Temporal cohesion of the structural, functional and molecular characteristics of the developing zebrafish heart

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Authors: Gianfranco Matrone, Kathryn S. Wilson, John J. Mullins, Carl S. Tucker, Martin A. Denvir

Running title: characterisation of zebrafish heart development

Affiliations:

University of Edinburgh/British Heart Foundation Centre for Cardiovascular Science, The Queen's Medical Research Institute, The University of Edinburgh, Edinburgh, EH16 4TJ

Author for correspondence:

Dr MA Denvir, University of Edinburgh/BHF Centre for Cardiovascular Science, The Queen's Medical Research Institute, The University of Edinburgh, Edinburgh, EH16 4TJ.

Tel. 0044 131 242 9236, Email: martin.denvir@ed.ac.uk

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Statement

Authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation. Authors declare to have no conflicts of interests.

Key words: zebrafish, heart, development

ABSTRACT

Heart formation is a complex, dynamic and highly coordinated process of molecular, morphogenetic and functional factors with each interacting and contributing to formation of the mature organ. Cardiac abnormalities in early life can be lethal in mammals but not in the zebrafish embryo which has been widely used to study the developing heart. However, studies have focused mainly on structural and molecular changes occurring during the first 48 hours post-fertilisation (hpf), whereas the mid-to-late developmental phase, where many heart defects occur in mammals, has been less well studied. In addition, little is known about functional changes during development and how these relate to architectural, cellular and molecular aspects of development. To address this we have carefully characterised cardiac structure, function, cardiomyocyte proliferation and cardiac-specific gene expression between 48 and 120 hpf in the zebrafish transgenic cmlc2:eGFP line. We show that the zebrafish heart increases in volume and changes shape significantly between 48 and 72 hpf accompanied by a 40% increase in cardiomyocyte number. Between 96 and 120, while external heart expansion slows, there is rapid formation of a mature and extensive trabecular network within the ventricle chamber. While ejection fraction does not change during the course of development other determinants of contractile function increase significantly between 72 and 96 hpf leading to an increase in cardinal vein blood flow. This study has revealed a number of novel aspects of cardiac developmental dynamics with striking temporal orchestration of structural and functional elements associated with very rapid changes in expression of developmental and maturational genes and provides novel insights into the complex temporal relationship between structure and function of the developing heart.

INTRODUCTION

Cardiogenesis is a highly complex process involving sequential heart primordia migration, folding, looping, septation and maturation to form the chambered heart (Fishman and Chien, 1997; Glickman and Yelon, 2002). The heart is the first readily identifiable organ to develop and function in the vertebrate. Fish and amphibian hearts are considered to be the ancestor of avian and mammalian hearts and have been used as prototypes for studying cardiac development of these organisms (Olson and Srivastava, 1996), particularly to understand the origin of the cardiac conduction system (Janse et al., 1976). Despite the evolutionary gap between fish and mammals, molecular data suggest that the essential regulatory elements of heart development are shared among vertebrates (Moorman and Christoffels, 2003; Xavier-Neto et al., 2007) and, in some cases, also with invertebrates (Perez-Pomares et al., 2009). Therefore common insights from the cardiac anatomy and physiology of many vertebrate groups is important to our overall understanding of cardiac development. According to the rules of Von Baer (VonBaer, 1828) (as cited by (Moorman and Christoffels, 2003)), it is the embryonic rather than the adult heart that should be compared since the general features of a vertebrate group appear earlier in development than the adult specialized features. In the last twenty years the zebrafish (Danio rerio) has emerged as a powerful and increasingly popular model to study cardiac development. Forward genetic screens have identified many novel regulatory mechanisms with essential roles in cardiogenic specification and differentiation, migration of cardiac progenitor cells, heart tube morphogenesis, and cardiac function (Harvey, 2002).

The study of these characteristics in the zebrafish has helped improve our understanding of heart formation in mammals (Stainier and Fishman, 1992; Stainier et al., 1993). Despite having only two chambers (Figure 1), the zebrafish heart retains many structural traits and developmental complexity of an amniote's heart, including a three-layer ventricular wall (epicardium, myocardium and endocardium) from 72 hours post-fertilization (hpf) (Serluca, 2008) and first and second heart fields (Hami et al., 2011). However, while early structural cardiac development up to 48 hpf has been well

studied there are few studies detailing the relationship between cardiac structure and function, cardiomyocyte proliferation and molecular markers of cardiac development beyond 48 hpf.

Here, we report the growth pattern in the zebrafish ventricle from 48 to 120 hpf, specifically in terms of changes in ventricle size, structure, function and cardiomyocyte proliferation. We have also analysed the expression of cardiac specific growth factors and genes associated with early cardiac development over the same time period in isolated embryonic hearts.

