Collagen XII Contributes to Epicardial and Connective Tissues in the Zebrafish Heart during Ontogenesis and Regeneration

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Abstract
Zebrafish heart regeneration depends on cardiac cell proliferation, epicardium activation and transient reparative tissue deposition. The contribution and the regulation of specific collagen types during the regenerative process, however, remain poorly characterized. Here, we identified that the non-fibrillar type XII collagen, which serves as a matrix-bridging component, is expressed in the epicardium of the zebrafish heart, and is boosted after cryoinjury-induced ventricular damage. During heart regeneration, an intense deposition of Collagen XII covers the outer epicardial cap and the interstitial reparative tissue. Analysis of the activated epicardium and fibroblast markers revealed a heterogeneous cellular origin of Collagen XII. Interestingly, this matrix-bridging collagen co-localized with fibrillar type I collagen and several glycoproteins in the post-injury zone, suggesting its role in tissue cohesion. Using SB431542, a selective inhibitor of the TGF-β receptor, we showed that while the inhibitor treatment did not affect the expression of collagen 12 and collagen 1a2 in the epicardium, it completely suppressed the induction of both genes in the fibrotic tissue. This suggests that distinct mechanisms might regulate collagen expression in the outer heart layer and the inner injury zone. On the basis of this study, we postulate that the TGF-β signaling pathway induces and coordinates formation of a transient collagenous network that comprises fibril-forming Collagen I and fiber-associated Collagen XII, both of which contribute to the reparative matrix of the regenerating zebrafish heart.

Introduction
The zebrafish heart provides a valuable vertebrate model for studying cardiac development, regeneration and disease [1–3]. In adult animals, this vital organ can completely regenerate within 1 to 3 months either after removal of up to 20% of the ventricle, after cardiomyocyte-specific genetic ablation that causes the loss of up to 60% of the myocardium, or after cryoinjury-induced cardiac infarction of 20–25% of the ventricle [4–6]. Among all the standard injury procedures, cryoinjury most resembles to myocardial infarction in the mammalian heart [7–9]. Indeed, freezing/thawing leads to cell death, which triggers inflammatory responses and
fibrosis in the damaged tissue. However, unlike in the injured mammalian heart, the remaining myocardium replenishes the lost tissue, while the fibrotic tissue is progressively resolved, giving space to the new myocardium.

Our laboratory has previously shown that the fibrotic matrix is essential for supporting the structure of the injured ventricular wall, as a reduction of collagen deposition results in a deformation of the ventricular wall [10]. Studies in various model systems established that the extracellular matrix not only provides a passive scaffold but also impacts cellular dynamics by regulating the availability of growth factors and cytokines and by transmitting the communication between adjacent cell types during tissue morphogenesis [11, 12]. One of the characterized ECM components in the zebrafish heart is fibronectin, which is deposited by both epicardial and fibrotic tissue cells [10, 13]. Genetic ablation of fibronectin 1 impairs heart regeneration, although not through the regulation of cardiomyocyte proliferation [13]. In addition, a de-adhesive matrix protein, Tenascin C is deposited at the interface between the leading edge of the regenerating myocardium and the provisional wound tissue [6, 10]. Surprisingly, the contribution of specific collagenous proteins has been poorly characterized in the zebrafish heart.

The ECM organization typically involves interactions between fibril-forming collagens and fibril-associated molecules, such as proteoglycans, glycoproteins and multi-domain proteins, called fibril-associated collagens with interrupted triple helical domains (FACIT) [14, 15]. Collagen XII (Col XII), one of the FACIT proteins, is thought to form flexible bridges between adjacent collagen fibers or between collagen fibers and glycoproteins. The phenotype of col12a1 knockout mice is characterized by muscle weakness and heavy disorganization of the bone matrix, suggesting that Col XII improves absorption of shear stress in the tissue, and consequently protects organs from mechanical distortions [16, 17]. Accordingly, human genetic studies revealed mutations in the COL12A1 gene in patients suffering from myopathy and joint hypermobility [18, 19]. In mammals and chick, Col XII is widely expressed in the embryonic mesenchyme of various tissues, but in the adult stage, it becomes restricted to a few places, such as dermis around hair follicles, cornea, periodontal ligaments and intramuscular connective tissue [16]. In zebrafish, expression of Col XII was identified during embryogenesis at 24 and 72 hours post fertilization in the connective tissue sheaths of various organs and in certain basement membranes [20]. In this study, we addressed the contribution and regulation of this distinct non-fibrillar Col XII in the zebrafish heart in homeostatic and regenerative conditions.

