collagen I and Collagen II (Table 2). The database (Uniprot) found identities with Collagen I from zebrafish and with Collagen II from rat/mouse (99% confidence). No *C. auratus* protein database is available. These data guided us to think that actinotrichia were composed by a mix of two collagens (types I and II).

To confirm that actinotrichia were formed by two different collagens, we proceeded to immunolocalize Collagen I and Collagen II in the developing and regenerating fins. We used antibodies previously tested in zebrafish for the specific detection of these collagens (Huang et al., 2009; Zhao et al., 2006). Collagen I and Collagen II were found to colocalize both in developing (Figs. 2A–D) and regenerating actinotrichia (Figs. 2E–H). Confocal projections showed the same localization of Collagen I and Collagen II at the whole fibers during development (Figs. 2A–C) and regeneration (Figs. 2E–G). In order to confirm colocalization of both antibodies, we performed confocal optical sections of 0.7 μm by sequential acquisitions of images in both channels (Figs. 2D and H). These results, together with the proteomic analysis, reveal that actinotrichia are composed of Collagen I and Collagen II.

Actinotrichia Collagens are encoded by col1a1a and a new chain of Collagen II, col2a1b

To further characterize the molecular nature of actinotrichia, we examined collagen gene expression in adult or regenerating fins. RT-

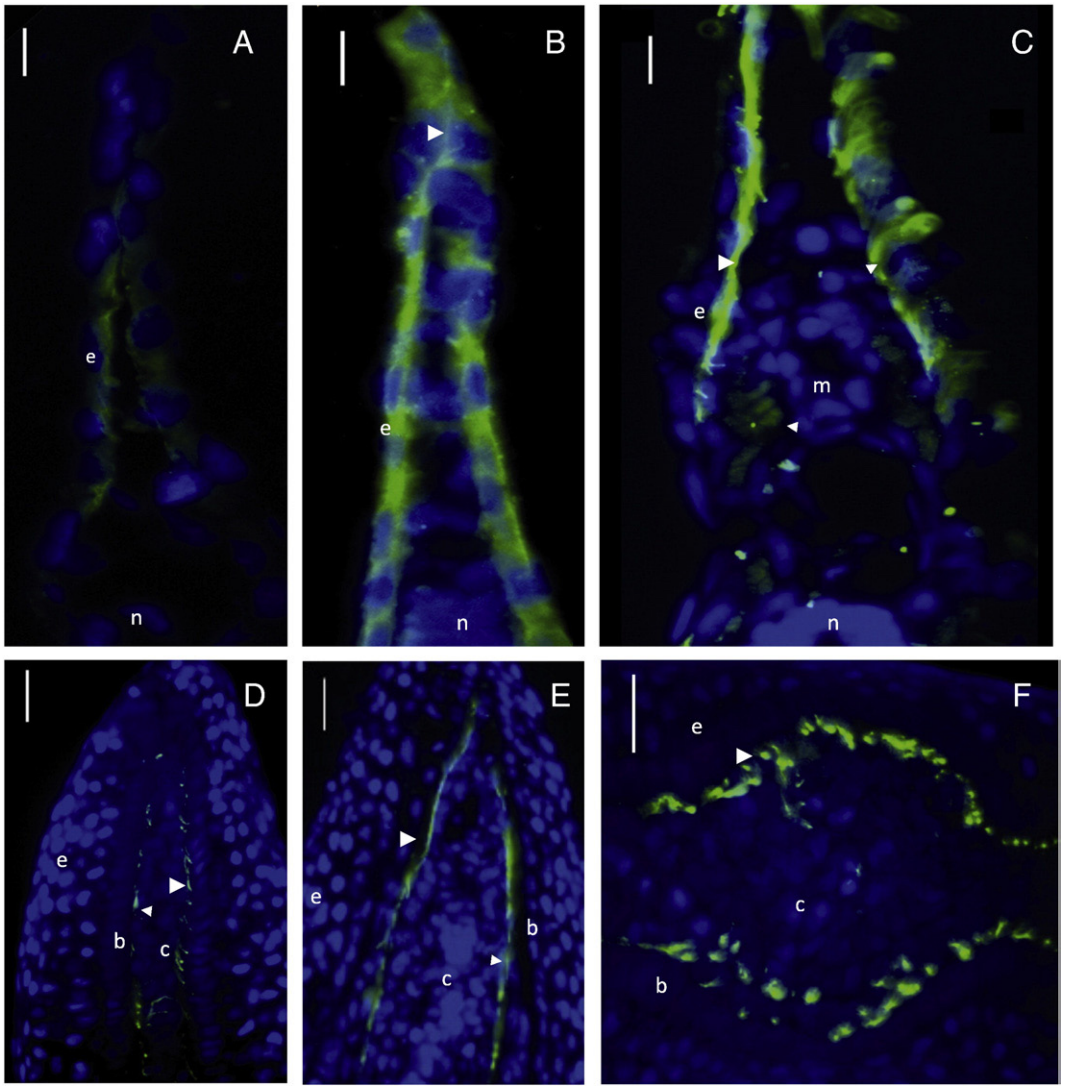


Fig. 1. Actinotrichia formation during fin development and regeneration. Immunofluorescence with JAS'96 antibody against actinotrichia and AFCs (green) and hoechst nuclei staining (blue). (A–C) Fin fold sections at 30 (A), 36 (B) and 72 hpf (C). (D–E) Longitudinal sections of regenerating fin at 2 dpa (D) and 3 dpa (E). Transversal section at 5 dpa (F). Bars represent 10 (A–C) and 25 μm (D–F). Arrowhead is actinotrichia. Arrow is actinotrichia forming cell (AFC). e is epidermis. b is basal epidermal layer. c is connective tissue. n is notochord. Connective tissue and basal epidermal layer are separated by white lines in F.

PCR analysis showed no expression of *col2a1a* in the fin, either normal or regenerating (Fig. 3A). Instead, we found the *col2a1b* transcript, which is encoded by a gene localized at chromosome 11. *col2a1a* is located in chromosome 8 and both collagens share a 74% of identity. The most different region between them is the non-collagenous domain at the N-terminal position. The analysis of the predicted sequence of type II alpha1b chain obtained from the Genebank revealed the presence of an early stop codon that resulted in a short amino acid sequence. That sequence lacked the G-X-Y triplet domains characteristic of collagen proteins. We cloned and sequenced the full-length *col2a1b* transcript found in the fin, finding a T for G substitution in the codon previously described as stop, which, according to our results, actually encodes for Gly-141 (Fig. S3).

Table 2
Protein identification of actinotrichia.

Name	Gen	Accession number	Conf	Number of peptides	Organism
Collagen 1	Col1a1	NP_954684	>99%	6	Zebrafish
Collagen 2	Col2a1	sp|P28481|	>99%	2	Mouse
Collagen 2	Col2a1	sp|P05539|	>99%	2	Rat

We performed *in situ* hybridization of *col2a1b* in embryos at different stages (Figs. 3B–D). During development, zebrafish *col2a1b* gene is expressed from 30 hpf stage and showed a similar gene expression pattern until 72 hpf (data not shown). At 48 hpf, *col2a1b* expression was observed in the nervous system and in the fin buds and folds (Fig. 3B). The expression at nervous system was restricted to neuromast cells of the lateral line primordium (Fig. 3C) and several head sensory organs (Fig. 3B). A diffuse expression was also observed in the brain, mainly at prosencephalic and mesencephalic derivatives (Figs. 3B and D). A conspicuous expression was observed at the anus (Fig. 3B), tail fin fold (Figs. 3B–C) and pectoral fin bud and fold (Figs. 3B and D). Expression at the tail fin fold was higher at distal locations (Fig. 3D), but it was also detected at proximal positions (Figs. 3B and D). A similar expression pattern was observed in the pectoral fin fold. A homogeneous expression was detected at the pectoral fin bud (Figs. 3B and D). Finally, a restricted expression was detected in association to the Cuvier duct positions (Fig. 3D).