RESULTS

Structural changes during development

Histology and immunostaining

Haematoxylin & Eosin stained sagittal histological sections (Figure 1) of zebrafish embryonic heart ventricle displayed a multilayered ventricular (V) texture, even at 48 hpf, with endocardial (Endo), myocardial (Myo) and epicardial (Epi) layers. At 72 and 96 hpf there was a significant thickening in the myocardial layer, which invagination at 96 and 120 hpf formed the trabeculae network.

Anti-DM-GRASP stained cardiomyocytes sarcolemma (Figure 2) showed the high variability in shape of the packed cardiomyocytes. For this reason, a precise measurement of in-situ single cardiomyocyte area was not reliable. In general, cardiomyocytes appear to pass from a round or square shape to a spindle-shape towards the AV junction. At 72 hpf the sarcomere structures become evident (Figure 2, red arrows), and are particularly clear at 120 hpf. Ventricle structure within the chamber itself showed a progressive increase in the trabeculation pattern (Figure 2, red arrows in the right images) first appearing at 72 hpf and increasing markedly by 96 hpf, By 120 hpf a dramatic increase of trabecular patterning was clear.

Proliferative changes

Total ventricle cardiomyocyte number (VCt) during normal zebrafish heart development (Figure 3, DAPI staining) increased by 30% between 48 and 72 hpf, 20% between 72 and 96 hpf and 50% between 96 and 120 hpf (Figure 3A). The number of mitotic cardiomyocytes (VCm), identified by

anti Phospho-histone H3 (PHH3) staining, increased significantly between 72 and 96 hpf (5.2 ± 1.2 vs 7.7 ± 1, $p<0.001$) and then decreased at 120 hpf (Figure 3B).

Functional changes during development

Cardiac function, measured by Ejection Fraction (EF), had values in the range 22-24±2% (Figure 4) between 48 to 120 hpf with no clear statistical trend over the developmental time period studied. The size of the ventricle (ventricular diastolic area, VDA) expressed in μm^2 increased significantly from 48 to 96 hpf ($p<0.05$), remaining stable by 120 hpf.

Heart rate increased gradually over the period form 48 to 120 hpf from a mean of 140 to 160 beats per minute (bpm). Other measures of cardiac function including contraction velocity and relaxation velocity increased significantly between 72 and 96 hpf ($P<0.01$ for all measures) with a general trend for increase between 48 and 120 hpf.

Tail blood flow, measured in the posterior cardinal vein, increased significantly between 48 and 72 hpf ($p<0.05$) and showed very similar values at 72 and 96 hpf before increasing further between 96 and 120 hpf (Figure 4).

Gene expression in isolated hearts during development

Real time PCR analysis of isolated embryonic and adult hearts showed a general increase in expression of genes associated with proliferation and growth (Figure 5) with GATA4, 5 and 6 showing similar increases between 48 and 120 hpf ($P<0.01$). GATA5 and GATA6 levels increased further in the adult heart, compared to 48 hpf. MEF2c also increased significantly between 48 and 120 hpf ($P<0.05$, versus 48 hpf) and increased further in the adult heart ($P<0.01$, versus 48 hpf). NKX25 and TBX5 showed a very similar pattern of expression, with a slight increase between 48 and 96 hpf followed by a significant reduction at 120 hpf and in the adult heart ($P<0.05$, versus 48 hpf).

DISCUSSION

While zebrafish cardiac morphology and function have been studied extensively in early developmental stages, prior to hatching (Stainier and Fishman, 1992; Stainier et al., 1996; Stainier et al.,

1993; Lee et al., 1994) there are few reports detailing cardiac structure, function and cardiac specific gene expression simultaneously during this key developmental window. The period of study selected here, 48-120 hpf, is characterised by fundamental developmental changes of the heart, from a basic two chambered organ at 48 hpf to a mature multi-layered heart ventricle, by 120 hpf, with a functioning AV valve, extensive trabeculation of the ventricle, regular sequential AV activation and rapid contraction and relaxation of the ventricle.

This study has provided detailed information on these developmental changes using a number of techniques including fluorescence microscopy, video image analysis, video edge detection tracking of the ventricle, counting of ventricle cardiomyocytes and proliferating cardiomyocytes, immunostaining and imaging of the ventricle by confocal microscopy and quantitative PCR of key cardiac genes in isolated embryonic hearts. To our knowledge, there have been no previous reports that have comprehensively assessed the embryonic zebrafish heart in such detail. The findings provide unique insights into the processes governing heart growth and development and provide novel information on the temporal relationship between structure, function and molecular signals.