**Materials and Methods**

**Animal procedures**

Wild-type adult zebrafish (AB, Oregon), Tg(wt1a(-6.8kb):GFP), also named Tg(-6.8kbwt1a:GFP) [21], and Tg(CD41:EGFP) [22] between 12–16 months were used in this study. Cryoinjuries were performed as previously described [7, 23]. Briefly, the fish were anaesthetized in 150 mg/L Tricaine (Ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich, 886-86-2) dissolved in water. When the animals stopped swimming and reacting to vibrations, they were transferred onto a moist sponge. First, a small (ca. 2 mm) incision above the heart was performed and then the silvery epithelial layer of the hypodermis was removed to give direct access to the beating ventricle. A cryoprobe, precooled in liquid nitrogen for 3–5 minutes, was then positioned on the surface of the ventricle for 20–25 seconds. After this, the cryoprobe was released by pouring 2–3 mL of system water (25°C) onto the chest, and the fish were transferred to a tank with system water. After surgery, the fish were monitored for 10 min in water, and repeatedly up to 3 hours. Swimming ability was used as criterion of well-being. The animals with signs of stress and pain, such as swirling and convolutions, were euthanized. Animals in experiments were observed daily, including weekends. To sacrifice the animals, the fish were
incubated for 10 min in water containing 300 mg/L Tricaine. We used loss of gill movement and tail-fin-pinched reflex to determine death. For the inhibition of TGF-β signaling, SB431542 (Tocris, 1614) was added to fish water at a concentration of 20 μM. Control animals were kept in water with 0.1% DMSO. The treatment was changed every 3 days. The cantonal veterinary office of Fribourg approved this experimental research on animals.

**Immunofluorescence**

Hearts were collected and fixed in 2% paraformaldehyde, embedded in Tissue-Tek OCT compound (Sakura Finetek Europe B.V.) and cryosectioned to 16 μm thickness. Immunofluorescence staining was performed as previously described [24]. To avoid cross-reactivity of antibodies in the multiple immunofluorescence procedure, sequential staining was performed in this study. Antibodies against Col XIIa were incubated after the immunolabeling with the other antibodies.

The following primary antibodies were used: mouse anti-tropomyosin at 1:100 (DSHB, CH1), rabbit anti-Aldh1a2 at 1:200 (GeneTex, GTX124302), rabbit, anti-αSMA at 1:2000 (GeneTex, GTX100034), mouse anti-Vimentin 40E-C at 1:50 (DSHB, 40E-C), rabbit anti-Tenascin at 1:500 (USBioLogical, T2550-23), rabbit anti-Fibronectin at 1:200 (Sigma-Aldrich, F3648), rabbit anti-p-Smad3 at 1:400 (Abcam, ab52903), mouse anti-embCMHC at 1:50 (N2.261; developed by H.M. Blau, obtained from Developmental Studies Hybridoma Bank), rabbit anti-Col1a1 at 1:200 (GeneTex, GTX124368) and guinea pig anti-ColXIIa1 at 1:1000 (Bader et al, 2009). Secondary antibodies (Jackson Immunoresearch) were used at 1:500 and Phalloidin 390 (ATTO, AD 390–81) was used at 1:500.

**In-situ hybridization**

In-situ hybridization on cryosections was performed as previously described [25]. The following forward (F) and reverse (R) primers (5’ to 3’) were used to synthesize antisense probes: 
- col12a1a (ENSDART00000154728) F: cctgatgtctcccttaccc and R: acctggaccatgtcctctg; 
- col12a1b (ENSDART00000025926) F: gagatctcgtattagatag and R: tcaacgcatagctgtgg; 
- col1a1a (NM_199214.1) F: aatggacctgcttggag and R: ggtctactggacatc; 
- col1a2 (NM_182968.2) F: gagatctcgtattagatag and R: aatggaccaagtctgttc; 
- tgf-β2 (NM_194385) F: acgtccgaagtgccag and R: ctgtccgtatctggagc. The PCR products served as templates to synthesize digoxigenin-labeled RNA antisense probes using the DIG labeling system (Roche).

In-situ hybridization combined with immunofluorescence was performed in a sequential manner. After completion of in-situ hybridization staining, the slides were rinsed for 30 minutes in PBST (PBS, 0.3% Triton X-100) followed by the normal immunofluorescence protocol.