In situ hybridization of *col1a1a* and *col2a1b* was performed in sections of adult regenerating fins at different stages (Figs. 4A–F). Both genes were observed in the same regions throughout regeneration. At 2 dpa, both genes were found at the epidermal basal layer

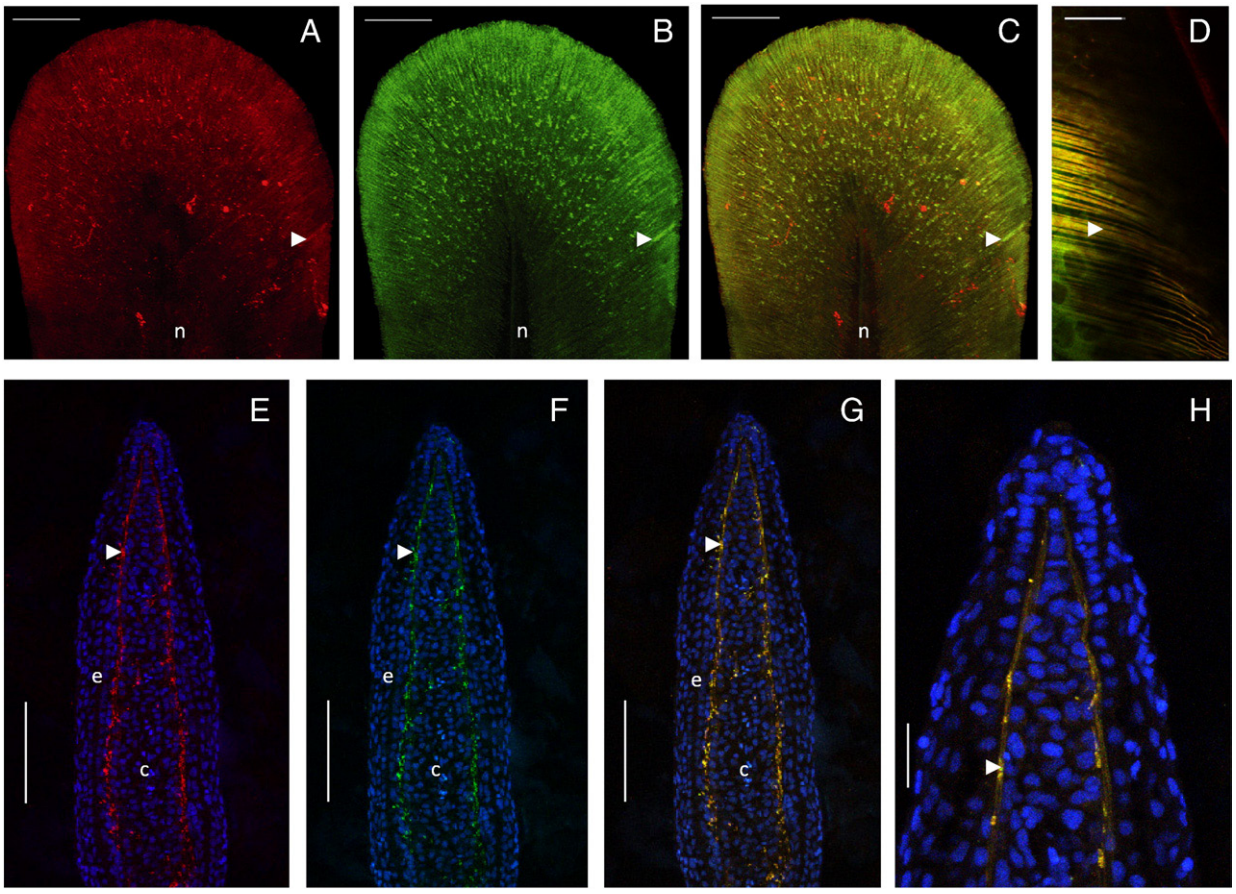


Fig. 2. Colocalization of Collagen type I and Type II in actinotrichia during development and regeneration. Immunofluorescence against Collagen I (red) and Collagen II (green). Sequential confocal sections were obtained in the Z-axis of the whole thick of the samples. Projections of the Z-stack are shown for the red (A), the green (B) and the merged channels (C) of the fin fold of a doubly immunostained 72 hpf embryo. A confocal plane of the merged red and green channels, at a higher magnification, shows the actinotrichia fibers in detail (D). Z-stack projections of all confocal sections .of a 4 dpa longitudinal fin section doubly immunostained and with nuclei counterstained in blue (E–G). E: Red and blue channels. F: Green and blue channels. G: All-merged channels. H: A detail of all merged channels shows colocalization of the green and red labels. Bars represent 100 μm in A–C and E–G; and 25 μm in D and H. Arrowhead is actinotrichia. e is epidermis. b is basal epidermal layer. c is connective tissue. n is notochord.

contacting the blastema, as well as in blastemal cells contacting the epidermis. At this stage, both collagen genes were also expressed in a group of cells surrounding the lepidotrichia at the amputation plane (Figs. 4A–B).

At 3 dpa, both collagens were expressed in AFC located around the newly formed actinotrichia (Figs. 4C and D). At 5 dpa (Figs. 4E and F) both genes showed two expression domains, as seen in transversal fin sections: a domain associated with the growing actinotrichia, formed by AFCs; and a second domain located around the regenerating lepidotrichia and formed by scleroblasts or lepidotrichia forming cells (LFC). These results suggest that *col2a1b* and *col1a1a* are expressed by AFC to form the actinotrichia and also by LFC to form the lepidotrichia.

Quantitative expression studies of the genes *col1a1a* and *col2a1b* showed similar patterns during fin regeneration (Figs. 4G and H). Basal levels were observed at 1 dpa, similar to those of a mature, non-regenerating fin. An upregulation of the expression was observed at 2 dpa when actinotrichia synthesis starts (10 times in *col1a1a* and 27 times in *col2a1b*). From 3 dpa, when lepidotrichia synthesis starts, col1a1a decreased gradually until reaching the lowest level at 7 dpa, when regeneration is finishing. These results suggest that *col1a1a* and *col2a1b* are strongly upregulated during actinotrichia synthesis whereas they are downregulated during the outgrowth of the lepidotrichia and the fin, almost reaching basal, homeostatic levels before regeneration is completed. The fact that each gene has a different degree of upregulation and reach their peak of expression levels at different stages might indicate that they have different contribution in fin skeleton formation.

col2a1b and col1a1a are essential for fin development

In order to elucidate the contribution of each collagen gene to actinotrichia and fin formation, knockdown morpholinos were used and morphant phenotype studied at 3 dpf (Figs. 5 and 6).