Changes in Structure and Anatomy

Cardiac growth was typified by a progressive increase in ventricle size between 48 and 96 hpf reaching a plateau by 120 hpf. The two chambers rotate and fold in relation to each other starting to reach an adult configuration by 72 hpf although further rotation occurs progressively towards 120 hpf. These changes in shape and anatomical relationship between atrium and ventricle are accompanied by the development of a complex trabecular network within the ventricle chamber (Singleman and Holtzman, 2012). At 48 hpf, the primitive ventricle resembles a thin walled chamber, few cell layers thick, with a smooth inner surface. Trabeculae form as structural extensions of the ventricle wall (Moorman and Christoffels, 2003; Sedmera et al., 2000) driven by directional delamination of cardiomyocytes into the ventricular lumen (Liu et al., 2010). This phase is associated with diminished external growth and a switch to internal myocardial growth creating numerous trabeculations that typically protrude inward and form a complex network of lacunae classically re-

ferred to as spongy myocardium. This spongiform appearance continues to form and develop into adulthood. The early formation of trabeculae is closely associated with the widespread formation of a mature sarcomeric patterning within cardiomyocytes.

Cardiomyocyte number increased progressively in the ventricle during development particularly between 48 and 72 hpf and again between 96 and 120 hpf. These two phases represent periods of significant structural change. The first phase between 48 and 72 hpf represents an increase in the cellular layering of the wall of the ventricle chamber while the second, between 96 and 120 hpf represents the newly forming trabecular network within the primitive ventricular cavity (Becker et al., 2014). During these phases the shape of the heart is not simply due to the accumulated cell mass but also a consequence of physical stress, as the myocardium experiences haemodynamic load and contractile stress and strain (Chicurel et al., 1998; Conlon and Raff, 1999; Day and Lawrence, 2000). As zebrafish cardiomyoytes changes from being predominantly spherical and rhomboid in shape to more mature spindle shaped cells they begin to loosely align with each other presumably as they become subject to the haemodynamic stress of chamber pressure and the shear stresses of cardiac contraction and relaxation. The acquisition of the heart's three-dimensional form, therefore, involves a complex interplay between cardiomyocyte shape, organization, physical forces and increasing cell numbers. This rearrangement of cellular architecture is likely to play a critical role in the final morphology of the heart (Auman et al., 2007).

Changes in Cardiac Function

As the ventricle switches from its early role as a conduit vessel contributing only a low level of pulsatile action until 48 hpf, it subsequently develops to become an active contractile organ by 120 hpf. In the zebrafish,this mature differentiated state allows it to act as an actively contracting “sponge” soaking up blood from the atrium and, with the AV valve now fully functional, ejecting it anterogradely through the bulbus arteriosus and on to the gills. Our observed changes in relaxation velocity by 96 hpf suggest that enhancement of cellular mechanisms linked to diastole, likely to be active calcium cycling, create an active filling process as the ventricle relaxes. These processes ma-

ture further towards 120 hpf and are accompanied by more rapid contraction and greater excursion of the ventricle wall contributing to the increased circulatory capacity required for increasing somatic growth.

Changes in structure of the heart are accompanied by a small but steady increase in heart rate over the same time period of development. Although ejection fraction does not change significantly during this time period we can infer that cardiac output (volume of blood ejected per minute) must increase since cardiac output is the product of heart rate and stroke volume. Taking the mean values for diastolic area for each developmental time point and assuming the ventricle is shaped as a prolate spheroid (Bagatto and Burggren, 2006) indicates a mean volume change of approximately 6% per day from 48 to 120 hpf. With this assumption the stroke volume in the zebrafish embryonic heart increases, on average, from 0.4 nL at 48 hpf to 0.7 nL at 120 hpf. With a heart rate of 120-160 bpm during this time period this yields a cardiac output of 50 nL/min at 48 hpf rising at 110 nL/min at 120 hpf. This is similar to data published by other authors (Antkiewicz et al., 2006; Jacob et al., 2002; Kopp et al., 2010).

These data are in agreement with the increasing metabolic demands in embryos during this developmental time period critical for newly forming organs requiring increased blood flow generated by the growing heart. The question arises, therefore, as to why the zebrafish heart begins to beat and propel blood containing haemoglobin to the growing tissues well in advance of the absolute need for a convective oxygen supply (Burggren WW, 1995). One theory proposes that nutrient and electrolyte transport could represent the limiting factor, rather than gas transport, during early development, because diffusional transport of metabolites and charged ions might be slower than diffusion of small gas molecules (Pelster and Burggren, 1996). In addition, there is evidence that the early beating heart is critical for normal angiogenesis, since pressurized pulsatile blood flow itself is crucial for growth and development of the vascular system (Davies and Tripathi, 1993; Hudlicka et al., 1992; Jacob et al., 2002).

