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FISHING FOR COLLAGEN FUNCTION: ABOUT DEVELOPMENT, REGENERATION AND DISEASE

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Abstract

Collagens are the most abundant vertebrate extracellular matrix proteins. They form a superfamily of 28 members that show a remarkable diversity in molecular and supramolecular organization, tissue distribution and function and mutations in collagen genes result in a wide range of inherited connective tissue diseases. In the recent years, unexpected and very diverse regulatory and mechanical collagen functions have been reported. But the structural and functional landscape of the collagen superfamily is still far from being complete. Zebrafish has emerged over the last decades as a powerful model to interrogate gene function and there are numerous advantages of using zebrafish for collagen research, including recent advances in genome editing technologies and the characterization of the zebrafish matrisome. One can confidently predict that zebrafish will rapidly become a popular vertebrate model to investigate the role of collagens in development, disease and regeneration as discussed in this chapter.

Keywords : extracellular matrix, collagens, zebrafish, collagenopathies, regeneration, development

1. Introduction

The extracellular matrix (ECM) is essential for the molecular mechanisms that determine cell survival, differentiation and movement in all multicellular organisms [1]. Cells in tissues are structurally and functionally integrated with their surrounding ECM through dynamic connections. The ECM harbors a remarkable versatility of organization in tissues that is supported by a relatively small set of structural proteins encoded by ~300 genes in vertebrates [2,3]. But the ECM, as defined by the matrisome [2], contains another category of proteins: the matrisome-associated proteins. The latter category encompasses the ECM regulators such as the matrix enzymes and represents no fewer than two thirds of the entire matrisome. The ECM regulators as well as the ECM secreted factors (*e.g.* growth factors and cytokines) can fine-tune ECM activities in a narrow window of time and thereby confer to them additional functions in a given tissue. How ECM molecules assemble to form organized tissues and how crucial interactive domains within ECM proteins are structurally organized to allow network formation or signaling through cellular receptor recognition remain important questions that are far from being fully addressed. It is particularly true for the collagen superfamily that contains large multi-domain proteins and are often expressed ubiquitously in tissues [4].

The collagen field undoubtedly regains attractiveness with the emergence of new concepts (cell niche, regulation of growth factor bioavailability, mechanotransduction, cell microenvironment sensing) and new tools to investigate their *in vivo* functions (atomic force microscopy (AFM), second harmonic generation (SHG) imaging, genome editing, gene reporter lines....). Growing evidence showed that they sustain functions that would have been difficult to predict based on *in vitro* and *in cellulo* data. In that, the use of diverse animal models has proven to be useful to fully understand collagen function in their entire diversity, from the simplest invertebrates like hydra to high vertebrates [5]. This has been beautifully illustrated with the story of the discovery of the collagen XVIII *in vivo* function. While the knockout of *Col18a1* in mice showed no obvious phenotype, lack of expression of its orthologs, *cle-1* in *C. elegans* or *Mp* in drosophila, was strikingly coupled to axon guidance defects [6,7]. This established a link with the Knobloch syndrome in humans and further allowed revisiting the *Col18a1*-null mouse phenotype [8].

More recently, zebrafish (*Danio rerio*) has proven to be a powerful animal model to interrogate ECM function in development and disease [9–11]. It came out as no surprise since, in a mere two decades, zebrafish has become one of the most popular model organisms used in basic and medical research [12]. Besides the genetic tractability of this model, zebrafish are unmatched in their maintenance, breeding, raising and feeding. Moreover, zebrafish is highly

amenable to live imaging approaches because of its small size, its external development and the transparency of embryos and larvae [13]. Lastly but not the least, this small vertebrate represents a good alternative model to implement the “3R” principles of ethical experiments in disease modeling and drug screening [14]. While zebrafish has largely gained its reputation on its amenability to forward genetics in the 90s, the development of reverse genetic approaches in this small vertebrate proved challenging. The real revolution arose from recent advances on the so-called Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/Cas9 genome editing technology that offered unprecedented possibilities for genome manipulation and the investigation of the specific physiological function not only during embryonic and larval development but also during adulthood and aging [15,16].

We believe that the recent advances in gene editing methods and the increasing availability of “omics” data together with our recent characterization of the zebrafish matrisome showing that ECM proteins are well conserved between mammals and fish [3] are factors, among others, propitious to rapidly propel zebrafish as one of the leading models in ECM research. Here we specifically discuss mounting evidence highlighting the suitability of the zebrafish model to investigate the collagen functions in development, tissue regeneration and disease.

2. The zebrafish extracellular matrix

Two main types of ECM organization can be observed in tissues: interstitial ECM and basement membrane (BM) that are both easily identifiable with transmission electronic microscopy (TEM) in vertebrates, and zebrafish is not an exception (Figure 1). Collagens are central components of these highly structured and tissue-specific supra-molecular aggregates. Interstitial matrices and basement membranes found in zebrafish tissues and organs are generally similar in composition and structure than the mammalian ones, but zebrafish ECM also exhibit a few unique features.

Specifically, skin ECM displays striking particularities. At early stage, epidermis is only composed of 2 cell layers and starts to thicken from 15 dpf (day post-fertilisation) to become multilayered in adults [17]. As in mammals, the epidermis is separated from the subjacent dermis by a BM whose structure and organization is indistinguishable at TEM from the mammalian one (Figure 1B). Along this line, a global time-course transcriptomic analysis of BM formation during caudal fin regeneration revealed that BM gene expression kinetics recapitulates that one described in mammals [18]. However, unlike other vertebrates, the lamina lucida contains electron-dense small aggregates aligned regularly between the inner surface of the

lamina lucida and the epidermal cell surface (Figure 1B) [17,19]. These structures, termed adepidermal granules, are not present in adults [20]. The epidermal cells are most likely responsible for their production, but their composition is unknown.