Knocking down *col2a1b* resulted in a ventral curled tail phenotype, and in a significant reduction of the fin fold and pectoral fin bud as early as 3 dpf (Figs. 5B, D, F and H). Due to the high similarity between *col2a1a* and *col2a1b* sequences, we decided to add a *col2a1a* morpholino to the *col2a1b* mismatch control. *col2a1a* control morphants showed a slight reduction of their body lengths and fin fold areas but no statistically significant differences were observed, as compared to normal development of controls (Figs. 6A–F). These results suggest that *col2a1b* is essential for actinotrichia formation and correct fin fold development.

Morpholino knockdown of *col1a1a* showed a delay in development similar to *col1a1a* heterozygous mutant (Fisher et al., 2003), and an affected caudal fin fold and pectoral fin bud development (Figs. 5A, C, E and G). These morphants showed reduced development of the fin fold, but the effect on the latter was not as striking as in the *col2a1b* morphant (Figs. 4G–I). Therefore, *col1a1a* seems to be needed for a correct fin fold development.

Collagen type I, type II and JAS'96 fluorescent immunostaining of both morphants showed that actinotrichia were present after knocking down both of the genes. However they seemed to be reduced and/or disorganized, or sometimes even absent. This phenotype was variable, depending on the degree of inhibition achieved by the

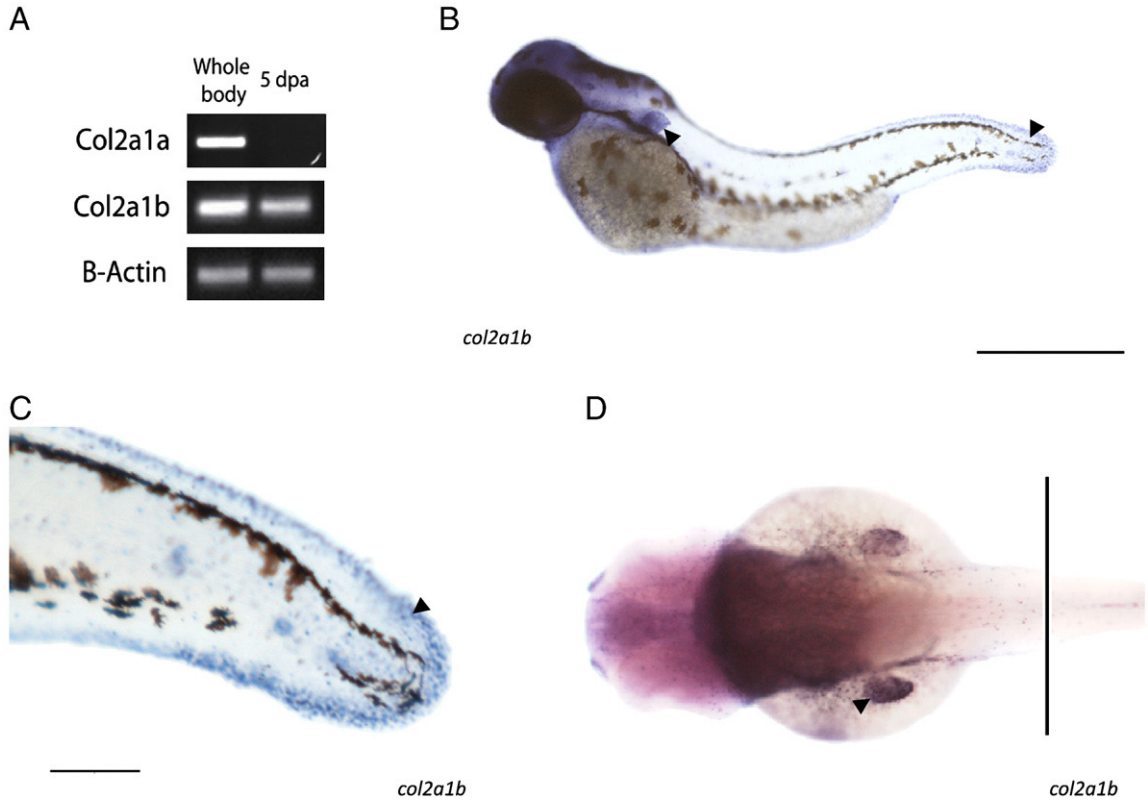


Fig. 3. *col2a1b* expression. (A) Semiquantitative RT-PCR of *col2a1a* and *col2a1b* in the whole body of 1 month postfertilization (left) and regenerating fin (rigth) tissues. b-actin is used as a control of constitutive expression. (B–D) In situ hybridization of a zebrafish embryo at 48 h stage. B. Whole mount in situ hybridization of *col2a1b* gene. Arrows point at the pectoral and tail fin folds. C. Detail of *col2a1b* gene expression at the tail bud. Note the expression at neuromast cells along the prospective lateral line. Arrow points at the distal portion of the tail fin fold. D. Dorsal view of *col2a1b* gene expression. Note the expression at the forebrain and midbrain. Arrow points to a labeled left pectoral fin bud. Bars represent 0.5 mm (B and D) and 0.2 mm (B).

morpholinos in each embryo (Fig. S4 and S5). In any case, the morpholino exerted a detrimental effect on the fin fold and the actinotrichia, resulting in a reduced immunostaining with the above-mentioned antibodies. The phenotypes observed in both morphants were then due to a correct inhibition.

The misregulation of col1a1a causes abnormal fin skeleton

To further dissect the role of collagen type I in the fin skeleton, we analyzed the phenotype of the mutant *col1a1*^{dc124} or *chihuahua* (*chi*). This mutant carries a mutation in *col1a1a* resulting in a change from glycine to aspartate at position 390, interrupting the Gly-X-Y domains typical of collagen. This change prevents the correct formation of the triple helix and results in aberrant Collagen type I molecules, which in turn result in skeletal dysplasia (Fisher et al., 2003). A study of the fin skeleton by the picrosirius red-polarization method (PSP) revealed that the fin skeleton is malformed in the mutant. We found that the mature adult fin was 20% shorter in *chi* than in the wildtype (Figs. 7A and B), the lepidotrichia were thicker, with only one bifurcation per ray, unlike the two observed in the wildtype, and the number and length of actinotrichia were significantly smaller (Figs. 7G and H). The morphology of regenerated fins was also different in *chi* mutants as compared to wildtype controls and *chi* non-regenerated fins. Eighty per cent of the rays in the regenerated *chi* fin had no actinotrichia and the lepidotrichia were wavy and not bifurcated. The remaining 20% or the rays, which had actinotrichia after regeneration, presented straight and normally bifurcated lepidotrichia (Figs. 7D and F).

The birefringence pattern observed with the PSP method, which is indicative of the fibrils arrangement within the tissues, was also observed to be different in *chi* and wildtype fins. Mature wildtype lepidotrichia displayed a homogeneous red-orange color that turned heterogeneous after regeneration (Figs. 7A, C and E). In contrast, mature chi lepidotrichia displayed a greenish color that turned to no color (i.e. no birefringence) after regeneration (Figs. 7B, D and F). These color differences indicate aberrant orientation of collagen molecules.