**Histological staining**

A triple staining with Aniline blue, acid Fuchsin and Orange-G (AFOG) was performed as previously described [7]. For the single-dye staining with Aniline blue, cryosections were refixed with 10% neutral buffered formalin solution for 15 min and washed in PBST for 10 min. The slides were transferred into preheated Bouins fixative (Lot. 1536576, Reactorlab) and incubated for 2.5 hours at 56°C and another hour at room temperature. The slides were washed twice for 20 minutes in tap water and then incubated in 1% phosphomolybdic acid for 5 minutes. After rinsing with distilled water, sections were incubated for 4 minutes with aniline blue staining solution (1 g aniline blue diammonium salt, Sigma Aldrich, 415049; 200 ml acidified distilled water) and again washed with distilled water. The sections were dehydrated in 95% ethanol and twice in 100% ethanol for 2 minutes. After passing through three consecutive xylol baths, the slides were mounted with Entellan (107961, Merck Millipore).
**Fig 1. Col XII is expressed in the epicardium of the adult zebrafish heart.**

(A) Aniline blue staining of a ventricle transversal section visualizes collagen (blue). Framed areas encompass parts with the atrio-ventricular valve (A/V-Valve) and ventricular wall (V-Wall). The thickness of the compact myocardial layer is depicted as a bar in this and subsequent panels. Ep, epicardium; CoM, compact myocardium; TrM, trabecular myocardium. N = 5. (B-D) In-situ hybridization of ventricle sections detected by a color reaction (purple). Probe names are to the left. Framed areas encompass the parts that are enlarged in the panels to the right. N > 4. (E) Superposition of a bright-field image with in-situ hybridization using col12a1b probe (purple) and fluorescent immunodetection of muscle protein Tropomyosin, TPM (red). col12a1b is expressed in the epicardium that is located externally from the myocardial border (dashed line). A few col12a1b-expressing cells are Tropomyosin-negative (arrows) and are interspersed within the compact myocardium (the thickness of the compact myocardium is indicated with a
Image analysis and quantification

Fluorescent images were taken using the confocal microscopes Leica TCS SP5 and Leica TCS SP-II. Image analysis was performed using the Fiji ImageJ software and Adobe Photoshop. To quantify the percentage of Col XII in the post injured myocardium, the area positive for Col XII expression was divided by the whole post-injury myocardium area, but excluding the epicardium. N represents a number of hearts. In order to obtain representative data, at least 2 sections of each heart at different time points were imaged and analyzed. Statistics were calculated with the Prism GraphPad software. All results are expressed as the mean ± standard error of the mean (S.E.M.). Bright-field images were taken using a Zeiss Axioplan 2 microscope coupled to an AxioCam Color camera.

Results

Col XII-reactive fibers outline the epicardium and the subepicardium of the ventricle

In the mammalian heart, the collagenous matrix plays an important role in myocardial architecture and function [11, 12]. This subject, however, has received relatively little attention in the fish [26, 27]. In the adult zebrafish heart, histological trichrome staining (Aniline blue, acid Fuchsin, Orange G (AFOG) detected collagen by blue coloration in the non-muscular structures, namely the atrio-ventricular valve and the bulbus arteriosus of the outflow tract (S1A and S1B Fig) [28]. By contrast, little collagen staining was observed in the ventricular wall. To optimize the detection method, we used a single dye, aniline blue, without counterstaining. As expected, collagenous leaflets of the valve displayed strong blue labeling (Fig 1A and 1A’). Remarkably, the outer layer of the ventricular wall, which encompasses the epicardium and the compact myocardium, also revealed the aniline blue reactivity (Fig 1A and 1A”). This finding indicates that collagenous matrix is present along the external surface of the ventricle, which is consistent with the published electron microscopy observations [28, 29].

To determine the type of collagen that contributes to the ECM of the superficial cardiac layer, we performed in-situ hybridization analysis of candidate genes. In the search for an epicardial expression pattern, we identified an ortholog of the mammalian collagen XII α1 (col12a1) gene, namely col12a1b (ZFIN database: ZDB-GENE-120215-116) (Fig 1B). The gene transcripts were detected in the outer sheaths of valve leaflets and the epicardium. The zebrafish genome contains another paralogous gene, called col12a1a (ZFIN database: ZDB-GENE-090728-1), which has a similar structural organization (S1D Fig). However, in-situ hybridization of col12a1a revealed weaker staining in the adult epicardium and the valve, compared to col12a1b (S1C Fig).