Changes in Isolated Heart Gene Expression

Very early heart development requires a battery of transcription factors switched on and off in a specific temporal and spatial pattern to orchestrate the key anatomical and functional processes leading to heart formation (Burch, 2005; Naiche et al., 2005; Patient and McGhee, 2002). The set of genes selected for analysis in this study are highly conserved in all Vertebrates including zebrafish at the level of sequence, expression pattern and function and are critical for normal cardiac development (Xavier-Neto et al., 2007). GATA4 regulatory mechanisms have been intensively studied and its levels and activity are crucial for normal heart specification and development (Zhou et al., 2012). GATA4 null mice display defects in heart morphogenesis and ventral foregut closure, resulting in embryonic lethality by E8.5 (Molkentin et al., 1997). Early cardiac-specific deletion of GATA4 results in myocardial thinning, abnormal endocardial cushion formation and right ventricular hypoplasia (Zeisberg et al., 2005). Cardiac-specific deletion of GATA4 at later time points results in decreased cardiac function and an inability to undergo hypertrophy in response to haemodynamic load (Xin et al., 2006; Oka et al., 2006). Indeed, GATA4 forms protein complexes with several other transcription factors expressed in the heart (Xin et al., 2006) and these synergistic interactions may act to regulate cardiac gene transcription. The hypertrophic response and sarcomeric reorganization induced by endothelin-1 and phenylephrine are dependent on an interaction between GATA4 and RhoA (Charron et al., 2001). Disruption of these interactions has been reported to underlie some cases of congenital heart disease (Zhou et al., 2012). Our data indicate that GATA4 mRNA becomes increasingly abundant in the heart at 96 hpf and remains high at 120 hpf. This rise in expression/abundance coincides with the marked trabeculation of the developing ventricle and with the appearance of clearly visible sarcomeric patterns in the cmlc2:eGFP labelled cardiomyocytes in our zebrafish hearts. Interestingly, the levels of GATA4 mRNA remain relatively high in the adult heart suggesting that it continues to play an important role possibly linked to the high background level of cardiomyocyte turnover (Laflamme and Murry, 2011).

GATA4 and 6 were found to have a similar pattern of expression during the course of development. This is consistent with a previous report showing that GATA4 and 6 co-express during early cardiac

development in the mouse with both being expressed in the precardiac mesoderm, the embryonic heart tube, developing endoderm (Morrisey et al., 1996) and ongoing low level expression in the adult heart. Indeed, GATA4 and GATA6 proteins have been shown to interact and can synergistically activate target gene transcription. In particular they activate the CEF-1 nuclear protein binding site in the cardiac-specific cTnC transcriptional enhancer (Morrisey et al., 1997). In contrast, GATA5 is expressed in a temporally and spatially restricted pattern in the early embryonic mouse heart and has very low levels of expression in late foetal mouse heart development (Morrisey et al., 1997). Our findings in the zebrafish are different and indicate an increasing level of GATA5 mRNA abundance between 96 and 120 hpf with a further increase in the adult heart. This may once again reflect the pattern of increasing levels of expression of GATA4 which is known to interact with GATA5 in supporting cardiomyocyte proliferation (Singh et al., 2010).

TBX5 and NKX2.5 showed a similar pattern of expression during development in our embryonic hearts showing a rise between 48 and 96 hpf and then reducing substantially at 120 hpf and remaining low into adulthood. TBX5 is known to influence cardiomyocyte proliferation and septation in the mammalian heart (Misra et al., 2014). Mutations in TBX5 are known to cause forearm deformities and septation heart defects in humans (Holt-Oram syndrome). In the zebrafish, TBX5 has been shown to modulate cardiac looping and laterality (Pi-Roig et al., 2014). NKx2.5 is known to modulate the formation of the second heart field (Guner-Ataman et al., 2013). The early rise and fall of NKx2.5 and TBX5 mRNA in our isolated hearts is highly consistent with the roles of these genes in early cardiac patterning. MEF2C is a transcription factor that influences early cardiac development (Vong et al., 2006) and in addition appears to modify the response of the adult heart to haemodynamic stress (Pereira et al., 2009). Overexpression results in dilated cardiomyopathy in mice (Xu et al., 2006). The gradual rise in mRNA levels of MEF2C during development support its role in heart development but more intriguing are the higher levels present in the adult heart where it may also support the high level of cell turnover typically seen in the zebrafish heart.

CONCLUSIONS

These findings highlight the dynamic nature of heart development in the zebrafish embryo and provide novel insights into the temporal cohesion between structural, functional and genetic changes occurring during mid to late developmental period of embryogenesis.

MATERIAL AND METHODS

Ethical approval

All experiments were approved by the local ethics committee and conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 in an approved establishment.

Zebrafish maintenance

Zebrafish husbandry, embryo collection and maintenance were performed according to accepted standard operating procedures (Nüsslein-Volhard, 2002). The cardiac myosin light chain 2:eGFP transgenic line (tg(cmlc2:eGFP)) (Burns et al., 2005) was used for all experiments, unless stated; embryos were maintained at 28.5°C on a 14h light/10h dark cycle and staged according to Kimmel (Kimmel et al., 1995). Embryos were kept in egg water until dechorionated and then in embryo medium (Westerfield, 2000). All experimental procedures were performed at room temperature (RT, 23°C).

