Cardiac Myocyte Diversity and a Fibroblast Network in the Junctional Region of the Zebrafish Heart Revealed by Transmission and Serial Block-Face Scanning Electron Microscopy

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Abstract

The zebrafish has emerged as an important model of heart development and regeneration. While the structural characteristics of the developing and adult zebrafish ventricle have been previously studied, little attention has been paid to the nature of the interface between the compact and spongy myocardium. Here we describe how these two distinct layers are structurally and functionally integrated. We demonstrate by transmission electron microscopy that this interface is complex and composed primarily of a junctional region occupied by collagen, as well as a population of fibroblasts that form a highly complex network. We also describe a continuum of uniquely flattened transitional cardiac myocytes that form a circumferential plate upon which the radially-oriented luminal trabeculae are anchored. In addition, we have uncovered within the transitional ring a subpopulation of markedly electron dense cardiac myocytes. At discrete intervals the transitional cardiac myocytes form contact bridges across the junctional space that are stabilized through localized desmosomes and fascia adherentes junctions with adjacent compact cardiac myocytes. Finally using serial block-face scanning electron microscopy, segmentation and volume reconstruction, we confirm the three-dimensional nature of the junctional region as well as the presence of the sheet-like fibroblast network. These ultrastructural studies demonstrate the previously unrecognized complexity with which the compact and spongy layers are structurally integrated, and provide a new basis for understanding development and regeneration in the zebrafish heart.

Citation: Lafontant PJ, Behzad AR, Brown E, Landry P, Hu N, et al. (2013) Cardiac Myocyte Diversity and a Fibroblast Network in the Junctional Region of the Zebrafish Heart Revealed by Transmission and Serial Block-Face Scanning Electron Microscopy. PLoS ONE 8(8): e72388. doi:10.1371/journal.pone.0072388

Editor: Leonard Eisenberg, New York Medical College, United States of America

Received March 1, 2013; Accepted July 9, 2013; Published August 23, 2013

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Funding: PJL is supported by the Fisher and Faculty Development Funds at DePauw University. A. Burns is supported National Institutes of Health/National Eye Institute grants EY017120 and P30EY007551. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The zebrafish heart consists of a spongy ventricular myocardium made of trabecular bundles projecting radially into the ventricular lumen, and of an outer compact heart layer that encases the spongy trabeculae. The proportion of spongy to compact heart varies significantly between fish and appears to strongly correlate to each species particular ecological physiology [1,2,3,4]. The hearts of fish with relatively low activity consist primarily or exclusively of a spong myocardium with a rudimentary compact myocardium fed by deoxygenated luminal blood flow (type-1 heart). More active fish display a thicker compact myocardium invested with vessels carrying oxygenated blood (type-2 heart). The two myocardial segments have been historically and empirically considered distinct anatomical structures that form a functional unit supporting the physiological demands of fish. Because of its hypothesized importance in fish ventricular function, the characteristics of the spongy-compact interface (SCI) between the two ventricular layers have been an area of considerable interest and controversy.

The zebrafish has emerged as an important model of heart development and regeneration. The adult zebrafish heart is a type-2 heart with a compact layer perfused by capillaries. An extensive number of studies have provided insights into the molecular mechanisms underlying the development of the zebrafish ventricle [5,6,7] and its spong trabeculae [8,9]. Other studies have explored the molecular mechanisms that orchestrate the zebrafish's remarkable ability to regenerate [10,11]. More recently, the emergence of the mature zebrafish ventricle has been demonstrated by genetic approach to be driven by clonally-dependent cardiac myocytes, illustrating diversity within the ventricular myocyte population [12]. A number of studies have also provided insight into the ultrastructural characteristics of the developing and adult zebrafish heart [13,14,15]. However to date the nature of SCI in the zebrafish heart has received little attention.

Studies in a variety of fish using different approaches have resulted in markedly diverse understandings and models of this
interface region. Early studies in the albacore tuna suggested that the two myocardial layers were attached by a connective tissue layer [16,17]. Transmission electron microscopy studies in four different fish species, not including the tuna, provided the first detailed evidence not only for connective tissue at the interface of the two myocardial layers [18,19], but of the presence of fibroblasts and a distinct set of flattened transitional cardiac myocytes occupying this complex junctional region (JR). On the other hand, in a more recent study of the sockeye salmon and rainbow trout, similar structures in the interface region were not observed [20]. Whether these studies underscore the structural diversity of the JR in fish, or reflect differences in study methodologies is not clear. The different findings in the salmon and trout heart may be indicative of the intra- and inter-species diversity in the architectural arrangements of the two layers.