The dermal ECM also shows specificities. During zebrafish embryogenesis, the epidermis is composed of the outer periderm and the inner basal keratinocytes, which are responsible for the initial deposition of ECM in the primitive acellular dermis [17]. The dermal endothelial cells, a species-specific cell type located at the interface between dermis and hypodermis, were shown to also contribute to the production of the developing dermal ECM. After metamorphosis (20-26 dpf), when juveniles seem miniature versions of the adults, fibroblast-like cells progressively invade the dermis. The epithelial ECM production ceases and dermal fibroblasts take over the synthesis and deposition of the collagen fibrils [17]. While dermis in mammals is composed of collagen fibrils showing no preferential orientation, the dermal ECM of the zebrafish comprises regular orthogonal layers of collagen fibrils [17] (Figure 1B). Strikingly, this plywood-like structure is reminiscent to the mammalian corneal stroma whose principal role is to contribute to the transparency of the cornea. The strict orthogonal organization of the dermal ECM might contribute, in the same way, to its optical transparency. Along this line, the deposition by basal epithelial cells of a primary stroma containing orthogonal layers of collagen fibrils, that act as a template for subsequent ECM deposition by invading fibroblasts, reminds us the development of the chick corneal stroma [21].

The development and physiology of cartilage and bones are highly similar in mammals and teleosts validating the zebrafish as a reliable vertebrate model in this field [22]. Expression of genes and the molecular pathways involved in the endochondral ossification are well conserved. For instance, as in mammals, Sox9, a key regulator of chondrogenesis was shown to regulate genes encoding cartilage-specific ECM in zebrafish embryos [23]. As such, the cartilage-specific collagens (types II, IX and XI) and proteoglycan, aggrecan, and the main matrix proteins, as matrilins and chondromodulin, are expressed in the zebrafish cartilage (review in Luderman *et al.* [24]). At the ultrastructural level, the organization and distribution of collagen and proteoglycan networks are highly similar to mammalian ECM cartilage (Figure 1A). Dermal bones that develop from the direct differentiation of mesenchymal cells into osteoblasts (a process known as intramembranous ossification) are found in fin rays, scales and gill covers [25]. The skeleton of zebrafish fins comprises the segmented parenthesis-shaped dermal bones called lepidotrichia and the unsegmented non-mineralized distal structures of ectodermal origin, the actinotrichia. Lepidotrichia is different from any other skeletal tissue that appears to be the result of a unique combination of collagen genes required for its development. Notably, *col10a1*, known as a specific marker of hypertrophic chondrocytes in

higher vertebrates is expressed in the zebrafish bony rays [25]. Actinotrichia are composed of collagen fibrils and non-collagenous proteins that aggregate into supramolecular structure referred to as elastoidin [26]. The nature of the ECM constituents of the zebrafish scales is less defined and the current knowledge on the matrix organization of the scales comes almost exclusively from TEM studies [27]. Elasmoid scales, the scale type of zebrafish and medaka, start to develop at 26 dpf, when fibroblast-like cells from which derived the scale-forming cells invade the dermis [27,28]. These mineralized structures made of orthogonal layers of collagen fibrils and elasmodin develop within the dermis and protrude into the epidermis. But the limiting layer of the scale, close to the epidermis, is devoid of collagen fibrils [27].

The trunk of the zebrafish embryo consists of a characteristic chevron-shaped block of muscles separated by the vertical myosepta. These thin connective tissue sheets transmit muscular forces to axial structures during swimming. In that, they are structurally and functionally equivalent to mammalian tendons. Muscle fibers insert into the myoseptal connective tissue *via* a highly specialized region referred to as the myotendinous junction (MTJ) [29,30]. The zebrafish MTJ is highly similar to that observed in mammals [30]. Specifically, this structure displays finger-like folds that increase contact between muscle and the adjacent myosepta (Figure 1C). Ultrastructural studies showed that myosepta remain acellular until 72 hours post fertilization (hpf) [30], suggesting that muscle cells are responsible for the synthesis and deposition of the ECM components present in the myosepta (mainly composed of collagen I fibrils) and the MTJ which contains BM zone components such as laminins and fibronectin [29], collagen XV-B [31] and the MTJ marker collagen XXII [32]. Later in development, fibroblasts are observed in myosepta and their presence coincides with the production of a dense network of collagen fibrils and the subsequent thickening of the myosepta [30]. The myoseptal fibroblasts were reported to express the classical collagenous tendon markers, collagens I and XII [33,34]. Even though their composition is highly similar, the ECM organization in myosepta and tendons are different. While mammalian tendons are composed of parallel bundles of collagen fibers, myosepta consist of tightly packed collagen fibers with an oblique arrangement adapted to the undulatory swimming of the fish (Figure 1C).

Notochord is a transient structure differentiating at early stage during embryogenesis that is at the origin of vertebral bodies in all vertebrates. In zebrafish, it is composed of vacuolated cells surrounded by a unique ECM structure improperly called notochordal BM. As in chicken and amphibians, this ECM sheath consists of 3 layers: a “true” BM inner layer, a dense fibrous medial layer and a fibrous outer layer [29,28]. It is not thus surprising that BM notochordal cells express both typical BM zone components, such as laminins [35], *col18a1* [36]

and *col15a1a* [37], and fibrillar collagen genes, such as *col2a1a* [38] and *col11a1* [39]. Expression of the collagen genes in the notochord is transient, decreasing by 24 hpf and ceasing after 48 hpf. We refer the interested readers to an existing excellent review on the composition, structure and function of the notochordal basement membrane [40].

3. The zebrafish collagen super family: common features and specificities

Collagens are the most abundant vertebrate ECM proteins. In mammals, they form a superfamily of 28 distinct members that show a remarkable diversity in molecular and supramolecular organization, tissue distribution and function. Filling the space between cells they play a unique role in providing a supportive scaffold to which cells attach and in maintaining the structural integrity of many tissues. In addition to their contribution to matrix biomechanical properties, collagens are also closely involved in various morphogenetic events and display highly specialized functions. Collagens clearly have both regulatory and mechanical functions in tissues during development and repair.