To verify whether col12a1b-expressing cells are non-mycocytes, we performed in-situ hybridization in combination with immunofluorescence staining against a muscle protein, Tropomyosin. This analysis demonstrated that col12a1b-positive cells were devoid of Tropomyosin immunoreactivity, suggesting that they were not cardiomyocytes (Fig 1E).
cells formed a continuous fine layer along the external surface of the myocardium and, additionally, were interspersed across and underneath the compact myocardium (Fig 1E'). This distribution pattern and the absence of overlap with the myocyte marker suggest that \textit{col12a1b} could be expressed not only in epicardial cells, but also in subepicardial interstitial cells.

To visualize Col XII protein distribution, we performed double immunofluorescence staining with Col XII and Tropomyosin of transgenic fish \textit{wt1a(-6.8kb):GFP}, which demarcates subepicardial fibroblasts [30]. Consistent with \textit{in-situ} hybridization, Col XII antibodies covered the epicardium and at the compact/trabecular myocardial junction, which contains fibroblasts expressing \textit{wt1a(-6.8kb):GFP} transgenic reporter (Fig 1F and 1F'). Thus, Col XII-reactive fibers are previously uncharacterized components of the epicardial and subepicardial tissues in the zebrafish heart.

\textbf{Col XII and Col I\textalpha} are detected in the non-overlapping compartments of the intact zebrafish heart

In mammalian and avian tissues, immunogold electron microscopy and expression analyses suggested that Col XII is associated with the surface of fibril-forming collagens, such as Col I\textalpha [31, 32]. To determine whether a similar co-distribution occurs in the zebrafish heart, we analyzed two Col I\textalpha genes, \textit{coll1a1} and \textit{coll1a2} (S1D Fig) [33]. \textit{In-situ} hybridization of transversal sections revealed that both collagens were expressed in the valve and in the epicardium (Fig 1C and 1D). However, double immunofluorescence analysis using anti-Col XII and anti-Col I\textalpha antibodies detected their non-overlapping expression patterns. While Col I\textalpha immunoglobulins strongly reacted with the interstitial ECM of the non-muscular bulbus arteriosus and atrio-ventricular valve, Col XII-positive fibers were present only at the outer surface of the heart (Fig 1G–1J”). We observed only a weak dot-like pattern of the Col I\textalpha immunoreactivity on the ventricular surface (Fig 1I’). Such a discrepancy between \textit{in-situ} hybridization and immunoreactivity has been already observed for structural protein complexes, as discussed below. Here, we focused on the expression of matrix-bridging Col XII, which unambiguously labels extracellular fibrils within the outer layer of the zebrafish ventricle.

\textbf{Col XII is associated with the ventricle surface during development}

To understand the developmental dynamics of Col XII in the zebrafish heart, we analyzed the embryonic, larval, juvenile and young adult stages, at 3, 14, 30 and 120 days post fertilization (dpf), respectively. Consistent with a previous study [20], 3-day-old embryos displayed Col XII expression in the connective tissue sheaths, but no labeling was present in the embryonic heart (Fig 2A). At this stage, nevertheless, we observed individual Col XII-positive fibrils invading the surface of the heart from the adjacent pericardial region (Fig 2A”). At 14 dpf, the outer layer of the larval heart was fully encased by Col XII-immunoreactive fibers (Fig 2B). This pattern was maintained in the juvenile and young adult zebrafish (Fig 2C and 2D). Interestingly, the Col XII-labeled structures were not only present on the surface, but also penetrated between
the cardiomyocytes within the compact layer of the myocardium (Fig 2B–2D). This observation suggests that Col XII might be associated with the morphogenesis of the epicardium and subepicardium during normal growth.