The purpose of our study was to ascertain the cellular and ultrastructural nature of the interface region between compact and spongy heart of adult zebrafish using light and transmission electron microscopy (TEM). Here, we provide evidence of a complex and previously unrecognized JR containing a network of fibroblasts, a subpopulation of phenotypically transitional cardiac myocytes at the base of the spongy myocardium, and describe the spatial distribution of adherens junctions linking the spongy and compact layers. The three-dimensional structure of the JR was confirmed by serial block-face scanning electron microscopy (SBF-SEM) and computerized segmentation. To our knowledge, this is the first detailed description of the JR of the zebrafish ventricle, as well as the first ultrastructural 3D reconstruction of this region using SBF-SEM.

Materials and Methods

Animals

Zebrafish were obtained from Aquatic Research Organisms (Hampton, NH), and maintained in 10-gallon tanks, with 15 to 20 fish per tank, at 28 degrees Celsius on a 14/10 hour day/night cycle. Experimental procedures were approved by the Committee for the Care and Use of Laboratory Animals at DePauw University.

Transmission Electron Microscopy

For studies of the myocardium in plastic sections and by transmission electron microscopy tissue samples were processed as described previously [21]. Zebrafish were euthanized using 0.2% MS Tricaine. Once all body and opercula movements had ceased, the heart was removed by grasping and pulling the aorta proximal to the bulbus arteriosus. The hearts were fixed in 100 mM sodium cacodylate buffer containing 2.5% glutaraldehyde overnight at 4 degrees C. The hearts were rinsed the next day in 100 mM sodium cacodylate. The hearts were post-fixed and heavy metal-contrasted with potassium ferrocyanide, osmium tetroxide, thiocarbohydra- zide, uranyl acetate and lead aspartate. Next, the hearts were dehydrated in ethanol followed by acetone and embedded in Durcupan resin. Approximately 2×2 mm, 100 nm thick sections were obtained, mounted on slot grids and surveyed by TEM. Blocks with optimal tissue orientation and easily identifiable landmarks in the region of interest were selected for further trimming and imaging. Serial block-face sectioning (at 50 or 100 nm imaging intervals) and imaging were performed using a Gatan 3View system (Gatan, Pleasanton, CA) mounted in an FEI Quanta FEG 200 SEM (FEI Company, Hillsboro, OR). The SEM was operated at an accelerating voltage of 2.0 kV and 30 cascals pressure in low vacuum mode. A solid state backscatter detector was used to acquire serial section images (stacks of 800, 632, and 500 sections) from three regions of interest. Three-dimensional segmentation and reconstruction of fibroblasts and cardiac myocytes was performed using Amira 5.2 software.

Results

I. Electron Translucent and Electron Dense Cardiac Myocytes are Organized into a Transitional Cell Ring

To determine the nature of the interface region between the compact and spongy heart of the adult zebrafish, we first studied the ventricle under light microscopy. Using two-micron thick toluidine blue-stained plastic embedded sections, we observed the expected organization: a relatively thin layer of the compact heart (approximately 10–20 um thick) framing the trabeculae which projected radially into the ventricular lumen (Fig. 1A). B. However closer inspection revealed an unexpected number of darkly-stained elongated structures, at irregular intervals at the interface between
Figure 1. Low magnification of the adult zebrafish heart by light and TEM. (A), light microscopy view of the apical region of the zebrafish ventricle showing a thin layer compact heart (CH) with trabeculae (Tr) projecting within the lumen (Lu). (B), higher magnification of the apex showing darkly stained linear structures (arrows) close to the spongy-compact interface of the ventricular myocardium. (C), low magnification TEM of compact ventricular myocardium constituted of four to five overlapping cardiac myocytes (CM) layers from epicardium (Epi) to lumen (Lu), with electron dense cells (arrow) within a complex junctional region (JR, brackets) at the interface of compact and spongy myocardium. (D), higher magnification show mitochondria (Mt) filled section of electron dense transitional cardiac myocyte (EDTCM). EDTCMs are apposed to trabecular cardiac myocytes and endocardial cells (Endo) on the luminal side. EDTCMs are separated from the compact cardiac myocytes (CCM) by a junctional space, an interstitial space within the JR. (E), low magnification TEM of the JR (brackets) showing flattened electron translucent transitional cardiac myocytes (ETTCM) in contact with trabecular and in proximity to endocardium (Endo) on the luminal side. (F), higher magnification of a flattened cells show presence of actin and myosin filaments, apposition to trabecular and endocardial cells as well as separation from the compact myocardium by the junctional space.

