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Characterization of spatial and temporal expression pattern of *Col15a1b* during zebrafish development

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ABSTRACT

In mammals, collagen XV is primarily expressed in skeletal and cardiac muscles, and loss of its expression in mice results in a mild skeletal myopathy. We recently identified *Col15a1a*, a zebrafish ortholog of the human collagen XV gene which expression was restricted to notochord in embryos. *Col15a1a* knockdown led to defects in muscle maintenance via Shh signaling. Here we report that zebrafish express a second ortholog *Col15a1b*. The identification of its complete primary sequence showed that the overall structure of collagen XV is well conserved between vertebrates. Whole mount *in situ* hybridization and RT-PCR analysis revealed that at 12 hpf *Col15a1b* is mainly expressed in slow muscle cell lineage and in nervous tissues, and, at later stages transcripts are detected in eyes, otic placodes and aortic arches. Based on the expression pattern of *col15a1b*, sequence alignments and synteny comparisons, we conclude that, contrary to collagen XVa, the zebrafish collagen XVb likely displays the same or similar function than the mammalian orthologs.

1. Results and discussion

Collagen XV forms with collagen XVIII a distinct subgroup among the collagen superfamily called Multiplexin for Multiple triple helix domains with interruptions. Both proteins are characterized by a central, highly interrupted triple helical domain (COL domains) flanked by large N- and C-terminal non-collagenous domains (NC domains). Both collagens XV and XVIII include a conserved thrombospondin N-terminal-like domain (TSPN) at their N-terminal ends and contain a C-terminal antiangiogenic fragment, restin and endostatin, respectively (for review see, Ricard-Blum and Ruggiero, 2005). Whereas collagen XVIII carries heparan sulfate chains, collagen XV has been shown to primary anchor chondroitin sulfate chains. In mammals, collagen XV transcripts are mainly expressed in heart and skeletal muscle (Hägg et al., 1997b) and *Col15a1* inactivation in mice causes skeletal myopathy and cardiovascular defects (Eklund et al., 2001).

In zebrafish, two orthologs of the human *COL15A1* gene have been characterized, called *Col15a1a* (GenBank accession number **AM259122**) and *Col15a1b* (accession number **FN432724**). In a previous study (Pagnon-Minot et al., 2008), we showed that expression of *Col15a1a* is mainly detected in the notochord and that its protein product is deposited exclusively in the peri-notochordal basement membrane. Morpholino-mediated knockdown indicated that collagen XVa is required for notochord differentiation and muscle development in zebrafish embryos and that it interplays with Shh signaling. In this study, the complete spatial and temporal expression of the second collagen XV gene, *Col15a1b* during zebrafish development have been characterized. Moreover, sequence alignments and synteny-based comparisons have been carried out.

1.1. Structure of zebrafish collagen XVb

Col15a1b corresponds to a partial cDNA (DW248793) previously found to be expressed in the marginal zone of the eyes and named mz98 (Pujic et al., 2006). The full length nucleotide sequence en-

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codes a protein of 1169 residues that is 45% identical to human collagen XV (AS:NM001855) and 46% identical to zebrafish collagen XVa. The structure of collagen XVb is similar to the human protein structure. Hence, the molecule comprises amino-(NC10) and carboxy-(NC1) terminal non-collagenous domains that flank a central highly interrupted collagenous domain (COL) which is composed of nine well-conserved collagenous domains, COL1–COL9 separated by eight short non-collagenous domains NC2 to NC9 (Table 1 and Fig. 1). The NC1 domain contains the typical restin domain of 181 amino acid residues including four conserved cystein residues. The restin fragment sequence presents higher identity to the human ortholog (61%) than to the collagen XVa zebrafish paralog (47%). A high degree of homology also exists between the zebrafish collagen XV restin sequence and the endostatin sequence of human and zebrafish collagen XVIII (57% identity). Several potential glycosylation sites were also identified (not shown).

The collagen XV zebrafish paralogs were found in two different linkage groups, LG24 and LG2 for *Col15a1a* and *Col15a1b*, respectively. Human *COL15A1* gene has been previously mapped to chromosome 9 region q21–q22 (Huebner et al., 1992). Higher degrees of conserved synteny were observed between human chromosome (Hsa) 9 and LG24 than with LG2 (Woods et al., 2005). Nevertheless, using ensembl for *in silico* chromosome mapping analysis, the orthologous gene pair *COL15A1/TGFBR1* and *Col15a1b/tgfr1* was found in Hsa9 and LG2, respectively. No conserved synteny was observed in the chromosomal region (LG24) containing *Col15a1a*.

1.2. Expression of Col15a1b during zebrafish development

The temporal expression pattern of *Col15a1b* was determined by RT-PCR (Fig. 2) and confirmed by whole-mount *in situ* hybridizations (Figs. 3 and 4). Expression was first detected at 12 h post-fertilization (hpf) and was maintained until 72 hpf, the latest stage examined in the present study (Fig. 2).