Col XII deposition is enhanced at the site of cryoinjury during zebrafish heart regeneration

The epicardium and the underlying cardiac muscle have been shown to interact during zebrafish heart regeneration [8, 34]. To determine whether Col XII is involved in this process, we used the cryoinjury model system to damage approx. 20% of the ventricle [23]. This method is based on the disruption of cell integrity by freezing/thawing of intracellular fluids that leads to cell death. We found that the original epicardial Col XII-positive fibers retained unaltered immunoreactivity along the entire ventricular circumference, including the site of injury, as shown at 0, 6 and 24 hours post-cryoinjury (hpci) (S2 Fig). This is not unexpected, as the muscle cytoskeletal proteins, namely actin filaments and associated Tropomyosin, were also detected at these time points (S2 Fig). Starting from 2 days post-cryoinjury (dpci), however, degradation of F-actin and Tropomyosin became visible, while the pre-existing Col XII fibers remained unchanged (S2 Fig). This finding indicates that certain matrix proteins, which are independent of cellular integrity, have not been destroyed by freezing/thawing, and may provide a scaffold for migration of new cells into the injury site.

The zebrafish heart repairs the cryolesion through the deposition of a transient collagenous scar as a replacement of the dead tissue [7–9]. At the same time, the remaining cardiomyocytes in the vicinity of the injury lose their differentiated character, proliferate and invade the post-infarcted area [35–37]. To assess whether Col XII contributes to the initial reparative and subsequent regenerative processes, we monitored the expression of this protein during 60 days after cryoinjury, until the zebrafish heart regeneration had been completed (Fig 3). During the reparative phase of heart regeneration, at 4 dpci, we found an increase in Col XII deposition in the epicardial region surrounding the damaged myocardium (Fig 3A–3B'). Consistently, in situ hybridization demonstrated that the rapid accumulation of Col XII after injury was associated with the enhanced expression of the \textit{col12a1a} and \textit{col12a1b} (S3 Fig). Thus, Col XII represents a new matrix component of the thickened epicardial cap, which surrounds the damaged ventricular wall [30, 38].

At 7 dpci, Col XII-positive fibers started to accumulate in the interface between the wounded area and remaining cardiomyocytes (Fig 3C–3D'). The amount of Col XII in the center of the post-infarcted tissue increased during the more advanced regenerative phase at 14 dpci (Fig 3E–3F'). During the late regeneration stage, at 28 dpci, when a large portion of the damaged myocardium had been replaced, Col XII-fibers persisted at the junction between the...
new cardiomyocytes and the remnants of fibrotic tissue (Fig 3G–3H’). In the completely regen-
erated hearts, at 60 dpci, the pattern of Col XII immunoreactivity was reminiscent of that in
uninjured hearts (Fig 3I–3J’). The dynamic deposition and resorption of Col XII indicates that
this collagen type is associated with the plasticity of the transient fibrotic tissue of the regener-
ating heart.

The muscle-wound junction is of particular importance because this area corresponds to
the regenerative leading edge [37, 39, 40]. To characterize the distribution of Col XII in relation
to dedifferentiated cardiomyocytes, we used an antibody that detects an embryonic isoform of
cardiac myosin heavy chain (embCMHC), which is re-expressed along the wound margin [37,
41]. Double immunostaining revealed that immature cardiac cells at the post-injury border
were associated with Col XII fibers (Fig 3K and 3K’). The shape of the cells relative to the pat-
tern of collagenous fibers suggests that the latter might contribute to the extracellular scaffold
for migrating cardiomyocytes. Taken together, our data suggests that Col XII contributes to
the dynamic reparative matrix that supports the regenerating myocardium.

Heterogeneous origin of Col XII in the epicardium and fibrotic tissue

Image analysis indicated that the largest area with Col XII immunoreactivity within the post-
cryoinjured myocardium was observed at 14 dpci (Fig 3L). At this time point, the ECM of the
damaged zone consisted of a mixture of collagen and fibrin-like material, which can be visual-
ized using AFOG staining (Fig 4A and 4D). Thus, we selected this phase to characterize the Col
XII expression in relation to participating cells and other matrix components.

To demarcate the activated epicardium, we analyzed Raldh2-expressing cells [34]. Closer
examination of the ventricular surface around the uninjured and injured sites revealed a co-
localization of Raldh2-positive epicardial cells and Col XII fibers (Fig 4B–4C”). Interestingly,
we observed few scattered Raldh2-labeled cells in the post-cryolesion area, suggesting that the
migrating epicardium-derived cells might at least partially contribute to the Col XII deposition
in the fibrotic tissue (Fig 4C”).