doi:10.1371/journal.pone.0072388.g001
Figure 2. Electron dense and electron translucent transitional cardiac myocytes interactions. (A), example of end-to-end interaction between EDTCM and electron translucent cardiac myocytes (ETTCM) forming a continuum at the spongy-compact interface. (B), higher magnification of contact area with complex adhesion junctions including desmosomes between the two cells. (C), example of interaction between mitochondria filled and actin-myosin filament (AMF) containing EDTCM cells and ETTCM via adhesion junctions (arrows). (D), higher magnification of EDTCM and ETTCM showing gap junctions (arrows), and (E), desmosomes. (F), AMF in a transitional cell. (G), higher magnification (inset in F, rotated) showing the hexagonal array of myosin in cardiac myocytes. (H) sarcomere and z-band in the same cell. AMF, actin-myosin filament; CCM, compact cardiac myocytes; CM, cardiac myocytes; EDTCM, electron dense transitional cardiac myocytes; ETTCM, electron translucent transitional cardiac myocytes; Endo, endocardium; Epi, IF, intermediate filaments; Lu, lumen; Mt, mitochondrion; Tr, trabeculae.

doi:10.1371/journal.pone.0072388.g002
the compact and spongy segments of the ventricle (Fig. 1. B). To elucidate the nature of these darkly-stained structures, we studied their characteristics and precise location within the zebrafish junctional region (JR) using transmission electron microscopy (TEM). Low magnification TEM reveals a compact myocardium of 4–5 layers of cardiac myocytes (CM) (Fig. 1. C). The compact and spongy interface is revealed as a complex JR containing a set of flattened CM as compared to the compact or trabecular CM. Ultrastructurally, the darkly stained structures appear as electron dense CM running along the adjacent base of the trabeculae (Fig. 1. D). We have called these cells: "dark cells" or electron dense transitional cardiac myocytes (EDTCM). Except for their high electron density, these cells display similar flattened and rectangular phenotypes as the transitional CM described previously [18]. In adjacent regions, the ventricle displays a similar arrangement but with electron translucent transitional cardiac myocytes (ETTCM). Except for their high electron density, these cells display similar flattened and rectangular phenotypes as the transitional CM described previously [18]. In adjacent regions, the ventricle displays a similar arrangement but with electron translucent transitional cardiac myocytes (ETTCM). Except for their high electron density, these cells display similar flattened and rectangular phenotypes as the transitional CM described previously [18].

II. Transitional Cell Ring and Trabeculae Contacts

The EDTCM were in direct contact with the ETTCM (Fig. 2. A). Within the plane of observation, shorter segments of EDTCM alternated with longer segments of ETTCM, and together these two cell populations appear to form a continuous ring of transitional CM within the JR. The transitional CM could be seen linked at their tapered ends through complex adherent junctions resembling fascia adherentes (Fig. 2. B). Additionally, the transitional CM were in direct contact or in close apposition to the base of CM sheets of projecting spongy trabeculae (Fig. 2. A, C), and in the intertrabecular lacunar spaces they were bounded in their luminal aspect by the endocardium (Fig. 2. G). However in contrast to their connections to trabeculae, no contacts were observed between transitional CM of the transitional ring and the adjacent endocardial cells. In some instances, EDTCM and ETTCM minimally overlapped at their tapered ends (Fig. 2 C), with connections occurring through discrete gap junctions (Fig. 2. D) and desmosomes (Fig. 2. E). Detailed observations of the cytoplasm of these cells show the presence of actin-myosin filament bundles oriented in different planes (Fig. 2. F). The presence of caveolae and the hexagonal array of equidistant thick myosin filaments characteristic of cardiac muscle cells can be appreciated (Fig. 2. G). Z-bands bordering sarcomeres oriented perpendicularly or obliquely in the plane of observation (Fig. 2. H) can also be seen in these cells, further confirming their cardiac myocyte phenotype. Finally the cardiac nature of these narrow width transitional cells was also confirmed using immunostaining with anti-myosin heavy chain-1 (MYH1) and anti-myocyte enhancer factor-2 (MEF) antibodies to specifically identify cardiac myocytes (Fig. 3).