Collagens are encoded by 44 genes in humans and by 58 genes in zebrafish [3]. Very recently, a fourth collagen XXVIII gene, *col28a2b*, was identified in the GRCz11 version of the zebrafish genome (<https://zfin.org/>) bringing the total number of zebrafish collagen genes to 59 (Figure 2A). The elevated number of collagen genes contained in the zebrafish genome results from a further round of whole-genome duplication (WGD) referred to as teleost-specific-WGD (TS-WGD) that took place 320-350 millions years ago and resulted in a massive increase of protein coding genes. Duplicated collagen genes can have different fates including non-functionalization of one of the duplicated gene as a consequence of lack of selective constraint or subfunctionalization, neofunctionalization or dosage selection, that make zebrafish fascinating from a phylogenetic perspective [41]. These mechanisms act through evolution for the loss, maintenance or functionalization of duplicated collagen genes resulting in specific features of the zebrafish collagen gene repertoire [3]. All in all, 16 collagen genes are duplicated in zebrafish compared to mammals and the function of only some of them have been interrogated so far. Specifically, the zebrafish genome contains 4 collagen XXVIII genes, *col28a1a*, *col28a1b*, *col28a2a* and *col28a2b*, consequential to the duplication of the $\alpha 1$ and $\alpha 2$ branch although the $\alpha 2$ branch was then lost in mammals [42] As many other teleost fish, zebrafish does not have any collagen III gene that was probably lost during early diversification of this infraclass [43]. Finally, some of the collagen paralogs have diverged during evolution and displayed distinct expression patterns and functions as we reported for collagen XV paralogs, *col15a1a* and *col15a1b* [31,37,44]. While collagen XV is expressed widely in the basement membrane zones of various organs in mice and humans,

col15a1b is specifically expressed in slow muscle precursors and *col15a1a* is mainly expressed in notochord [37,44]. Exploiting the existence of collagen paralogs for discovering new functions will undoubtedly bring new insights in the multiple roles of collagen genes in human development and disease.

Collagen genes encode long polypeptidic chains called α -chains that contain unique structural signature, the presence in their primary structure of the [Gly-X-Y] amino-acid repeats, where X and Y are often a proline and a hydroxyproline respectively. This results in the formation of a triple helix, hallmark of the collagen superfamily (Figure 2B). Collagens are secreted proteins and their biosynthesis ends in the extracellular space where they can form different supramolecular aggregates (banded fibrils, beaded filaments, hexagonal networks, anchoring fibrils) [4]. This main extracellular step in collagen biosynthesis is mediated by fine-tuned combination of self-assembly and cell-driven processes that are still far from being completely understood. Overall, zebrafish collagen fibrils are structurally indistinguishable from the ones of other vertebrates with TEM (Figure 1) and they can be revealed by the classical histology and imaging technics such as SHG microscopy [45–48]. Although collagen chain assembly is rarely studied in zebrafish, the presence of duplicated genes can lead to differences compared to mammals. Collagen I mostly occurs as a heterotrimer with two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain, but zebrafish contain an additional α -chain, initially called $\alpha 3$ (encoded by the *col1a1b* gene) that may form with the two other chains the heterotrimeric molecule $\alpha 1(I)\alpha 2(I)\alpha 3(I)$. These chains are expressed in connectives tissues (skin, bones, tendons...) as in other vertebrates. In addition, the actinotrichia contains another molecular species, the homotrimeric form $[\alpha 1(I)]_3$ [49]. Interestingly, high amounts of collagen I homotrimer was also reported in fetal calf bones [50].

Although the presence of at least one triple helix domain is typically the collagen signature, collagens are modular proteins composed of a succession of collagenous (COL) and non-collagenous domains (NC) that are both involved in collagen functions and in receptor binding. COL domains are linked by short linker regions of about 20 residues, while larger NC domains are found at the N- and/or C-terminal ends of the α -chains and can represent more than two-third of the entire collagen molecule (Figure 2C). Protein sequence alignments revealed that NC domains are generally well conserved in zebrafish [3,19,34,39,44] but their specific functions in zebrafish await further studies. Collagen molecules undergo a series of post-translational modifications both intracellularly and extracellularly that are most often mediated by key enzymatic activities, as the collagen prolyl 4-hydroxylases (P4Hs) and the lysyl-hydroxylases (LHs) that both reside within the endoplasmic reticulum and the lysyl oxi-

dase (LOX), a matrix crosslinking enzyme (Figure 2B). Most of the post-translational modifications are shared with all collagen types, but specificities exist. In zebrafish such modifications are also present. For instance, the *plod2* mutant carrying a mutation in the lysyl-hydroxylase-2 encoding gene showed a severe disruption of the three-dimensional collagen I fibril network in vertebral bones [51]. Another hallmark is the extremely large size of collagen molecules, the huge amount of collagen molecules that are secreted in the extracellular space in a very short time window and their rapid secretion during development or repair. The secretory vesicles have thus to accommodate the large size of collagen molecules. It has been shown that the fibrillar collagens are transported from the ER to the Golgi complex in a COPII (coat protein II complex)-dependent manner. Additional ER associated proteins were shown to assist collagen macromolecules transport into vesicular carriers. Specifically, the auxiliary protein Sar1 and the recruited heterodimers Sec23/Sec24 proteins are proven to be essential for vesicle biogenesis and collagen molecule exportation [52]. Interestingly, collagen trafficking is impaired in the *crusher/sec23a* mutant. This prevents collagen II secretion and causes severe cartilage defects [53]. The *feelgood* mutant also manifests severe skeletal defects. Melville and coworkers [54] reported that the *feelgood* mutation caused a single amino acid substitution within the sequence encoding the DNA-binding domain of transcription factor Creb3l2, which specific targets are genes encoding the COPII proteins, *sec23a*, *sec23b* and *sec24d*. Despite intracellular accumulation of collagen, no activation of ER stress response genes was observed [54].