To examine the distribution of fibroblast-like cells, we visualized Vimentin and αSMA,
which our laboratory previously described in the context of heart regeneration (Fig 4E–4F”) [7,
10]. High magnification of the uninjured site of the ventricular surface revealed that both cyto-
skeletal markers are expressed in non-overlapping cell populations, namely Vimentin was
detected in the outer-most epicardial layer, whereas αSMA labeled the junctional zone between
the compact and trabecular myocardium (Fig 4F”). We found that both cell types were associ-
ated with Col XII-labeled fibers, suggesting that they might account for the deposition of Col
XII along the ventricular wall. In the post-cryolesion area, Vimentin- and αSMA-positive cells
were present in a scattered manner within the ColXII-positive region (Fig 4F”).

To genetically identify cardiac fibroblasts, we analyzed the cryoinjured hearts of transgene-
fish wt1a(-6.8kb):GFP. We found that the transgene-expressing cells, which were present at
the junction between compact and trabecular myocardium and in the cryoinjured zone, coloca-
lized with Col XII fibrils (Fig 5A). We concluded that Col XII is produced by distinct cell

Fig 4. Collagen XII distribution correlates with the activated epicardium and fibroblast-like cells.
(A-F) Analysis of transversal heart sections at 14 dpci. (A and D) AFOG staining of the sections used for
immunostaining. (B and C) Raldh2 expression (red) demarcates the activated epicardium and endocardium.
(C’) Col XII (green) and Raldh2 are colocalized in the intact epicardium (epicard). (C”) Raldh2-positive cells
invade the post-cryoinjured area that is labeled by Col XII expression. Cardiac muscle is detected by
Tropomyosin antibody staining (blue). N = 5. (E and F) Triple antibody staining against Col XII (green),
intermediate filament Vimentin (blue) and alpha-Smooth Muscle Actin (αSMA; red). (F’ and F”) αSMA- and
Vimentin-positive cells are non-overlapping cell populations in the epicardium and post-cryoinjured area.
Both of them are associated with Col XII-labeled fibrils. N = 6.

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Fig 5. The multi-component matrix of the fibrotic post-cryoinjury tissue is rich in Collagen XII. (A) Heart section of transgenic fish wta(-6.8kb):GFP (red) at 14 dpci. (A') Subepicardial fibroblasts (red) co-localize with Col XII.
populations, such as the epicardium, epicardial-derived cells and cardiac fibroblasts, indicating a heterogenous origin of collagenous matrix in the regenerating zebrafish heart.

**Col XII colocalizes with Col Iα in the post-cryolesion area**

Mammalian and avian studies suggested that one function of Col XII is to regulate organization and mechanical properties of collagen fibril bundles to optimize the mechanical property of the connective tissues in conditions of high pressure [32, 42, 43]. Accordingly, we performed double immunofluorescence analysis between Col XII and other known matrix components of the fibrotic tissue, which is detected by histological staining in the post-cryolesion area (Fig 5B, 5D and 5F and S4 Fig). Unlike in the uninjured hearts, we found that Col XII and Col Iα displayed a nearly overlapping distribution in cryoinjured hearts, suggesting that they are part of the same network (Fig 5C). Interestingly, Col XII also partially co-localized with the tissue remodeling protein Tenascin C and with the adhesive matrix protein Fibronectin (Fig 5E and 5G). Thus, the fibrous tissue that repairs the damaged myocardial wall consists of a combination of various collagenous and non-collagenous matrix components, which might interact to ensure appropriate biomechanical properties of the beating zebrafish heart.

**Regulation of Collagen XII expression by TGF-β signaling in the post-cryolesion zone**

Next, we asked which regulatory mechanisms account for such an intense and specific expression of Col XII in the cryoinjured part. Our laboratory has previously characterized the role of TGF-β signaling in the stimulation of collagenous matrix deposition in the post-cryolesion zone [10]. *tgf-β* genes are expressed in the post-cryoinjured tissue, as exemplified by *tgf-β* at 14 dpci (Fig 6A). To investigate whether the Col XII expression pattern correlates with the TGF-β activity, we analyzed the presence of nuclear p-Smad3, which is an activated signal transducer of this pathway [44]. We found that p-Smad3 immunoreactivity was present in the fibrotic tissue containing Col XII, suggesting a possible link between TGF-β signaling and Col XII deposition (Fig 6B–6C').

To perform a functional study, we used a specific pharmacological inhibitor of the TGF-β type I receptors, SB431542, which has been previously validated in the context of fin and heart regeneration in zebrafish [10, 24]. Treatment with 20 μM SB431542 was sufficient to significantly reduce phosphorylation of Smad3 in the heart, as compared to control hearts treated with 0.1% DMSO, which is the final concentration of the SB431542 solvent (S5 Fig). Remarkably, the exposure to 20 μM SB431542 suppressed Col XII expression in the post-cryoinjury zone without affecting the epicardial expression (Fig 7A–7D').