Lysyl hydroxilase in the formation of actinotrichia

Since fibril formation depends on the activity of Lysyl hydroxilase, we studied the expression of these enzymes in fin skeleton development. We focused on lh1 as it is expressed in the fin fold during zebrafish development (Schneider and Granato, 2007). In situ hybridization of *lh1* in sections of regenerating fins revealed that lh1 is expressed only by AFCs. Unlike *col1a1a* and *col2a1b*, *lh1* expression was not found in the epidermis or in LFC associated to the lepidotrichia (Figs. 8A–D).

Since the expression of *lh1* in AFC suggested that this enzyme might be implicated in the post-translational modification of the actinotrichia collagens, we performed morpholino knockdown to test this idea. *lh1* morphants showed a dorsal curled tail phenotype, no actinotrichia development and defective formation of the fin fold (Figs. 8F–G). This phenotype was not observed in the 5-mismatch controls, which displayed well-developed fin fold and actinotrichia (Fig. 8E). Therefore, a functional Lh1 is essential for actinotrichia and fin development.

Discussion

Actinotrichia are synthesized by both the epidermis and the mesenchyme

The ontogenetic origin of actinotrichia had always been elusive, as no study had identified the origin, epidermal or mesenchymal, of the

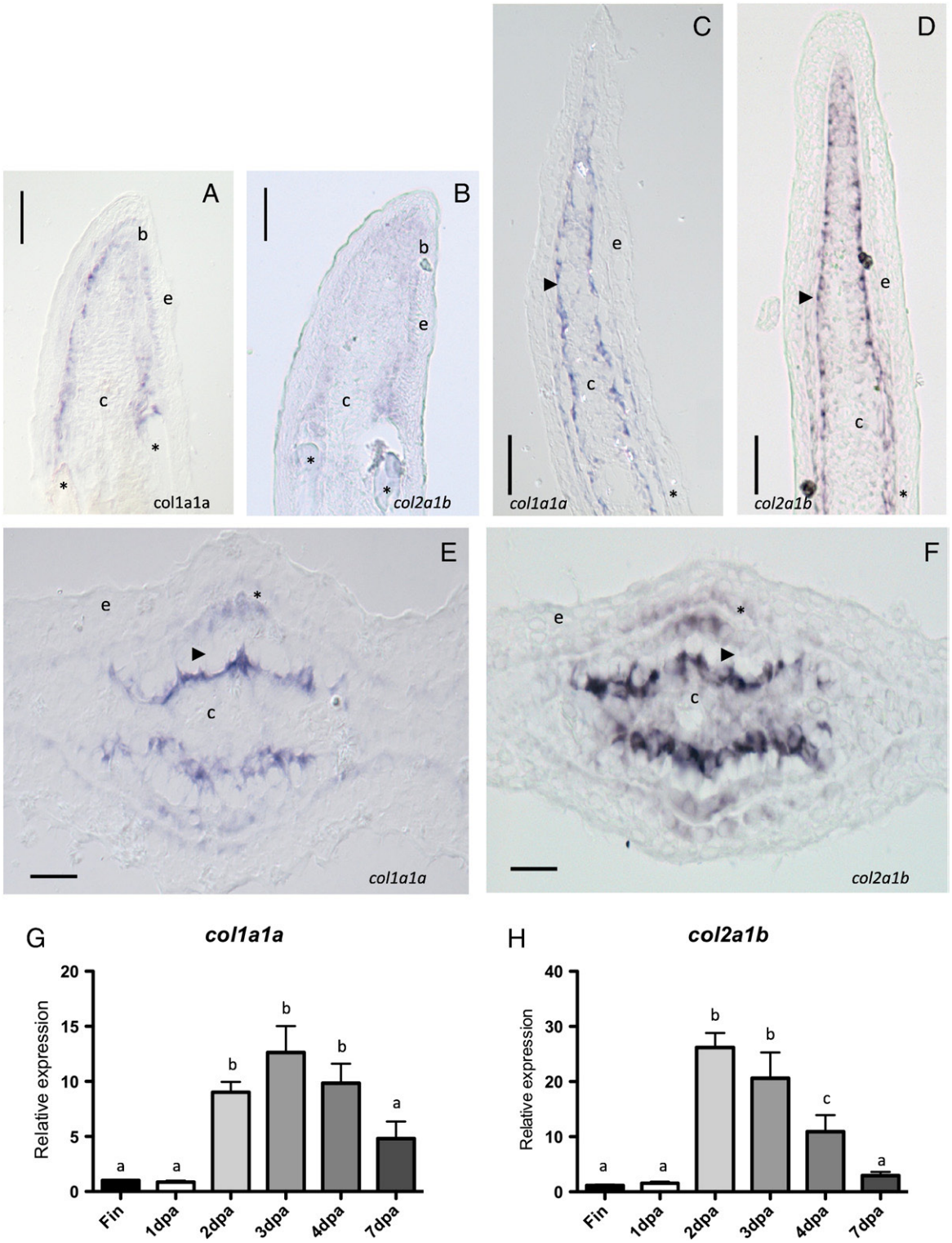


Fig. 4. Collagens type II alpha 1 b and type I alpha 1 are constituents of the fin skeleton. (A–F) In situ hybridization of *col1a1* (A, C and E) and *col2a1b* (B, D and F) in longitudinal (A–D) and transversal (E–F) sections of regenerated fins. A–B: Ray blastema at 2.5 dpa. C–D: Ray blastema at 3 dpa. E–F: Ray blastema at 7 dpa. Bars represent 25 μm (E–F) and 50 μm (A–D). Arrowhead is actinotrichia. Asterisk is lepidotrichia. e is epidermis. b is basal epidermal layer. c is connective tissue. (G–H) Expression analysis of *col1a1a* and *col2a1b*. Total RNA was extracted from different stages of fin regeneration and the relative transcript levels of *col1a1a* and *col2a1b* were determined by qRT-PCR. *col1a1a* (G), *col2a1b* (H) in different regeneration stages: Fin, non regenerating fin; 1 dpa, 1 days post amputation; 2 dpa, 2 days post amputation; 3 dpa, 3 days post amputation; 4 dpa, 4 days post amputation; 7 dpa, 7 days post amputation. Bars represent the mean of two independent biological samples ± SE. Different letters indicate a significant difference between samples according to the corresponding ANOVA ($P<0.05$).

cells that synthesize the actinotrichia, herein called actinotrichia-forming cells (AFC). Wood and Thorogood (1984) concluded that actinotrichia were synthesized by the ectodermal epithelium, after observing that they were formed inside the ectodermal fin fold before mesenchymal cells migrated into this space. Our results confirm an early ectodermal origin for actinotrichia, but find a second tissue