4. Collagen function(s) in zebrafish development

Among the zebrafish community, collagen genes were first valued as tissue markers. For instance, the *col10a1* gene, encoding collagen X, was first reported as a reliable marker of both osteoblasts and chondrocytes in zebrafish [55] and the *col2a1* gene was commonly used as a notochord marker in early embryos [38]. Finally collagen I genes were used to probe fibrosis as in all vertebrates [56,57]. Thanks to the development of gene modulation technologies (morpholino (MO)-knockdown, mutagenesis, transgenesis and targeted genome editing), the description of collagen expression patterns and the investigation of collagen gene function in developing zebrafish embryos have largely expanded, giving significant insights into their functional diversity and most importantly, the underlying mechanisms (Table 1). Along this line, the generation of specific antibodies to the zebrafish protein has proven, in our lab and others, to be instrumental for the analysis of collagen function in developing zebrafish [19,31,32,34,56,58] (Table 1). The recent generation of fluorescent collagen I-fusion zebrafish lines represents unique tools to investigate collagen dynamics [59] and opens up new avenue for the study of collagen biosynthesis and remodelling in living ani-

imals. Our objective here was to report on the main advances in collagen function research, and we now apologize that other important functional studies have not been quoted due to word limitation.

4.1. Nervous system and neuromuscular system

Although ECM of nervous system is mainly composed of hyaluronic acid, associated glycoproteins and proteoglycans, non-fibril forming collagens are expressed by neuronal cells and other cell types and participate to neural circuit formation [60]. Zebrafish has proven to be essential to highlight the *in vivo* role of collagens both in peripheral and central nervous system. For instance, the zebrafish mutant line *dragnet* that exhibited defects in retino-tectal projection, was then reported to carry a premature stop codon in *col4a5* identifying collagen IV α 5 as a novel contact-mediated guidance cue [61]. To reach their target, axons are instructed during their extension by attractive and repulsive guidance cues that are secreted in the extracellular space. *In vivo* genetic interaction and biochemical analysis using mammalian recombinant or purified proteins revealed that the zebrafish BM collagen IV acts on target selection of retinal ganglion cells (RGC) through its interaction with the conserved guidance molecule slit2 [62]. However, more recent studies showed that, in both *dragnet* and *shio-maneki* (*col4a6*) mutants, collagen IV rather acts on axogenesis of RGC and granule cells of the cerebellum by regulating the integrity of the BM [63]. Whether collagen IV operates as an instructive protein or a structural protein (or both) is thus still unclear.

Several collagens (*stumpy/col19a1*, *col18a1* and *col15a1b*) expressed in the peripheral nervous system were reported to provide motor neuron axons with important cues during their navigation towards their muscle target [31,64,65]. The above-mentioned myotomal collagens, *stumpy/col19a1*, *col18a1* and *col15a1b*, are all produced by adaxial cells as revealed by *in situ* hybridisation. These cells are slow muscle progenitors that are apposed next to the notochord in early somitogenesis. They then migrate radially towards the surface of the myotome where they differentiate into superficial slow muscle fibers (SSF). Thanks to the generation of specific antibodies to zebrafish collagens, our group was able to demonstrate the unexpected physical presence of one of these contact-mediated guidance cues, collagen XV-B, in the axonal motor path [31]. We showed that the protein is secreted then deposited specifically all along the motor axon path before the onset of adaxial cell migration, through a two-step mechanism involving two independent signaling pathways, Hedgehog and *Unplugged*/MusK. As such, these cells leaved a specific matrix fingerprint that delineates motor axon path behind them a few hours before motor axons project from the spinal cord. This key result was also instrumental in deciphering the unique function of collagen XV-B in motor axon navigation [31]. 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 [66] and cardiovascular and nerve defects [67]. Expression of the second paralog, *col15a1a*, was restricted to notochord in embryos, and its knockdown provoked defects in muscle maintenance through Shh signalling [37]. Based on the expression patterns of the two paralogs, their sequence alignment and synteny comparison, it has been suggested that collagen XV-B could fulfil similar function than the mammalian ortholog [44]. However, none of the functions identified for zebrafish collagen XV genes match so far with the known functions of the mouse gene.

Myoblasts are another source of collagens. Developing skeletal muscles spatially and temporally shapes collagen-containing matrices that are critical for proper function of the neuromuscular system as exemplified above with collagen XV-B. The myotomal collagen XXII is a marker of the MTJ that was first characterized by Koch and co-workers in 2004 [68]. Its *in vivo* function remained uninvestigated for several years owing to a lack of available genetically-modified animal models. Again, zebrafish was helpful to dissect the function of this collagen. We showed that *col22a1* transcripts were expressed throughout the myotome at 22-24 hpf and transcripts progressively and specifically localized at muscle ends when MTJ formation is initiated. The protein product, collagen XXII, followed the same pattern and integrated the junctional ECM as transcripts started to be expressed at the muscle cell ends. *Col22a1* knockdown in developing zebrafish resulted in muscular dystrophy-like phenotype with massive muscle fibre detachment from their myoseptal insertion in response to repeated muscle contractions showing that collagen XXII strengthened skeletal muscle attachments during contractile activity [32]. As in humans, zebrafish muscle fibres attach to the MTJ using two linkage systems: the dystrophin glycoprotein complex and the $\alpha7\beta1$ integrin complex [69]. Synergistic gene interaction experiments further suggested that collagen XXII is part of the $\alpha7\beta1$ integrin complex. Although the muscle phenotype was reminiscent to reported ECM related myopathies [69], no human disease has been associated to mutation in the human gene so far.