Considering that matrix proteins are typically stable structures, they do not always reflect the actual transcriptional regulation. To examine the *col12a1b* gene expression, we performed *in-situ* hybridization that was followed by the sequential immunofluorescence assay and AFOG staining (Fig 7E–7I). In control hearts, *col12a1b* transcripts were detected in the cryoinjured part of the myocardium and in the epicardium, which corresponded to the pattern of Col XII protein distribution (Fig 7E–7G). The inhibition of TGF-β signaling resulted in the
suppression of *col12a1b* expression specifically in the cryoinjured part without affecting the epicardial region (Fig 7H–7J). This suggests that the inductive mechanisms for Col XII expression might be differential in the outer and the inner tissue of the injured ventricle. However, we cannot exclude the presence of a residual TGF-β activity in the epicardium, despite the inhibitor treatment.

Interestingly, we observed a similar effect on the deposition of fibrillar Col Iα, which was absent after the inhibition of the TGF-β signaling pathway (Fig 8A–8D). Consistently, *in-situ* hybridization analysis revealed a lack of *colla2* transcription within the fibrotic tissue, although a normal expression was observed at the surrounding epicardium (Fig 8E–8H). Taken together, TGF-β signaling may directly or indirectly trigger both fibrillar Col Iα and non-fibrillar Col XII in the post-cryoinjured tissue, which repairs the damaged myocardial wall during heart regeneration.

**Discussion**

Adult mammalian hearts fail to regenerate and heal by irreversible scarring, which is accompanied by cardiac hypertrophy and dilation. By contrast, zebrafish cardiomyocytes remain competent for dedifferentiation and proliferation during the entire lifespan, while the reparative fibrotic tissue beneficially supports the damaged ventricle, being able to progressively resolve in order to give space for new cardiac muscle. Which collagenous components are unique to this reversible fibrotic and dynamic epicardial matrix that render it so suitable for regenerative processes? In this study, we identified that FACIT-type Col XII, is expressed in the intact epicardial region and becomes abundantly upregulated in the reparative tissue. FACIT collagens play a particular role in the matrix, because they do not form fibers on their own, but they regulate fibril size and spacing, as well as link collagenous fibrils to each other, and to other ECM components [14, 45]. Col XII is thought to modulate the matrix arrangement and its morphogenetic flexibility, especially under biophysical stress [16]. This function is particularly important during organogenesis, and consistently, Col XII deposition is abundant in embryonic tissues of fish and tetrapods [16, 20]. To our knowledge there are no reports of Col XII deposition in either the mammalian epicardium or a fibrotic scar. Thus, the presence of this FACIT protein in the zebrafish analogous tissues might significantly influence the matrix properties that are particularly beneficial for heart regeneration.

Despite a common origin of the vertebrate heart, substantial structural differences are apparent between the adult myocardium of fish and mammals. One of the main anatomical distinctions is the architecture of the heart chambers. In contrast to the human heart, the zebrafish heart is composed mainly of a trabecular myocardium that is surrounded by a thin compact layer [46]. A recent electron microscopy study provided evidence that both myocardial compartments are interconnected by a complex junctional region, which contains a network of flattened fibroblasts and collagen fibrils [29]. Here, we show that this region is demarcated by *wt1a(-6.8kb):GFP* positive fibroblasts and Col XII-positive fibrils. Thus, Col XII might contribute to the flexible matrix that differentially oriented cardiac muscle cells of the compact and trabecular myocardium. Further studies are required to determine the role of Col XII in the junctional regions of the zebrafish heart.
The main focus of this study was on the accumulation of Col XII during heart regeneration. Our immunofluorescence analysis revealed that Col XII tightly colocalized with fibrillar Col Iα and several other glycoproteins at the wound site of the ventricular wall. To obtain mechanistic insights into the regulation of Col XII expression, we investigated the impact of TGF-β signaling on the induction of col12 and col1a after myocardial infarction. We found that the TGF-β signaling pathway is required for the upregulation of both these genes in the inner injury zone of the ventricle. By contrast, the epicardial expression of col12 and col1a were not affected by the TGF-β inhibition (Fig 9). Thus, the regulation of collagens is differential in mesothelial and fibrotic tissues of the regenerating zebrafish heart.