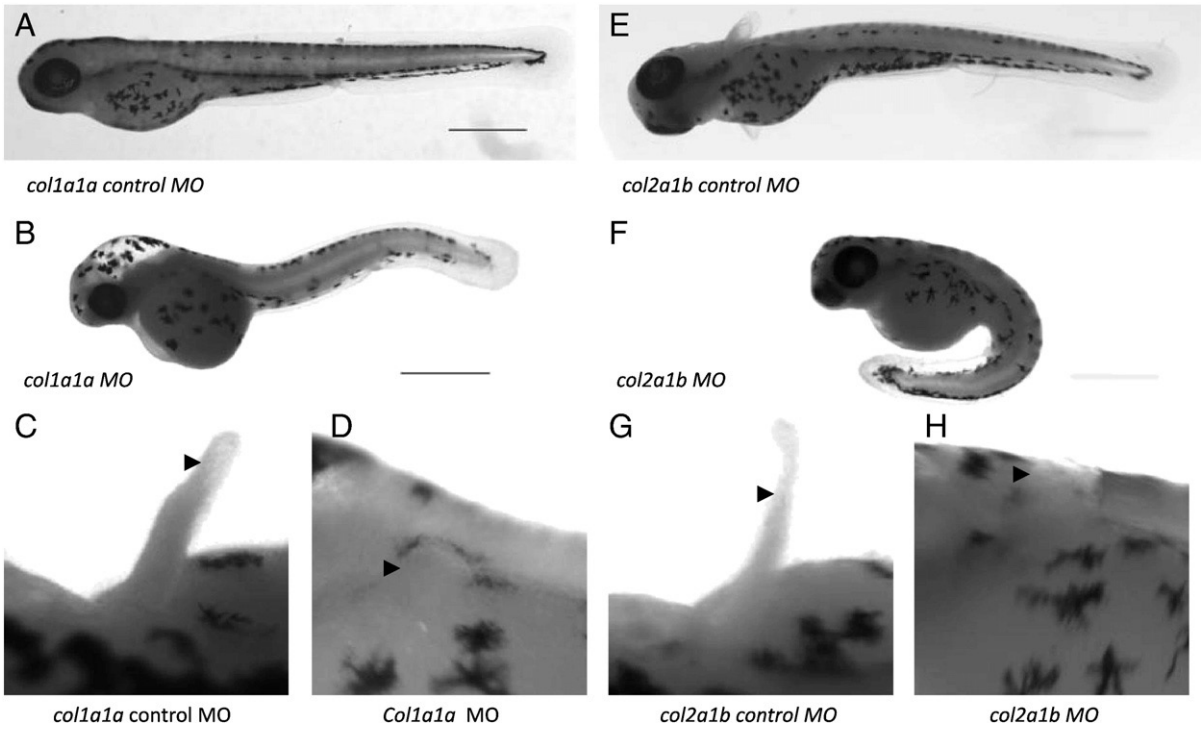


Fig. 5. *col2α1b* and *col1α1a* knockdown. Morpholino knockdown of *col1a1a* (A–D), *col2a1b* (F–H). Morphants of these genes (B, F, D and H) are compared with their corresponding 5-mismatch morphant control (A, E, C and G). Bars represent 0.5 mm (A, B, E and F) and 0.2 mm (C, D, G and H).

involved in its development: the mesenchyme that colonizes the fin fold. The temporal sequence of events suggests that actinotrichia synthesis is initiated by the ectoderm, which would be responsible for the early fibrillogenesis, while growth of the fibrils in length and thickness to form mature actinotrichia would be due to the mesenchyme populating the fin fold (Fig. 9A).

Similarly, during regeneration, constituents of actinotrichia are sequentially detectable in both the basal epidermis at the beginning and in the fin connective tissue later (Fig. 9B), suggesting an orchestrated interplay between these two tissues to form and maintain the actinotrichia. This interplay seems to involve a functional transition from the ectoderm/epidermis to the mesenchyme/blastema during development and regeneration, respectively.

Actinotrichia are constituted by heterotypic fibrils of Collagen I and a new form of Collagen II

The molecular nature of actinotrichia is not clear and has been debated ever since it was first studied. Since most authors agree on the homology of actinotrichia and ceratotrichia, a similar biochemical nature was assumed for both of them, and most of the authors assumed that actinotrichia are made of Elastoidin (Geraudie and Meunier, 1980). Elastoidin is a hypothetical protein complex proposed by several authors to explain the pretty unique physical and biochemical properties of ceratotrichia, which reminded to those of Collagen and Elastin (Damodaran et al., 1956; Kimura and Kubota, 1966; Ramachandran, 1962; Sastry and Ramachandran, 1965). Electron microscope studies eventually showed that actinotrichia are hyperpolymerized collagen macrofibrils, but the unusual size of the actinotrichia collagen fibrils lead the authors to the idea that actinotrichia might contain a different, unknown type of collagen (Montes et al., 1982).

We have analyzed actinotrichia collagen by proteomics finding then to be formed by Collagens type I and type II. This result was confirmed by immunostaining, which also showed that both Collagen types colocalize in the actinotrichia.

In situ hybridization showed the expression of *col1a1a* chain in the fin fold of the zebrafish. However, no expression of *col1a2* was found (Thisse et al., 2004). The collagen type I alpha 1 and alpha 2 chains usually assemble to form heterotrimers. The absence of *col1a2* expression during fin development means that Collagen type I is not formed by the normal heterotrimer composed of two type I alpha 1 chains and one type I alpha 2 chain, $[\alpha1(I)]_2+[\alpha2(I)]_1$. Hypothetically, the unique expression of *col1a1a* suggested a homotrimeric molecule $[\alpha1(I)]_3$ (Kimura et al., 1986), this kind of composition is the same as that present in collagen II $[\alpha1(II)]_3$. However, our results show the colocalization of *col1a1* expression with another gene, *col2a1b*. This might mean that heterotypic fibrils are formed in the fin due to co-polymerization of both collagen types. Type I and II chains in heterotypic fibrils have previously been described in the cornea to form fibrils of hybrid features between those of Collagen type I and Collagen type II, with unique properties as to elasticity and diameter (Fitch et al., 1995; Hendrix et al., 1982). Interestingly, these fibrils associate with Collagens type IX and type V (Birk et al., 1990; Olsen, 1997). Similarly, cells surrounding actinotrichia express *col9a1* and *col5a1* genes (Huang et al., 2009). These genes could mediate Collagen types I and II fibrillogenesis and interactions with other molecules of the extracellular matrix (Birk, 2001; Eyre et al., 1987). This mixture of Collagens I and II, together with actinodins, might be responsible for the unusual properties (elasticity, solubility, etc.) that drove former authors to hypothesize a new protein named elastoidin.

A further particularity found in actinotrichia is the form of collagen II they are made of. Several studies had previously described the presence of collagen II in the fin skeleton of the zebrafish (Smith et al., 2006; Yan et al., 2005). According to our results, the collagen type II found in the zebrafish fin is *col2a1b*, a new collagen gene that is expressed in actinotrichia and lepidotrichia forming cells. Genome duplication in actinopterygian fishes (Amores et al., 1998; Postlethwait et al., 1998) might be responsible for the emergence of *col2a1b*. According to our results, *col2a1a* and *col2a1b* can be found in the body of the fish, and they seem to complement some functions (as