Zebrafish whole-mount immunostaining and histology

Embryos were euthanized in Tricaine 1 mM and fixed in 4% paraformaldehyde (PFA, Sigma) and hearts isolated by micro-dissection. Isolated hearts were pre-incubated in proteinase K (10 μg/ml), washed in PBS and Triton X100 (0.1%) and then Bovine Serum Albumin (5% for 3 h) before being incubated with anti Phospho-histoneH3 antibody (Millipore 05-670; rabbit, 1:200), a marker of mitosis, followed by incubation with anti-rabbit antibody (Alexa fluor, Dako, 1:500). The whole ventricle structure was also analysed by observing the GFP signal. In order to visualize the shape of in-situ single cardiomyocytes, hearts analysed for ventricle structure were additionally stained with an

antibody against DM-GRASP, a sarcolemmal integrin, called zn-8 (mouse; produced by Developmental Studies Hybridoma Bank) diluted 1:200 in PBS containing BSA 5%.

Subsequently, hearts were incubated in DAPI (Sigma, 1:1000), washed in PBS and then mounted in glycerol (100%). Confocal microscopy (Leica SP5) was used to capture z-stack images of isolated zebrafish heart ventricles at 3 μm intervals. The total number of ventricular cardiomyocytes (VCt) and the number of mitotic ventricular cardiomyocytes (VCm) were counted using ImageJ software, by marking each nucleus with a tag while moving progressively through the z-stack. Only cardiomyocytes were included in the counting process by ensuring that each nucleus was located within a GFP positive region of the heart. Counting excluded the atrium and bulbus arteriosus, and was performed by a single individual (GM) and the intra-observer variation for a sample of 25 hearts was ±4.5%.

Haematoxylin & Eosin (H&E) staining of whole embryos was performed according to standard protocols (Sabaliauskas et al., 2006).

Assessment of cardiac function and tail blood flow

Images of the beating heart were captured by video camera (IonOptix CCD100 MyoCamtm, Milton, MA, USA) mounted on a microscope (Zeiss, Axioscope II MOT Plus, Thornwood, NY, USA) linked to a computer. Ejection fraction (EF) and heart rate (HR) were determined using image analysis software (ImageJ, NIH, Bethesda, USA). Ejection fraction was estimated using a simple area subtraction method which subtracts ventricular systolic area from diastolic area and expresses this as a percentage of diastolic area (Shu et al., 2003). In addition, digital video analysis was used to track ventricular wall motion and heart rate in embryonic hearts in-situ between 48 and 120 hpf using a technique previously validated in our laboratory (Denvir et al., 2008). Briefly, a frame-grabber card in a computer continuously digitizes output from a video camera mounted on a low power microscope and displays it on a monitor. The user optimises the image for contrast and then selects video lines on the edge of the heart corresponding to the ventricular wall close to the apex using a small cursor. The cursor tracks the movement of the ventricle wall and displays it as a con-

tinuous trace indicating contraction and relaxation movements of the ventricle. The trace is calibrated using a standard graticule and then stored digitally. The trace is analysed off-line using commercial software (SoftEdge™, IonOptix Corporation) for ventricle wall motion amplitude (WMA, μm), ventricle contraction velocity (CV, μm/s) and ventricle relaxation velocity (RV, μm/s). Heart rate is also obtained from these traces under each experimental condition and at each time-point.

Caudal Vein blood flow velocity was estimated in the posterior cardinal vein (Isogai et al., 2001) by assessing frame-by-frame motion of single erythrocytes determined from video images. Four erythrocytes per fish (at least 4 embryos per group) over 10 frames at video frame-rate of 30 frames per second were analyzed using ImageJ to determine mean erythrocyte cell velocity (μm s−1).

Gene expression analysis

Transcription factors were chosen on the basis of their importance in cardiac development of Vertebrates (Brand, 2003). Real time PCR was used to assess gene expression of target genes in the embryonic and adult heart (6 months). Gene and related primer sequences were as follows:

GATA4 (NM 131236.1148) F:cctgtggactctaccataagatga; R:gctgacagtttgtgcaggataa; GATA5 (NM 131235.2148) F:gcggcctttaccacaagat; R:gctcgtcgtgatgtgcttt; GATA6 (NM 131557.1162) F:cagtccttcgtcaacacaggt; R:acgtcaagcctacattggtgt; MEF2c (NM 131312.2) F:gaaacacagaggtctgatgg; R:gtggtttccgtacccgttt; TBX5 (NM 130915.1) F:ccactgcatcaagaggaaagt; R:ccacatacggcttcttataggg; NKX2.5 (NM 131421.1) F:ggacaaaggcaacaaaatcaa; R:cctgacaaaacccgatgtct.