We further investigated the nature of the arrangement between the transitional CM ring and the CM forming the trabeculae. Transitional CM can be found linked to trabecular myocytes through a variety of adherens junctions. These junctions included fascia adherentes (Fig. 4. A, B) that link actin-myosin filament bundles of adjacent cells. Various amounts of amorphous material were observed along transitional CM and trabecular CM areas of apposition (Fig. 4 C, D). However discrete electron dense areas...
with a sub-domain containing desmosomes were also observed (Fig. 4 E, F).

III. Fibroblast Network and Collagen in the Junctional Region

The transitional ring of myocytes on its abluminal side is bounded by a narrow (0.5–1 um) connective tissue space. This space separates the EDTCM and ETTCM from the adjacent CM of the compact layer, creating a junctional space within the JR (Fig. 5 A). The junctional space appears to create an extensive structural discontinuity between the spongy and the compact myocardium. It contains numerous cells arranged longitudinally and running parallel to the transitional CM ring. The absence of basement membranes, their elongated shape, and their location within this interstitial space suggest they are fibroblasts (Fig. 5. B, C). These fibroblasts contained ovoid nuclei with scant perinuclear cytoplasm that appeared mostly devoid of organelles, except for occasional vesicles and polyribosomes. In the perinuclear region of the fibroblasts, the junctional space widens to 2–3 microns. The cytoplasm of these fibroblasts is stretched bi-directionally into long thin filopodia-like processes. These fibroblasts mostly form a single layer in the junctional space with little to minimal overlap of their cytoplasm. The fibroblasts and their cytoplasmic processes fit tightly within the junctional space, however a measurable space was always observed between the fibroblast and the adjacent transitional and the compact ventricular CM.

The fibroblasts were also found in close proximity or in direct contact with parallel or perpendicularly-oriented collagen fibers (Fig. 5. B, C). At irregular intervals, terminal profiles displaying long cytoplasmic extensions were in close proximity with the membranes of long cytoplasmic processes originating from another fibroblast (Fig. 5. D). In some instances, cytoplasmic terminal ends of fibroblasts overlapped, or could be seen in contact with the same bundle of collagen. Occasionally fibroblasts terminal ends were found in close contact; however we could not unequivocally confirm specialized adhesion junctions between interacting terminal ends. Occasionally close approximation between fibroblast filipodia and CM could be seen; however no direct contact between fibroblasts and CM and no specialized myocytes-fibroblasts junctions were observed. Close observation of many of the transitional CM however revealed the presence of caveolae within the membranes orientated toward the fibroblasts within the junctional space (Fig. 5. C). Across the junctional space, the compact CM membranes were observed to have numerous caveolae facing toward the fibroblasts (Fig. 5. D, E). More examples of caveolae could be seen on the membranes of CM facing the junctional space at a distance from the fibroblast processes (Figure S1). While most fibroblast nuclei and cytoplasmic processes resided within the JR, some cytoplasmic projections could be seen invested in the compact myocardium adjacent to small vessels, suggesting that fibroblasts may not be locally restricted to the junctional space.

Figure 4. Transitional cardiac myocytes ring and trabecular cardiac myocytes contacts. (A), trabecular cardiac myocytes (Tr) in direct contact with two CM of the transitional ring. Trabeculae and transitional CMs are quasi-perpendicularly oriented. Contacts are mediated by electron dense adhesion junctions (arrows). (B), higher magnification of (A), with fascia adherentes junction (thick arrow) and desmosome (thin arrow) between the Tr and ETTCM. Well organized actin and myosin filaments are oriented at an approximately 145 degree angle. (C, D), region of approximation of trabecular CM and transitional CM with amorphous material (AM) interposed between the two cell membranes. Note a number of caveolae in the trabecular CM membrane (C,*) on their abluminal side, and in the transitional CM membrane (D,*) on the luminal side. (E), another example of desmosome linking a trabecular to a transitional CM. (F), higher magnification of (E). doi:10.1371/journal.pone.0072388.g004
Figure 5. Fibroblasts network in the junctional region of the zebrafish ventricle. (A), TEM of a fibroblast (Fb) cytoplasm and its thin extended process/filopodium (FP) 100 to 200 nm thick spanning the junctional space (JS) in between the transitional CM ring and the adjacent compact cardiac myocyte (CCM). Note vesicle (*) in an enlarged region (~300 nm) of the fibroblasts filopodium (FP). (B), fibroblast profile with nucleus (Nu) and its cytoplasm extending into a long thin process, and collagen fiber (CF) bundles running perpendicular to the section’s plane. (C), another fibroblast and its nucleus (Nu) and with cytoplasm extending into a long process, and collagen fiber (CF) bundles running parallel to the section’s plane. (D), fibroblast filopodial termini in close approximation. Electron dense region in the close approximation area suggestive of specialized adhesion junction (arrow). Note the presence of caveolae (*) on the membrane of the CCM facing the fibroblast in the junctional space.