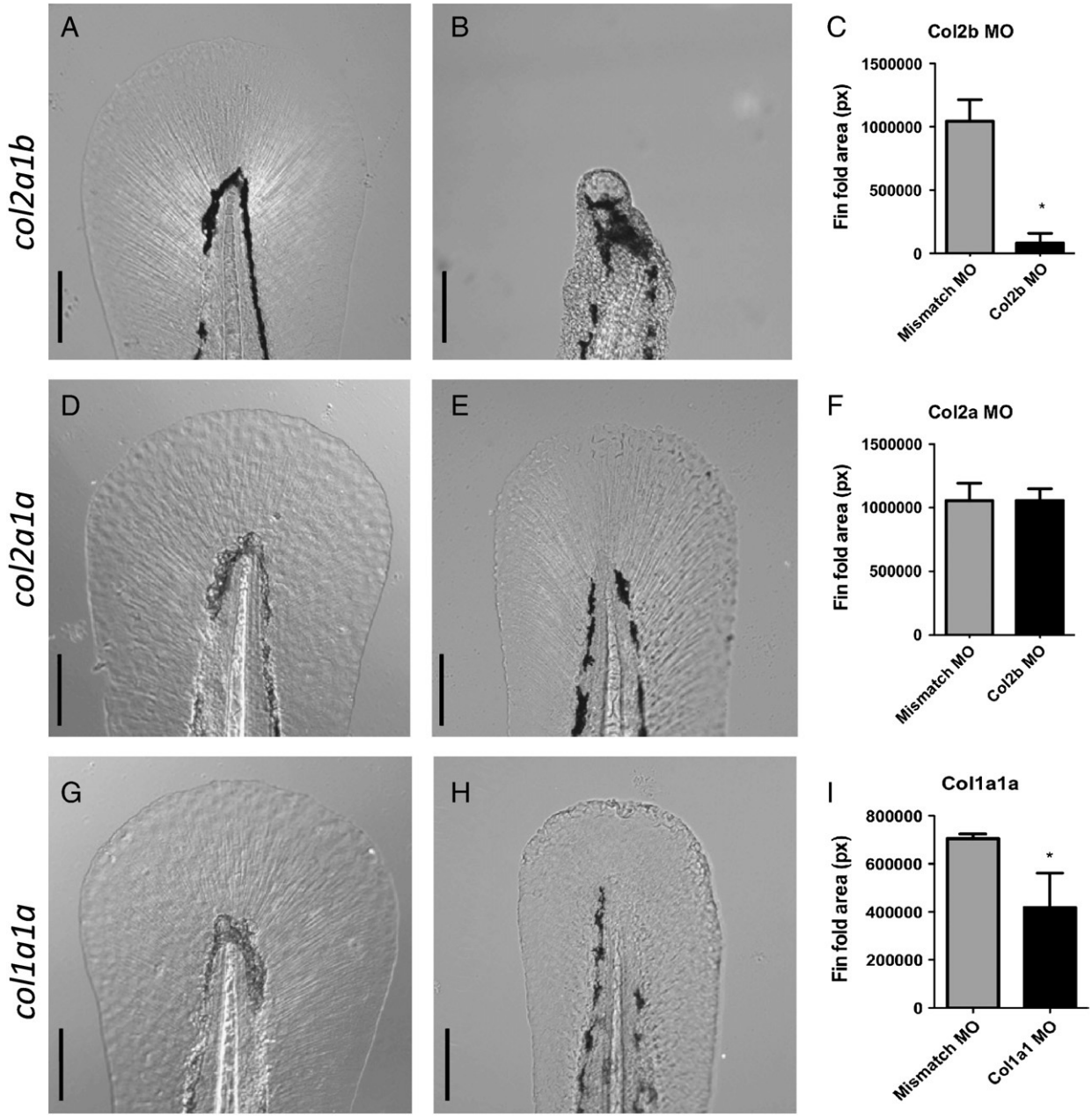


Fig. 6. *col2α1b* and *col1α1a* knockdown in fin development. Morpholino knockdown of *col2a1b* (A–C), *col2a1a* (D–F) and *col1a1* (G–I). Morphants of these genes (B, E and H) are compared with their corresponding 5-mismatch morphant controls (A, D and G). Fin fold area (in pixels) of each gene experiment were statistically compared (C, F and I). Asterisks represent significant differences after Mann–Whitney *u*-test. Note: E shows the strongest phenotype induced by col2a1aMO.

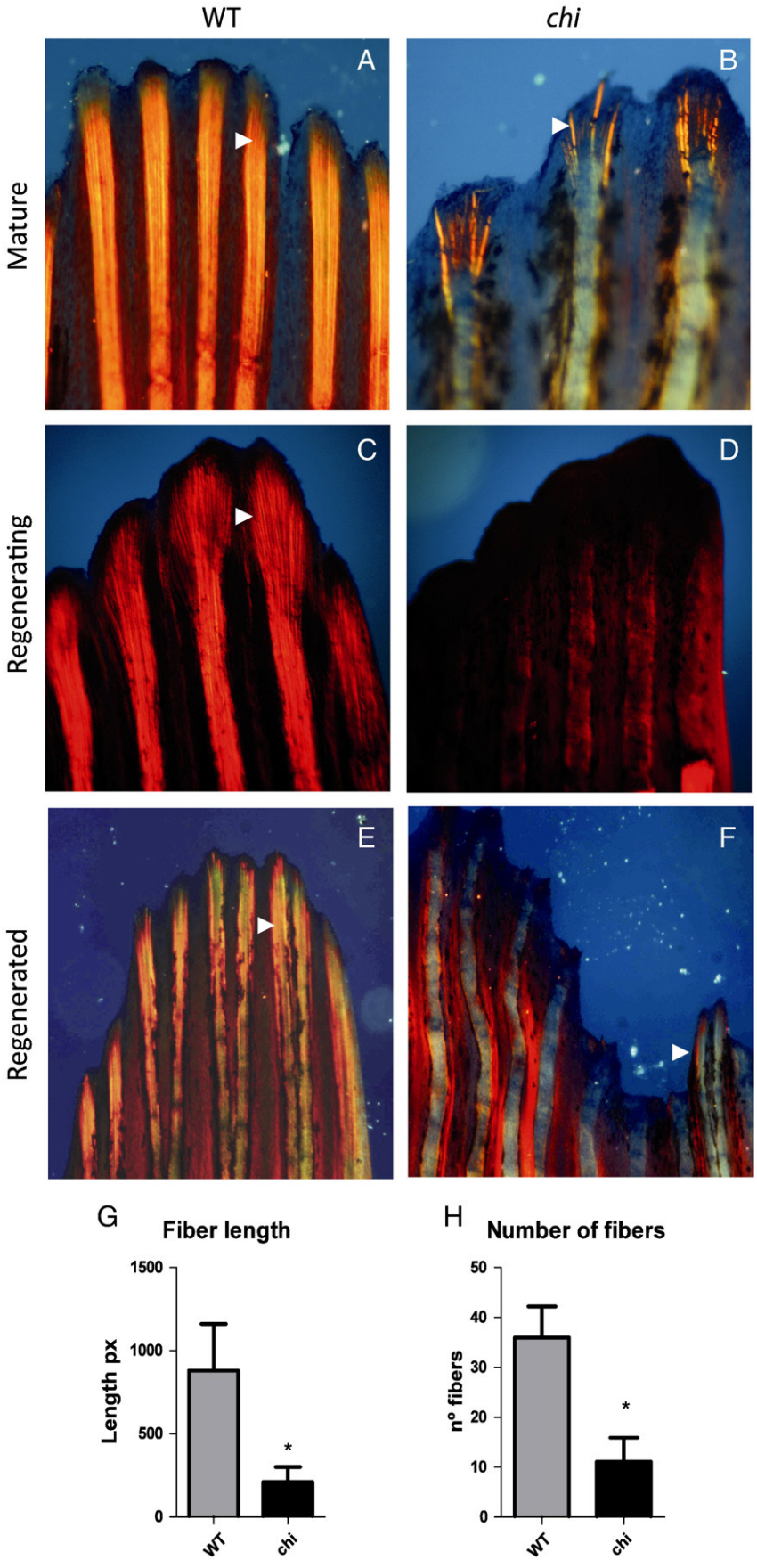
seen in *col2a1a* morphant), but only *col2a1b* is expressed in the fin and is therefore participating in fin formation.