4.2. Cartilage and Bones

Cartilage is a connective tissue in which the fibrillar collagens type II and XI are highly represented. As such, collagen II probes were heavily used as a notochord marker in zebrafish [37] (Table 1). Several transgenic lines were recently created after isolation of *col2a1a* regulatory elements that finally allowed to directly visualize the notochord along with craniofacial cartilage, ear, floor plate and fins [70,71]. The regulatory elements of the *col10a1* promoter were also isolated to create a reporter line that monitors the presence of this collagen in bones [71]. As highlighted by Mitchell and collaborators, such tools can be now used to study

genes involved in osteoarthritis [71]. Besides, the function of collagens in developing bones and cartilage was investigated using MO-knockdown or ENU-mutants. Thereby, *col1a1a* and *col2a1b* that are both expressed in the skeletal elements of the fins were shown to be required for proper fin fold morphogenesis [72]. Two mutants, called *gulliver* and *leviathan*, were isolated in a mutant screen searching for genes involved in notochord morphogenesis. These mutants that exhibited notochord distortion showed complementation as the mutations both mapped to the *col8a1a* gene [73,74]. Zebrafish also allowed establishing the importance of another collagen gene in notochord development although it was not expected to play such function regarding its expression in mammals. As mentioned above (paragraph 4.1), the expression pattern of *col15a1a*, one of the paralog of the human gene *COL15A1*, was restricted to the notochord and deposited in the ECM of the peri-notochordal sheath [37]. Ultrastructural analysis of *col15a1a* morphants revealed that collagen XV-A is important to maintain the integrity of the notochordal BM. Remarkably, despite the transient effect of MOs, Christiansen and collaborators showed that knockdown of *col27a1a* and *col27a1b*, two paralogs expressed in the notochord during zebrafish embryogenesis, provokes the curvature of the notochord at early developmental stages but also scoliosis in older animals at 21 dpf [75]. Finally, loss of function of *col11a1* in zebrafish was shown to act on spatial organization and maturation of chondrocytes resulting in important defects in craniofacial cartilage reminiscent to collagen XI-related chondrodysplasia in humans [39].

4.3. Skin

Only a few functional studies have been conducted so far and most of them have interrogated the role of epidermal BM-associated collagens. Collagen XVII is an integral component of the hemidesmosome, a structure that ensured the dermo-epidermal cohesion by anchoring the basal epidermal cells within the underlying BM. In zebrafish, *col17a1a* is expressed in a punctuated pattern within the skin while its paralog *col17a1b* is restricted to the lateral line, a fish specific sensory organ [76]. Knockdown of *col17a1a* in embryos caused defects in the BM structure that resulted in a skin blistering phenotype with occasional detachment of the epidermis [76]. Remarkably, similar defects were observed after injection of *col14a1a* MOs in zebrafish [19]. An intriguing additional observation was made in embryos knocked-down for collagen XIV-A in which an uneven distribution of the adepidermal granules was observed with the presence of frequent gaps [19]. To our best knowledge, this represents the first evidence for a gene involved in the formation of the adepidermal granules. The *col14a1a* paralog is transiently expressed in proliferative epithelia and the corresponding protein persisted in BM at later stages [19] where staining partially overlaps with the closely related mesenchymal collagen XII [34]. Collagen XII is ubiquitously expressed in all connective tissues in zebrafish [34] but its function in skin has not been investigated yet.

All these studies underscored the importance of BM-associated collagens for its formation and integrity and confirmed that zebrafish is a relevant model to study BM associated disorders [11]. In 2013, Richardson and collaborators have identified zebrafish as a new valuable model for cutaneous wound healing thus paving a new way for skin research [77].

5. Collagens as new drivers of zebrafish tissue regeneration

Contrary to mammals, zebrafish possess very high regeneration capacities and thereby represent a versatile model to study tissue and organ regeneration. Adult fish can fully reconstruct their brain, spinal cord, retina, kidney, liver, pancreas and all its appendages [78]. For evident reasons related to human regenerative medicine, the heart reconstruction was highly studied as well as the caudal fin regeneration for practical reasons.

Collagen IX was involved in the formation of the vascular plexus during adult zebrafish caudal fin regeneration, a structure that is abnormally persistent at 10 days post-amputation (dpa) in *persistent plexus* mutants (*prp*). This mutant carries a point mutation causing a single leucine (176)-to-histidine change in the collagen IX TSPN subdomain. This mutation in the collagen IX gene resulted in smaller fins with rounded edges and osteoblast mispatterning. Both *prp* embryo and adult animals exhibited defects in fin skeleton regeneration, a collagen II-rich structure to which collagen IX is associated [79]. The possible role of collagen IX in angiogenesis is intriguing and has not been described in mammals.

Several fibrillar collagens were expressed in fin skeleton, the collagen types I, II, V, XI and XXIV and XXVII [43]. As mentioned above, fish bones also expressed collagen type X. Collagen I is encoded by four genes in zebrafish: *col1a1a*, *col1a1b*, *col1a2a* and *col1a2b*. Analysis of collagen I gene expression during fin skeleton regeneration suggested that, while *col1a2a* was not detected, *col1a2b* is highly expressed and could assemble with one of the two other chains to form collagen I heterotrimers [43]. *In situ* hybridization at 4 dpa showed that *col1a1b*, *col5a1*, *col10a1*, *col24a1*, *col27a1* are restricted to the proximal regenerate that will form the collagenous lepidotrichia part of the fin skeleton while *col1a1a* and *col2a1b* are also present in the distal outgrowth corresponding to unmineralized actinotrichia bundles [43]. The fin skeleton regeneration is also regulated by the Hsp47 procollagen chaperone. Its knockdown reduced cell proliferation and the length of the different segments that compose the fin skeleton, resulting in smaller fins [80]. As illustrated by collagen II staining, the regeneration of fin skeleton is compromised in MO-injected fins. This suggested that Hsp47 is re-

quired for proper fin growth and patterning probably through its collagen chaperone activity. Finally, the collagen-based ECM was critical for proper wounding and its formation and reorganization at the leading edge of the wound was dependent on reactive oxygen species and vimentin [81].