doi:10.1371/journal.pone.0072388.g005
Figure 6. Transitional CM ring and compact CM contacts. (A), ETTCM and Compact CM direct contacts mediated by adhesion junctions (arrows). Note the JS with fibroblast (Fb) on the right and narrow JS of the left of the contact region. (B), higher magnification of inset in (A) showing adhesion junctions associated with fascia adherentes (thick arrow), and also with desmosomes (thin arrow). Note the abundance of caveolae (*) in the transitional CM membrane in the contact region. (C), another narrow region of contact (arrow) between a transitional and a compact cardiac myocyte...
Discussion

This paper documents a previously unrecognized connective tissue space located at the interface of the spongy and compact myocardial of the adult zebrafish heart. It extends our understanding of the cellular and ultrastructural nature of the ventricular JR of an important research model. The existence of a connective tissue layer in the junctional region of type-2 fish hearts has remained controversial. A continuous fibrous membrane interposed between the compact and spongy layers has been reported in the hearts of tuna [16,17], Atlantic salmon and rainbow trout [23]. These observations led to the hypothesis of the connective tissue as an important adhesive substrate between the two myocardial layers. In these early studies, detailed composition of the connective tissue layer was not described. Midttun using TEM demonstrated in four type-2 fish hearts species the presence of fibroblasts and collagen fibers within a 6 to 7 μm wide space existing in the junctional region [18]. By contrast in a study by Pieperhoff et al., using light and scanning electron microscopy, a connective tissue layer at the interface of the two myocardial layers in the sockeye salmon and trout was not observed [20], beyond small focal regions of extracellular matrix enrichment. Unlike the previous studies, these authors challenged the hypothesis of a connective tissue layer separating the two myocardial layers. In both Midttun’s and our studies, the junctional space and its content were not readily detected by light microscopy; however, they could be observed by transmission electron microscopy, suggesting that ultrastructural thin sections imaging may be necessary to describe the structure of the JR of fish ventricles.

Our ultrastructural observations reveal a junctional space measuring for the most part less than one micrometer wide, populated by a single layer of fibroblasts organized into a network, and framed by CM belonging the compact myocardium distally and the spongy myocardium proximally. Fibroblasts are an important component of the mammalian heart and play an essential role in heart structure and physiology. They are a source of extracellular matrix molecules and growth factors, and they serve as sensors for mechanical signals [24,25,26,27,28]. While it has been suggested in early studies that collagen accumulation modulates regeneration in the zebrafish ventricle [10], fibroblasts in the fish heart have received little attention. More recently studies of cardiac injury in the zebrafish [29,30,31] and giant danio [21] demonstrated accumulation and resorption of collagen during fish ventricular regeneration, suggesting an important role for fibroblasts. Indeed the presence of activated fibroblasts was observed during ventricular remodeling in the zebrafish [31] and giant danio heart [21]. The presence of fibroblasts has previously been noted in the sub-epicardial space of fish species, including the zebrafish [14]. In the present study, the fibroblasts’ arrangement suggests a highly organized three-dimensional network exists at the interface of the compact and spongy ventricle. The function of this fibroblast network is presently unclear.