Despite of our findings, it is still unclear how Collagen type I and Collagen type II are interacting to form these fibrils. Morphants of both genes, *col1a1a* and *col2a1b*, showed different phenotypes on fin fold. *col1a1a* morphant develops a reduced but almost complete caudal fold (Fig. 6H). During regeneration, when actinotrichia synthesis is starting, *col2a1b* expression increases around 25-fold at 2 dpa, whereas *col1a1a* increases less than 10-fold (Figs. 4G and H). However, at 3 dpa, the difference in the increase of expression of both genes is smaller than at 2 dpa, possibly due to lepidotrichia synthesis. Because of the dual role of these collagens, we cannot conclude exactly how both proteins interact, but functional analysis might suggest that *col1a1a* has less contribution than *col2a1b* during actinotrichia fiber formation.

Lh1 function in actinotrichia synthesis

The Lh1 enzyme hydroxylates lysine residues of the collagen G-X-Y domains. Each collagen type is usually composed of a relatively

Fig. 7. *col1a1a* dominant *chi*/+ mutant shows aberrant fin exoskeleton. Actinotrichia and lepidotrichia pattern in mature (A–B, E–F) and regenerating fin at 4 dpa (C–D). Wild type (A, C and E) and heterozygous *chihuahua* mutant (*col1a1a*$^{dcl24/+}$; B, D and F). Arrowhead represents actinotrichia. (G–H) Statistical comparisons of length and number of actinotrichia per fin ray between *chi* mutant (black column) and wild type (gray column). All images were obtained under polarized optics following staining with Picrosirius red. Asterisks represent significant difference by Mann–Whitney *u*-test.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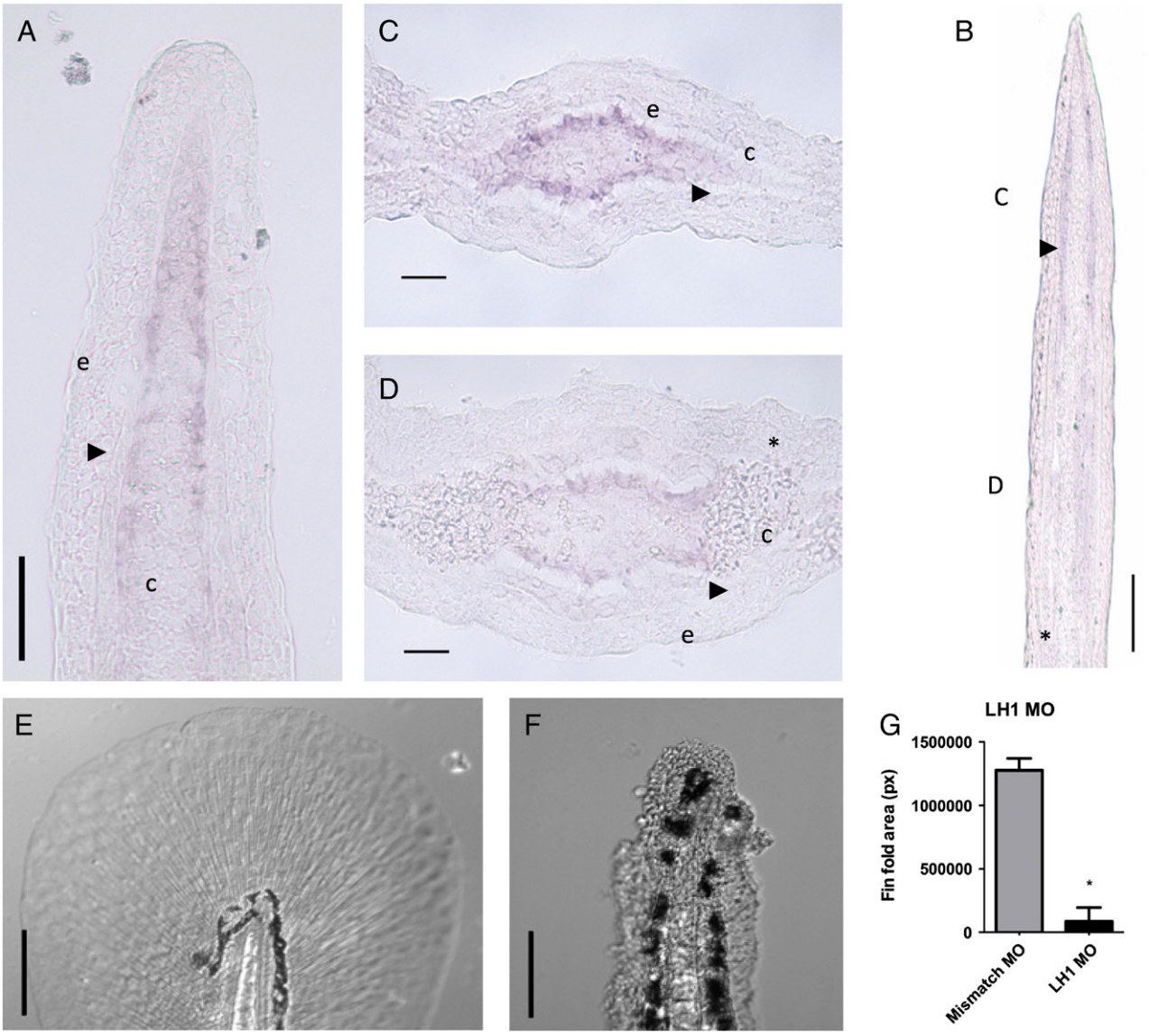

Fig. 8. *lh1* is implicated in actinotrichia synthesis. (A–D) In situ hybridization of *lh1* in fin sections. Longitudinal sections of ray blastema at 3 (A) and 7 dpa (B). (C–D) Transversal sections of a 7-day regenerated fin at the positions outlined in (B). Arrowhead is actinotrichia. Asterisk is lepidotrichia. e is epidermis. c is connective tissue. (E–F) Morpholino knockdown of *lh1* at 3 dpf (F) and 5 mismatch morphant control at 3 dpf (E). Fin fold areas (in pixels) are statistically compared following by Mann–Whitney *U*-test (G). Asterisk represents significant difference. Bars represent 25 (C and D) or 50 μm (A and B).

stable number of hydroxylysine residues. The abundance of these residues determines the number of attachment sites to carbohydrates and cross-linking degree during fibrillogenesis (Risteli et al., 2004). Collagen chain binding degree must be essential for actinotrichia formation. A previous report by (Schneider and Granato, 2007) shows that *lh1* is expressed at AFCs in the fin fold, and our results have shown that it is also expressed in AFCs during regeneration. *lh1* morphant phenotype suggests it plays an important role in fin fold morphogenesis and is essential for actinotrichia formation. Regulation by *lh1* function may modulate binding degree of collagen chains. Our results suggest that Lh1 might be as well responsible for post-translational modifications that provide the cross-linking capacity needed for hyperpolymerization of collagen chains.