Collagens are also critical for spinal cord regeneration. A conditional heat-inducible mutant for lysyl hydroxylase 3 (*lh3*), encoding a glycosyltransferase required for collagen secretion and deposition has been generated. *Lh3* invalidation caused defects in axons target-selective regeneration through its main substrate in Schwann cells, collagen IV $\alpha 5$ chain [82]. Interestingly, the collagen IV $\alpha 5$ chain was shown to destabilize mistargeted axons during regeneration in order to ensure target-selective regeneration through interaction with the axonal repellent *slit1a*. Collagen IV $\alpha 5$ could sequester Slit1a to form a repulsive barrier that directs axons onto their original path.

The FACIT collagen XII is also part of the zebrafish embryo axonal regenerative program. Its expression was induced in fibroblast-like cells by Wnt/ β -catenin signalling after spinal lesion in zebrafish larvae [56]. The collagen XII-containing ECM was necessary for axons navigation within the damaged site to promote axonal bridging through the lesion and functional recovery. Strikingly, *col12a1a* overexpression was sufficient to rescue the effects of Wnt/ β -catenin inhibition showing that collagen XII actively promotes axonal regeneration. Similarly, a collagen XII-rich ECM was critical for adult zebrafish heart regeneration. After cryoinjury, collagen types XII and I were highly upregulated at the wound site upon TGF- β signalling [83]. In the regenerating edge of the myocardium, collagen XII partially co-localized with tenascin C and fibronectin that composed the transitional regenerative ECM. Together, these studies positioned collagen XII as a new actor of the provisional ECM. Overall, ECM components actively contribute to regeneration. This has been illustrated by a recent study showing that decellularized ECM from zebrafish heart promotes cardiac functional recovery in mammals [84] opening a new area of future regenerative medicine.

Recently, we have created CRISPR/Cas9-mediated *col14a1a* knockout fish to interrogate the function of *col14a1a* during regeneration using the caudal fin model. Using AFM, we showed that collagen XIV-A transiently acts as a molecular spacer responsible for BM biomechanics possibly by helping laminins integration within regenerative BM. These data indicated that 3D-organization of BM is thus fine-tuned by minor components of the BM zone while the integral components of BM are involved in the assembly of its backbone [18]. In addition, our study has unexpectedly provided new insights into the analysis of the epidermal BM topography and mechanics [18]. AFM and 3D reconstruction revealed that the epidermal

BM is organized as a checkerboard structure made of alternation of soft and rigid regions. Interestingly, in absence of collagen XIV-A, the checkerboard organization is compromised leading to a more compact structure suggesting that this collagen may act as a molecular organizer of the BM network [18].

6. Zebrafish is a versatile model for collagenopathies

The critical roles of collagens have been clearly illustrated by the wide spectrum of diseases that affect various tissues: osteogenesis imperfecta (OI, bone), Alport syndrome (kidney), Ehlers-Danlos syndrome (skin, joints and vessels), Bethlem myopathy and Ulrich congenital muscular dystrophy (UCMD) (skeletal muscle), epidermolysis bullosa (skin), Knoblock syndrome (retina)... [5,85]. These diseases are caused by more than 1500 mutations identified in genes encoding more than 12 different collagen types. In common diseases, such as fibrotic disorders, tumor stroma, fibrosis, atherosclerosis inflammation, and neurodegenerative disease, the imbalance in the rates of collagen synthesis and breakdown is critical [85]. Mutations in collagen genes often led to intracellular assembly defects that induced ER stress if proteins are not properly degraded [85]. Glycine substitutions are the most common mutations found in collagens. They result in interruptions in the Gly-X-Y sequence that subsequently affect triple helix assembly. Frame exon skipping mutations are also a frequent cause of disease. They result in the synthesis of shorter collagenous domains that, in turn, affected proper assembly of the three α chains [86].

In the last 15 years, zebrafish has become an emerging model for studying human diseases (Table 1). Random mutagenesis has generated many mutants that can model a particular disease and even sometimes have help connecting a particular locus with a human disease [12]. In order to identify new genes involved in skeletal dysplasia, an adult fish skeletal defect screen of mutants generated by ENU mutagenesis was performed using X-ray radiography [87]. Over the 2000 fish examined, only one mutant that was 20% shorter than wildtype fish was identified and named *chihuahua*. Mutation in *col1a1a* gene leading to a replacement of a glycine by an aspartate (G574D) in collagen I $\alpha 1$ chain was shown to be responsible for *chihuahua* phenotype. Collagen I is the most abundant protein in bones and mutations in any of the collagen I genes led to classical OI. This disorder is characterized by bone fragility and growth deficiency, as reported for the *chihuahua* mutant [88,89]. At the cell level, *chihuahua* mutant also recapitulated the human disease since ER stress caused by intracellular retention of misfolded collagen in ER [85] was observed in fin bones. Interestingly, this model

opens new perspectives of investigation for OI treatment as the *chihuahua* phenotype could be improved in adults by treating embryos with the chemical chaperone 4PBA [88].