Fibroblast networks have been documented in a variety of organs and species, with direct fibroblast-fibroblast contacts being observed in mammalian corneas [32,33]. Fibroblast interactions spectroscopy of a network have also been reported in the mammalian heart [34]. Fibroblasts have also been implicated in physiological mammalian cardiac muscle growth [27] as well as in injury responses [35,36,37,38]. Our study by TEM and the reconstruction following segmentation of the SBF-SEM micrographs demonstrate that fibroblasts form a three-dimensional network in the JR of the adult zebrafish heart. In addition to fibroblast-
fibroblast interactions, direct CM and fibroblast contacts have also been reported in normal and diseased hearts and in culture [39,40,41]. In this study however, we did not uncover direct CM–fibroblasts contacts in the uninjured zebrafish heart. When fibroblast processes were seen in close proximity and in apparent apposition to myocytes, the intervening myocyte basement membrane was always present. Yet we cannot rule out that these contacts exist and may occur at a frequency too low to be easily
detected. Nevertheless, we found a remarkable number of caveolae in the sarcolemma of CM bordering the junctional space and facing adjacent fibroblasts. This suggests possible mechanisms by which paracrine signaling may take place between adjacent CM and fibroblasts occupying the junctional space.

The present study also establishes that the junctional space occupies the majority of the overall SCI, and creates an extensive volume of non-contact between the two myocardial layers. Given the sparsity of the collagen fibers observed in the junctional space, we question whether in the zebrafish, the connective tissue would provide sufficient support to maintain the structural integrity of the two layers. We speculate that the connective tissue compartment may not be the primary mechanism by which adhesion between the two myocardia is maintained. Indeed, the junctional space is interrupted by contact bridges between the two layers at discrete intervals through membrane apposition of adjacent CM, and that desmosomes and fascia adherentes are frequently found within the contact regions between transitional zebrafish CM and compact CM. These findings are consistent with the demonstration of highly enriched adhesion junction molecules between the two myocardial layers observed in salmon and trout [20].

An important finding is the transitional ring of cardiac myocytes similar to that previously described by Midtunn, that underscores the phenotypic diversity of cardiac myocytes present in the zebrafish heart, suggesting specific structure-function relationship. The notion of cardiac myocyte diversity is apparent in recent work demonstrating the clonal contribution of cardiac myocytes to the emergence of the zebrafish heart [12]. In that study two populations of peripheral cardiac myocytes were described by genetic labeling and spatio-temporal characteristics: a primordial and a cortical set. The localization of the primordial layer suggests it occupies a space similar to the transitional ring population described in the present study. Consequently, it would be interesting to determine whether these two populations spatially interact or are one and the same. Another intriguing finding is the discovery of a subset of “dark” electron dense myocytes within the transitional CM ring that supports the trabeculae. To our knowledge, these electron dense cardiac myocytes have not been reported in fish or mammalian hearts. A set of dark cells was observed in the ventricle of icefish; however, they were believed to be non-contractile in nature [42]. Observations of the zebrafish electron dense cells at high magnification show clear evidence of myofibrils. In addition these cells form a continuum with adjacent electron translucent CM as well as other electron dense CM in the transitional ring and myocytes of adjacent trabeculae via adhesive junctions. These observations raise the intriguing possibility of further phenotypic specialization within the transitional CM population bordering the JS. We speculate this particular phenotypic change may be regulated by regional stress distribution within the myocardium or may reflect their metabolic state. The understanding of the functional and the molecular differences between the dark and light cells within the transitional ring warrants further study.

Conclusions

In conclusion, this study sheds light on the phenotypic diversity of CM at the JR of the adult zebrafish heart. It documents the existence of a fibroblast network that contributes to the architectural complexity of the region (Fig. 8). We suggest the transitional CM form the main anchoring substrate for the trabecular and compact heart. It is possible that in type-2 fish hearts, both the connective tissue layer and the adherens junctions account for the integrity of the spongy-compact interface. It is also possible that in fish, the ratio of connective tissue and direct CM adhesive contact, as well as the distribution of adherens junctions might be dependent on the species, their age and ecological...
physiology. In the end it is intriguing to consider how the articulation of the compact and spongy interface progresses during development and how the re-articulation occurs during regeneration of the zebrafish heart.

Supporting Information

Figure S1  Transient myocyte abluminal caveolae. (A), Junctional space JS with a fibroblast process FP. (B), Higher magnification of inset in (A) showing caveolae (*) on transitional CM facing the JS.

(TIF)

Movie S1 Architecture of the junctional region of zebrafish heart. The sheet-like nature of fibroblasts can be appreciated.

(MP4)

Movie S2  Segmented fibroblasts and transitional CM.

(MP4)

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