For each sample, 300-500 hearts were extracted by mechanical-agitation method (Burns and MacRae, 2006). Briefly, embryos were anaesthetized, transferred to a 1.5-mL microfuge tube (Eppendorf UK Limited, Stevenage, UK), washed with embryo disruption medium [EDM; Gibco Leibovitz's L-15 Medium (Life Technologies Ltd, Paisley, UK) and 10% fetal bovine serum (Life Technologies Ltd, Paisley, UK)] and resuspended in 1.25 mL of fresh EDM. Approximately 1 mL EDM containing embryos was drawn into the needle (19-gauge) and syringe (5mL) and immediately expelled back into the microfuge tube 25 times at a rate of 3 seconds per syringe motion. Fragmented

embryos were applied to a 100 μm nylon mesh (Cell strainer, Becton and Dickinson, Oxford, UK) and the flow-through was collected in a 35 mm Petri dish. The flow-through was subsequently applied to a 40 μM nylon mesh (Cell strainer, Becton and Dickinson, Oxford, UK). The 40 μM mesh was inverted and the retained material was washed off with EDM into a clean 35 mm Petri dish. Intact GFP positive ventricles, many of them still beating and many of them with the atrium still attached, were identified under fluorescent light and collected selectively with a p100 micropipette under transmitted light. To minimize contamination by non-cardiac tissues, the hearts were subsequently expelled into a 35 mm Petri dish containing fresh EDM and selectively retrieved a second time before being accumulated in a microfuge tube on ice. Hearts were pelleted, the media was decanted, and the preparations were frozen in a dry ice bath prior to storage at -80 °C.

RNA was extracted according to the Qiagen RNeasy mini kit (Qiagen, West Sussex, UK). Each eppendorf containing hearts received 600 μL buffer RLT and with two small metallic beads, these had been previously autoclaved and wiped with RNase Zap (Life Technologies Ltd, Paisley, UK). Efficient disruption and homogenization was performed by milling for 45 s at 30 Hz (Mixer Mill 301 model, Retsch, Haan, Germany). Beads were removed and the samples were centrifuged at 13,000 g for 3 mins at 4 °C. The supernatant was placed in a new 2 mL eppendorf tube and the pellet discarded. Later step were performed according to the Qiagen RNeasy mini manufacturer's manual.

RNA was quantified using a Nanodrop Spectrophotometer (Thermo Fisher, West Sussex, UK), whereas the quality of RNA was assessed by electrophoresis on agarose (Lonza, Berkshire, UK) gel (1 % w/v in 0.5x TBE).). RNA integrity was assessed on basis of 18S and 28S ribosomal RNA (rRNA) bands. RNA was reverse transcribed in cDNA by using high capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). For the PCR, the DNA intercalating SYBR Green fluorescent dye method was used.

Statistical analysis

Experiments were performed in triplicate with on average 20-30 embryos per experiment, unless otherwise stated. Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 5. One-way or two-way repeated measures ANOVA followed by Bonferroni post-hoc test, were used to compare means within and between groups. P values <0.05 were considered significant.

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COMPETING INTERESTS STATEMENT

Authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation. Authors declare no conflicts of interests.

AUTHOR CONTRIBUTIONS

G.M. helped conceive, design and perform the experiments and in the analysis and interpretation of data and in the writing of the manuscript. K.S.W. helped to conceive, perform the experiments and in interpretation of the data. J.M. helped conceive and design the experiments. M.D. and C.T. helped conceive and design the experiments and in the interpretation of data and in writing the manuscript. All authors approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1 – Histology of developing zebrafish heart (48-120 hpf).

A. Transgenic cardiac myosin light chain (cmlc)2:eGFP zebrafish embryo (72 hpf). The GFP positive two chambered heart is shown in the lower panel.

B. Longitudinal sections of wild-type (Wik) zebrafish embryonic heart ventricle between 48 hour post-fertilization (hpf) and 120 hpf (Haematoxylin & Eosin), showing the significant morphological changes during this time period. Key: V- ventricle; A- atrium, BA – Bulbus arteriosus, SV – Sinus Venosus.

Figure 2 – Immmunostaining of developing Zebrafish ventricle (48-120 hpf).

Confocal images of cmlc2:eGFP zebrafish embryo heart ventricle at different developmental stage, stained with anti-DM-GRASP (sarcolemmal integrin) in red, and counterstained with DAPI. Each time-point reports images of the same heart, of the ventricle surface (left side) and internal ventricle structure (right side). Red arrows show trabecular formation inside the ventricle and sarcomere structure evident from 72 hpf. Key: V - ventricle, BA-bulbus arteriosus, A-atrium.

Figure 3 – Cardiomyocyte growth and proliferation during heart development.