Zebrafish was also used to model collagen VI-related myopathies. The use of splicing MOs is a method particularly adapted to recapitulate type VI collagen disease as many exon skipping mutations in the 3 main collagen VI genes are present in the human genome of patients [86]. Depending on the exon deleted in the zebrafish *col6a1* gene, Telfer and co-workers were able to induce either a severe or a mild muscle phenotype in agreement with the degree of severity observed in the two targeted myopathies, UCMD and Bethlem myopathy [90]. Zebrafish larvae also showed the typical ultrastructural features of UCMD and Bethlem myopathy such as the abnormal structure of mitochondria and the swollen sarcoplasmic reticulum. The mitochondrial defects have been rescued after treatment with the mitochondrial permeability transition pore (PTP) inhibitor cyclosporin A [90] as in the mouse model [91]. This drug has been successfully tested in clinical trials, but unfortunately it cannot be used for long term treatment due to its immunosuppressive properties [92]. In an attempt to find new treatment, another PTP inhibitor (NIM811) with non immunosuppressive properties was tested in zebrafish embryos and showed even stronger phenotype rescue than cyclosporin A [93]. Nevertheless, all these studies could be only performed during early development due to the transient efficiency of MOs. Using TALEN genome editing strategy, Radev and co-workers were able to create a stable line harboring a deletion in a splice donor site that led to the skipping of *col6a1* exon14 that is the most frequent mutation found in BM patients [48]. They also observed dilated sarcoplasmic reticulum and mitochondria defects and, most importantly, better recapitulated the BM progressive disease. Such a stable line will be useful to dissect the pathophysiological mechanisms of the disease as well as to fully interrogate the function of collagen VI in muscle physiology and regeneration.

In frame exon 41 skipping of *COL6A3* has been suspected to be responsible for isolated dystonia in several human kindreds without muscular defects [94]. In order to validate their hypothesis, the authors used zebrafish to selectively knock down several exons in *col6a3* and found a negative impact on motor neuron pathfinding only after exon 41 ortholog deletion. This observation suggested that the C3-C4 domains encoded by exon 41 are not important for muscle physiology but in peripheral nervous system in humans as well as in zebrafish. This paper nicely underscored the usefulness of zebrafish model in linking gene mutations to human disease. However, some mutants do not recapitulate the corresponding human disease. This is the case of the *COL4A5* gene that was reported to be responsible for the Alport Syndrome that mainly affects kidney function whereas the *dragnet/col4a5* mutant does not manifest renal defects. Further investigation is clearly needed to clarify such discrepancies.

7. Outlook

Beside the fundamental point of view, understanding the function of collagens and their mechanisms of action has significant and broad impact on human health issues such as connective tissue disease treatment, wound repair, tissue regeneration and tissue ageing prevention. Zebrafish has proven to be a relevant animal model to interrogate collagen gene functions. The generation in zebrafish of reporter gene lines and transgenic lines expressing fluorescent fusion collagen will allow to further develop an imaging approach unmatched in its ability to image in real-time the dynamics of collagen synthesis, secretion, extracellular assembly and remodeling. Mutations in 34 human collagen genes result in disease [85]. Apart from a fundamental point of view, zebrafish models for the various human diseases associated with collagen deficiency are also needed to enable a clear understanding of the pathophysiological mechanisms and to provide a suitable preclinical model for drug screening. We have no doubt that this will be rapidly achieved. The generation of zebrafish stable transgenic lines that represented a long and difficult task until a very short time ago is now largely facilitated thanks to the recent advances in genome editing technologies.

Abbreviations

ECM extracellular matrix; AFM atomic force microscopy; SHG second harmonic generation; CRISPR Clustered Regulatory Interspaced Short Palindromic Repeats; BM basement membrane; TEM transmission electronic microscopy; dpf day post-fertilisation; MTJ myotendinous junction; WGD whole genome duplication; ER endoplasmic reticulum; MO morpholino; ENU ethylnitrosourea; TALEN Transcription Activator-Like Effector Nuclease; RGC retinal ganglion cell; UCMD Ullrich congenital muscular dystrophy; OI osteogenesis imperfect; PTP mitochondrial permeability transition pore; hpf hour post-fertilisation

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Table 1. Function of zebrafish collagens .

Collagen genes	Genetic tools	Tissue affected	Expression pattern ^{a,b}	References
<i>col1a1a</i>	MO ENU, <i>chihuahua</i>	Skeleton, fin	ISH, IF	[72,87,88]
<i>col2a1a,b</i>	MO	Fin	ISH, IF	[72]
<i>col4a5</i>	ENU, <i>dragnet</i>	Tectum, cerebellum, motor nerve	ISH	[61,82,63]
<i>col4a6</i>	ENU, <i>shiohane</i>	Cerebellum	ISH	[63]
<i>col6a1</i>	MO TALEN	skeletal muscle	IF	[48,90,93]
<i>col6a3</i>	MO	motor axon		[94]
<i>col6a4a,b</i>	MO	skeletal muscle	ISH, WB	[95]
<i>col8a1</i>	MO ENU, <i>leviathan</i> <i>gulliver</i>	notochord	ISH	[73,74]
<i>col9a1</i>	MO ENU, <i>persistent plexus</i>	regenerated fin vessels	ISH	[79]
<i>col11a1</i>	MO	craniofacial cartilage	ISH	[39]
<i>col12a1a,b</i>	<i>vivo</i> -MO transient CRISPR	regenerated spinal cord	ISH, IF	[34,56]
<i>col14a1a</i>	MO	basement membrane	ISH, IF	[19]
<i>col15a1a</i>	MO	notochord, muscle	ISH, IF	[37]
<i>col15a1b</i>	MO TILLING	motor axon, muscle	ISH, IF	[31,44]
<i>col17a1a,b</i>	MO	Skin, neural system,	ISH, IF	[76]
<i>col18a1</i>	MO	motor axon	ISH	[36,65]
<i>col19a1</i>	MO ENU, <i>stumpy</i>	motor axon	ISH	[64]
<i>col22a1</i>	MO	myotendinous junction	ISH, IF, WB	[32]
<i>col27a1a,b</i>	MO	notochord, head cartilage	ISH	[75]

^aThe reference of the original paper is mentioned when no expression pattern was performed in the functional study.