1.2.1. Expression of Col15a1b in developing skeletal muscle

At 13 hpf, *Col15a1b* was homogenously expressed in adaxial cells, which lie on each side of the notochord, as shown in dorsal view (Fig. 3A) and caudal cross-section (Fig. 3B). At 15 hpf, when the differentiation of adaxial cells towards slow muscle lineage is engaged, *Col15a1b* expression was progressively restricted to two anterior and posterior regions of adaxial cells (Fig. 3C). At 18 hpf, the expression of *Col15a1b* differed along the anterior–posterior axis according to the anterior-to-posterior wave of somite formation. At this stage, when radial migration of adaxial cells starts in the most anterior somites, the restricted expression of *Col15a1b* was clearly observed in posterior somites whereas its expression extended to the lateral mesoderm in anterior somites (Fig. 3D–F). Simultaneous detections of *Col15a1b* and *myoD* (Fig. 3G–I) or *smhc* (*slow myosin heavy chain*) (Fig. 3J–L) were performed to monitor *Col15a1b* expression at 18 hpf, when slow and fast muscles differentiate. Double *in situ* hybridization with *Col15a1b* and *myoD* antisense riboprobes (Fig. 3G–I) revealed that *Col15a1b* was expressed in all the eighteen somites, whereas *myoD* was exclusively expressed in the ten posterior somites as previously reported (Weinberg et al., 1996). In the posterior developing somites, the *myoD* muscle marker was expressed in adaxial cells and paraxial mesoderm that contains the fast muscle precursors (Fig. 3H). *Col15a1b* only co-localized with *myoD* in adaxial cells as shown in dorsal view (Fig. 3H) and transverse sections (Fig. 3I). Double *in situ* hybridization with the antisense riboprobes for *Col15a1b* and the slow fiber differentiation marker *smhc*, showed that *Co15a1b* is expressed in all smhc-positive cells (Fig. 3J–L). At 19 hpf, *Col15a1b* expression decreased in differentiated adaxial cells (data not shown) and at 24 hpf signal was restricted to somites of the posterior part of the tail (Fig. 3O).

Commitment of adaxial cells to slow muscle lineage depends on Hedgehog signaling (Hirsinger et al., 2004). In absence of Hedgehog signaling pathway, adaxial cells still form but differentiate into fast muscle fibers. To demonstrate that *Col15a1b* was exclusively expressed in slow muscle cells precursors, embryos were treated from 5.3 hpf to 18 hpf with cyclopamine, a specific inhibitor of Hedgehog signaling and processed for *in situ* hybridization with the *Col15a1b probe*. No signal was observed in these somites of cyclopamine-treated embryos (Fig. 3M and N). Note that the signal located in rhombomere 5 was not affected by the drug treatment.

1.2.2. Expression of Col15a1b in other tissues

Col15a1b was also expressed in several other tissues. A *Col15a1b* labeling was detected in nervous tissues from 12 hpf to 72 hpf, the latest stage examined in this study, and in eyes, otic placode and aortic arches from 18 hpf to 72 hpf (Fig. 4).

A strong expression of *Col15a1b* was found in the medial region of the developing otic vesicles of 24 hpf embryos (Fig. 4A). At 30 hpf, the expression was restricted to the ventro-medial epithelial cells surrounding the central cavity (Fig. 4B). At 48 hpf, only epithelial cells located in the posterior region of the otic vesicle were labeled (Fig. 4C).

At 24 hpf, the optic cup is well developed and the lens detaches from the overlying epidermis (Schmitt and Downling, 1999). The undifferentiated retina is constituted by the retinal pigmented epithelium and by the primordium of the neural retina, in which *Col15a1b* expression was observed (Fig. 4D). At 48 hpf and 72 hpf (Fig. 4E and F), a strong *Col15a1b* expression was detected in the ciliary marginal zone containing neuroepithelial cells, as previously reported (Pujic et al., 2006).

The aortic arch system consists of six pairs of vessels, including the vestigial arch 2 that connect the ventral aorta to the lateral dorsal aorta (Isogai et al., 2001). They are located within the corresponding branchial arches. At 48 hpf and 72 hpf, aortic arches 1, 3, 4, 5 and 6 expressed *Col15a1b* (Fig. 4G–I). Consistent with *Col15a1* expression in mammals, zebrafish *Col15a1b* transcripts were detected in the heart at 72 hpf (Fig. 4H).

Col15a1b was also expressed in the developing brain (Fig. 4H–J). In 24 hpf embryos, a weak signal was detected in the medial part of the midbrain–hindbrain boundary and in rhombomere 5 (Fig. 4J). At 72 hpf, a labeling was additionally detected in the dorsal telencephalon area closed to the diencephalon (Fig. 4H). Finally, *Col15a1b* transcripts were also detected in primary mouth (Fig. 4K).

1.3. Conclusion

The expression pattern of the second zebrafish collagen XV gene described here (*Col15a1b*) does not overlap with the one previously

Table 1
Comparison of the different domains between the zebrafish and human Collagen XV proteins. Numbers represent the percentage of identity (% Id) obtained from alignments using ClustalW. Hum: human Collagen XV.

Domains	% Id ColXVa/b	% Id ColXVa/hum	% Id ColXVb/hum
Full length	46	41	45
TSPN	54	54	54
COL9	76	52	52
COL8	69	61	53
COL7	86	60	50
COL6	54	48	46
COL5	65	50	49
COL4	63	40	46
COL3	64	55	50
COL2	59	67	65
COL1	83	75	73
Restin	46	47	61