A multi-factorial regulation of Col XII expression has also been shown in mammalian systems. In tenocyte cultures, TGF-β signaling has been shown to upregulate col12 expression [47, 48]. There are also several lines of evidence from in-vivo and in-vitro studies that tensile force stimulates Col XII deposition [16]. It is likely that the mechanisms controlling the expression level of the FACIT protein involve a combination of tissue-specific transcriptional regulators, paracrine signals and biomechanical cellular sensors. Further biochemical and transgenic studies of the col12 promoter will be required to dissect the variety of regulatory responsive elements. The elucidation of the mechanisms regulating the expression of FACIT proteins in specific tissues is essential to understand matrix biology during organ development and regeneration.

Association between fibrillar Col Iα and non-fibrillar Col XII

Our in-situ hybridization analysis revealed that two homologous FACIT collagens, col12a1a and col12a1b, and the fibril-forming collagens, col1a1a and col1a2, are co-expressed in the tissue sheath covering the intact zebrafish ventricle. Despite a partially overlapping transcription pattern of both genes on the heart surface, antibody staining against Col Iα and Col XII failed to reveal a co-localization of both proteins. Furthermore, the immunoreactivity appeared to be nearly exclusive: Col XII labeling was restricted to the superficial layers of all heart structures, while Col Iα staining filled the interstitial matrix of non-muscular structures. A differential Col Iα and Col XII protein localization was also reported during atrio-ventricular valve remodeling in mice [43]. The discrepancy between in-situ hybridization and immunofluorescence staining is not unusual for matrix components, and can be attributed to differences in transcript stability and protein turnover, as well as to posttranslational modifications, multimerization and agglomeration with other partner molecules, which might interfere with the exposition of immunoreactive epitopes. In amniotic vertebrates, immunogold and immunofluorescence labeling provided evidence that Col XII tightly associates with collagenous fibril surfaces and with basement membranes [20, 31, 32, 49, 50]. Thus, it is possible that Col XII proteins that are bound to the surface of Col Iα-fibrils could hinder the accessibility of certain Col Iα epitopes.
for immunoreactivity. Further studies are required to investigate interactions between zebrafish fibril-forming and fibril-associated collagens.
Col XII contribution to the provisional scaffold of the regenerating heart

Col XII deposition is dynamically regulated in the post-infarcted tissue. Importantly, the cryoinjury procedure did not destroy the original collagenous layer of the epicardium. This is
an expected finding, as membrane-independent structures are more resistant to freezing/thawing than cells. After death of cardiac cells, the persisting ECM could serve as a ready fibrous skeleton for the establishment of transient connective tissue during the reparative phase. Indeed, previous characterization of the post-cryoinjury tissue revealed a trabecular organization of the fibrotic scar tissue that emerges after resorption of fibrin and dead cells [6–9]. Here, we identified that Col XII accumulates in the post-injured area with the most abundant appearance at 14 dpci. Importantly, Col XII fibers closely associated with cardiomyocytes of the regenerative leading edge. Thus, Col XII could be involved in bridging the provisional fibrotic tissue to the regenerating edge of the myocardium. This could be achieved indirectly, through binding to other adhesion and de-adhesion matrix proteins, such as Fibronectin and Tenascin C, both of which partially co-localize with Col XII. This finding is consistent with studies in other species, in which Col XII was found to bind collagenous fibrils and other matrix components, namely Decorin, Tenascin X, Fibromodulin and Cartilage oligomeric matrix protein COMP [32, 50–53]. Thus, in the zebrafish heart, as in mammalian tissues, Col XII might interact with various matrix components to generate unique biomechanical properties in the post-cryolesion tissue. Further functional studies are required to address the function of this FACIT protein during heart regeneration.

Supporting Information

S1 Fig. The adult zebrafish heart and its collagenous components. (TIF)

S2 Fig. Cryoinjury does not damage the pre-existing Col XII protein in the epicardium. (TIF)

S3 Fig. col12a1a-expressing cells accumulate at the site of injury at the onset of heart regeneration. (TIF)

S4 Fig. Complete ventricle sections that were used for analysis of fibrotic tissue at 14 dpci in Fig 5. (TIF)

S5 Fig. Treatment with 20 μM SB431542 reduces the activity of the TGF-β signaling pathway in injured hearts. (TIF)

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