Mutations in genes like *laminin alpha 5*, that prevent the correct formation of the fin fold, cause an altered arrangement of the actinotrichia, but not their synthesis (Webb et al., 2007). In turn, by inhibiting the expression of *lh1*, that is specifically expressed in AFCs, we have shown that actinotrichia are indeed essential for the correct formation of the fin fold and, therefore, for the development of the adult fin. During regeneration, AFCs distribution further suggests that actinotrichia are laterally synthesized as previously proposed by our group (Marí-Beffa et al., 1989).

Do actinotrichia play a morphogenetic role in fin ray patterning?

Although they seem to carry out a mere secondary structural function in the adult fin, actinotrichia have been proposed to play important morphogenetic roles. One of such roles is the correct patterning of the ray, a role that would be achieved by inducing the differentiation of the scleroblasts or LFC (Santamaría and Becerra, 1991).

Mutants for *col9a1* and col1a1a (*col1a1*$^{dc124/+}$) (Fisher et al., 2003; Huang et al., 2009), where actinotrichia are defective, may help us understand the role played by these fibrils. Both mutants develop abnormal lepidotrichia, with a wavy and non-bifurcated pattern. While in the *col9a1* mutant the actinotrichia are not correctly formed at all, in the *col1a1*$^{dc124/+}$ mutant rays may form with or without actinotrichia. In this mutant, rays that regenerate with actinotrichia grow straight and bifurcated, while rays without actinotrichia

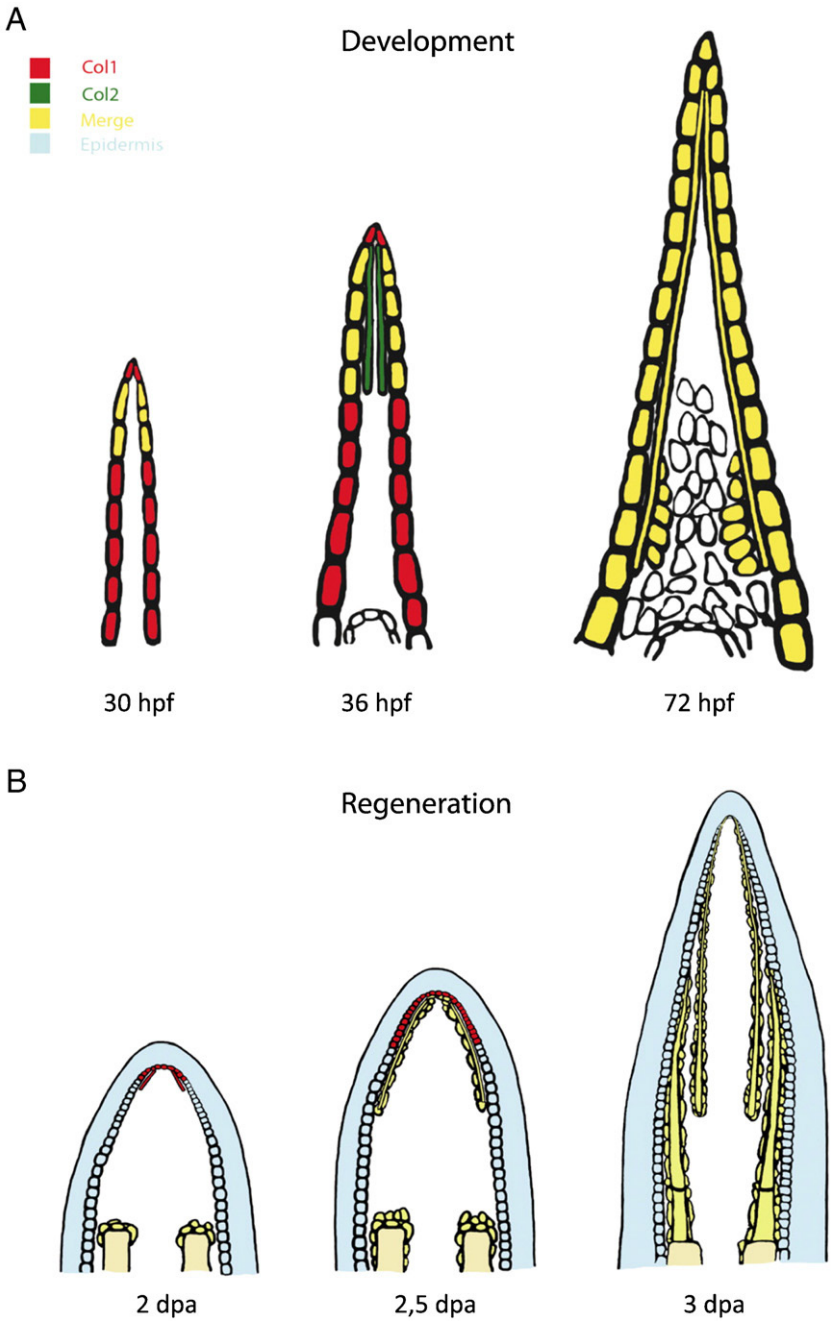

Fig. 9. Model of actinotrichia formation during fin development and regeneration. Collagen expression is represented in red (*col1a1*) and green (*col2a1b*), except when they are co-expressing in yellow (merge like), only relative to actinotrichia collagenous expression. (A) Actinotrichia development starting like cellular expression at 30 hpf; first fibrils at 36 hpf and elongation at 72 hpf. (B) Actinotrichia regeneration starting like cellular expression and first fibrils at 2 and 2.5 dpa and elongation at 3 dpa. Connective without actinotrichia collagenous expression is represented in blue.

regenerate curved and lacking bifurcations, as those found in the *col9a1* mutant. All these data suggest that the actinotrichia are critical for the correct patterning of the lepidotrichia.

In conclusion, the present study contributes to elucidate the origin and molecular nature of the teleostean actinotrichia, and supports that actinotrichia are needed for the correct development and regeneration of the fin ray, thus proving a morphogenetic role for these fibers.

Evolution of actinotrichia constituents

Ceratotrichia and actinotrichia appeared as an evolutive solution to the need for a supporting tissue within the fin. Lower chordate fins, such as those of amphioxus, consist of an epidermal fold with a supporting tissue inside. All the mentioned "skeletal" structures are immunoreactive to JAS'96 antibody raised against ceratotrichia, even amphioxus (Fig. S6). Immunoreactivity of all of these fin skeletons to JAS'96 antibody suggests biochemical conservation. Zhang and colleagues have shown that a new family of genes, actinodins, are components of actinotrichia. These genes are exclusive of fish lineages and their disappearance is related to fin disappearance and limb evolution. In this report, we describe another gene product exclusive of the fin, whose presence in the actinotrichia seems to be essential for a correct fin formation. The gene encoding this collagen (*col2a1b*) may have arisen as a result of *col2a1* gene duplication and further

neofuncionalization in actinopterygii. According to Zhang et al. (2010) and our own results, actinopterygii fin skeleton contains unique gene products, present in actinopterygian, but absent in tetrapods genomes. All these genes are essential for fin fold formation and may have gradually arisen through chordate evolution. Their absence might have been a requisite for the evolutionary disappearance of the fin dermoskeleton during vertebrate limb evolution.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.03.014.

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