Ventricle cardiomyocyte number during normal development from 48 to 120 hpf. **A.** Pattern of increase in total cardiomyocyte number in cmlc2:eGFP zebrafish embryo heart ventricles. At each time point, image captured under confocal microscopy show DAPI stained nuclei (right panel top) counted as single cardiomyocytes on the GFP background shown in the right panel top in a ventricle 120 hpf, containing the highest number of cardiomyocytes. **B.** Changes in mitotic ventricle cardiomyocyte numbers over the same time period. At each time point, image captured under confocal microscopy show phosphohistone H3 (PHH3) positive nuclei, counted as single proliferating cardiomyocytes on the GFP background, shown in the right panel bottom in a ventricle at 96 hpf, that is the highest cardiomyocyte proliferative stage.

Figure 4 – Heart Function during development.

A. Still images extracted from a video captured under fluorescence microscopy representing the beating cmlc2:eGFP zebrafish embryo heart at different developmental stages. B. Assessment of cardiac function. Diastolic area was measured in video images captured under fluorescence microscopy at end-diastole (n=8 embryos per time point). Ejection fraction was measured by subtracting ventricular systolic area from diastolic area and expressing this as a percentage of diastolic area. Cardinal vein blood flow was measured by assessing red blood cell velocity in high speed video images (n=8 embryos per time point). Heart rate, ventricle contraction and relaxation velocity were measured using video-edge tracking of the ventricle apex (n=10 embryos per time point). Analysis were repeated every day in the same embryos from 48-120 hpf. Key – BA=bulbus arteriosus, A=atrium, V=ventricle.

Figure 5 – Gene expression in isolated embryonic hearts during development.

mRNA levels of genese linked to cardiac development and growth were assessed by Q-PCR during normal development at 48, 96 and 120 hpf, and in the adult (6 month age) heart. mRNA was normalised against the housekeeping gene Ef1α.

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Figure 1 – Histology of the developing zebrafish heart (48-120hpf)