^b ISH *in situ* hybridization, IF immunofluorescence, WB western blot.

Figures legends

Figure 1: Transmission electron microscopy of zebrafish extracellular matrices. A. Ultrathin section of cartilage at 50 hpf showing the characteristic stacking of chondrocytes (upper panel); zoomed image of the cartilaginous extracellular matrix that surrounds the chondrocytes (lower panel). B Ultrathin section of the skin at 48 hpf, arrow indicates adepidermal granules (left panel) and at 5 dpf (right panel) showing the epidermal basement membrane and the dermis composed of orthogonal layers of collagen fibrils. C. Ultrathin section of vertical myosepta at 5 dpf; (upper panel) Arrows point to finger-like folds, (lower panel) zoomed image of the oblique organization of the collagen fibrils (arrows) in the vertical myosepta. Ch, chondrocyte; ep, epidermis; de, dermis; BM, basement membrane; mu, muscle; myo, vertical myosepta; hpf: hour post-fertilisation; dpf, day post-fertilisation.

Figure 2: A. Zebrafish collagen genes - Multidimensional pie chart showing the distribution of collagen genes that are present in the zebrafish (blue) or human (orange) genomes. Genes that are duplicated in zebrafish genome are in shaded colors. B-C Collagen biosynthesis: from intracellular to extracellular events. (B) Diagrammatic representation of biosynthesis of fibril-forming collagens and key regulators. (C) Collagens are modular proteins made of collagenous domains (COL) linked by short linker regions and flanked by N-terminal and C-terminal non-collagenous domains of various natures. FN3, Fibronectin type III repeat TSPN, Thrombospondin N-terminal like domain; CCR, Cysteine Rich Repeats; vWA, von Willebrand factor A domain; Frz, Frizzled; C-pro, C-propeptide.

Figure 1

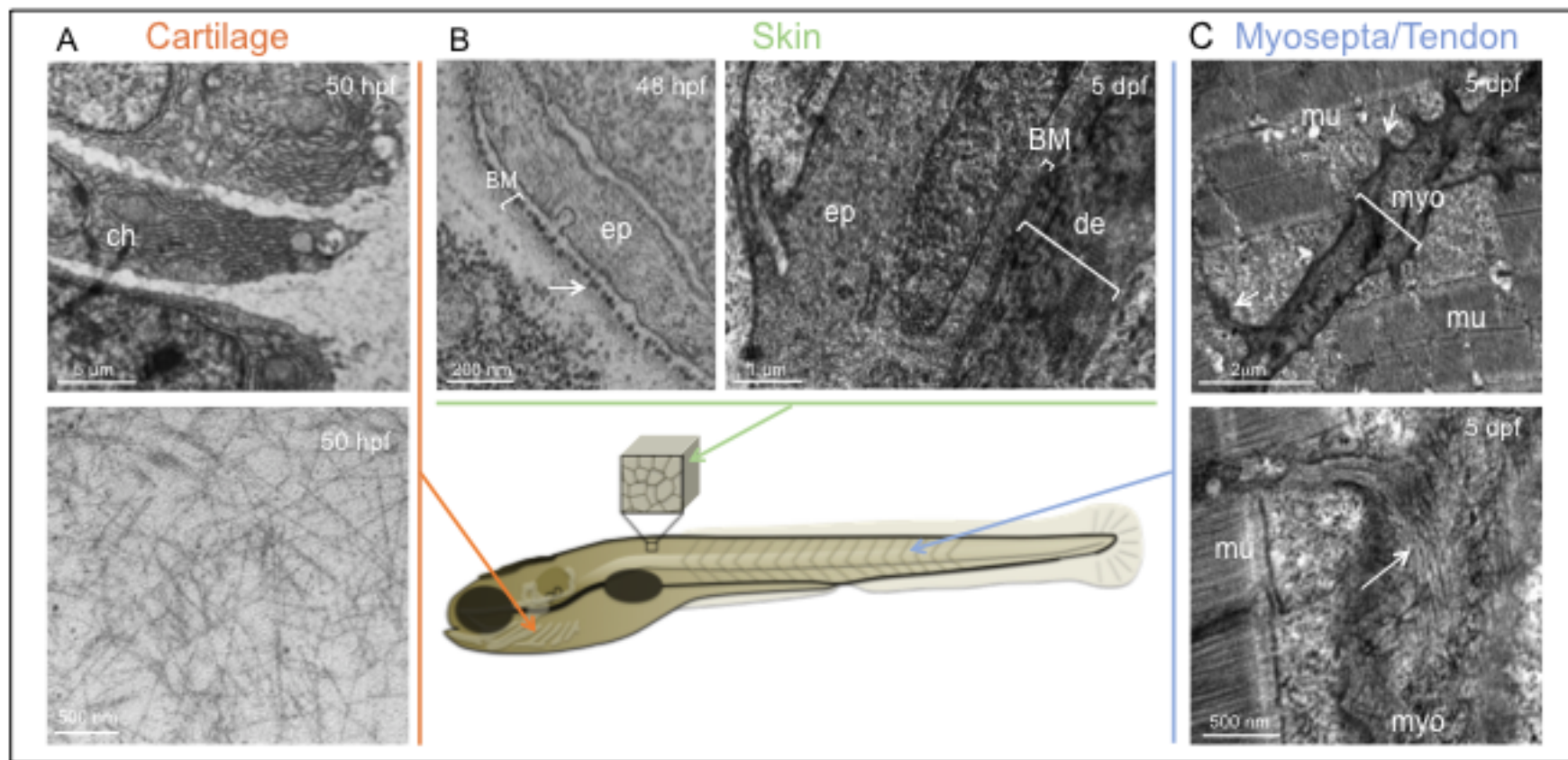


Figure 2