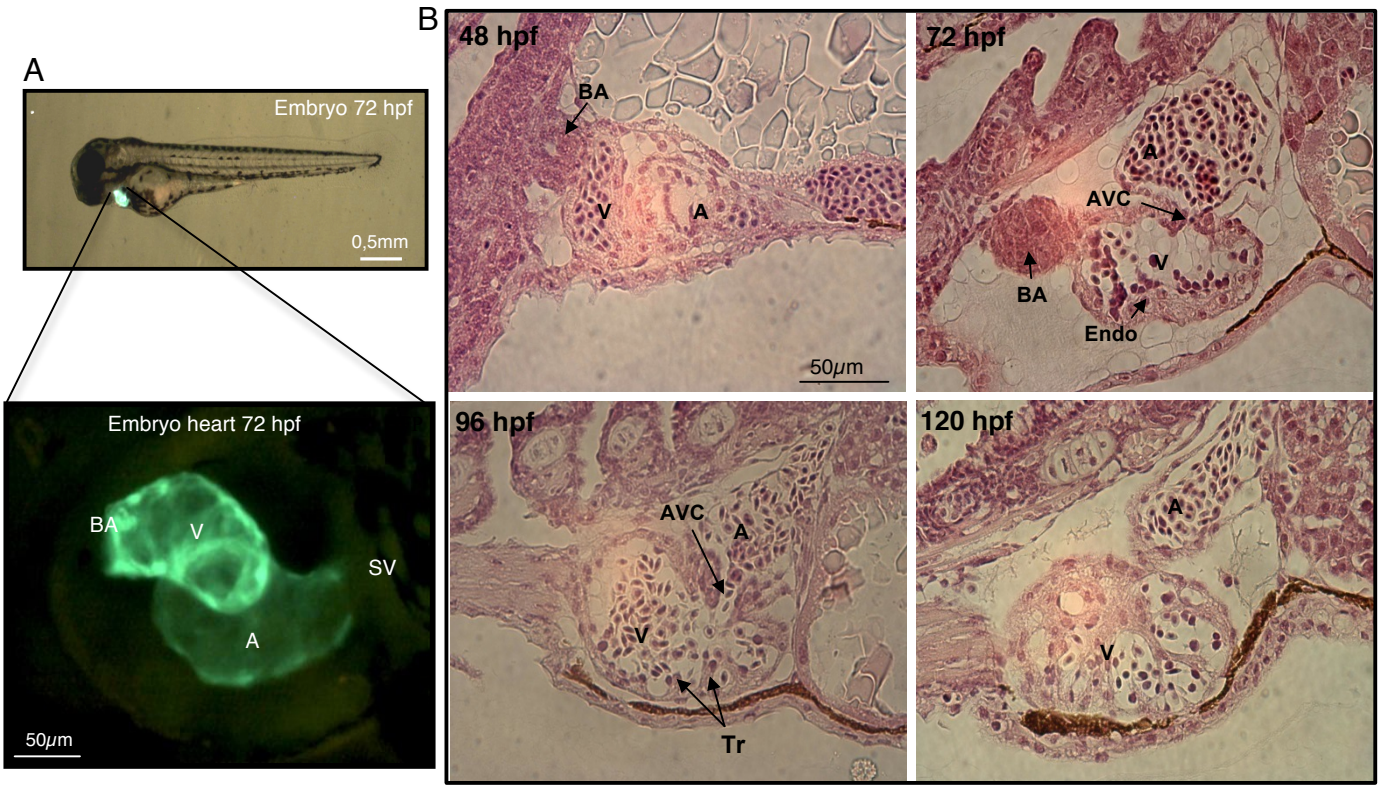

Figure 2 – Confocal images of the developing zebrafish heart (48-120hpf)

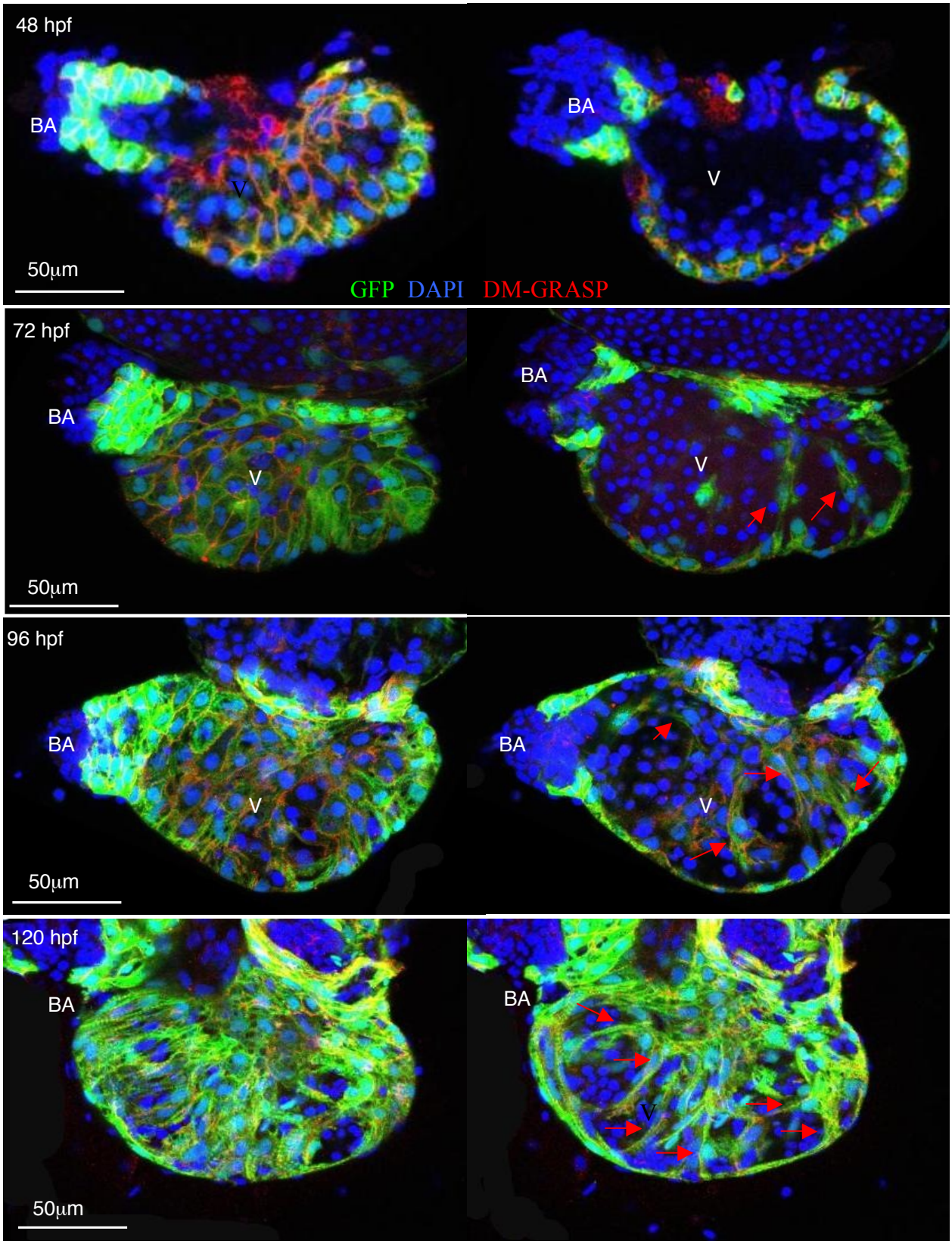

Figure 3 – Ventricle cardiomyocyte number during normal development from 48 to 120 hpf

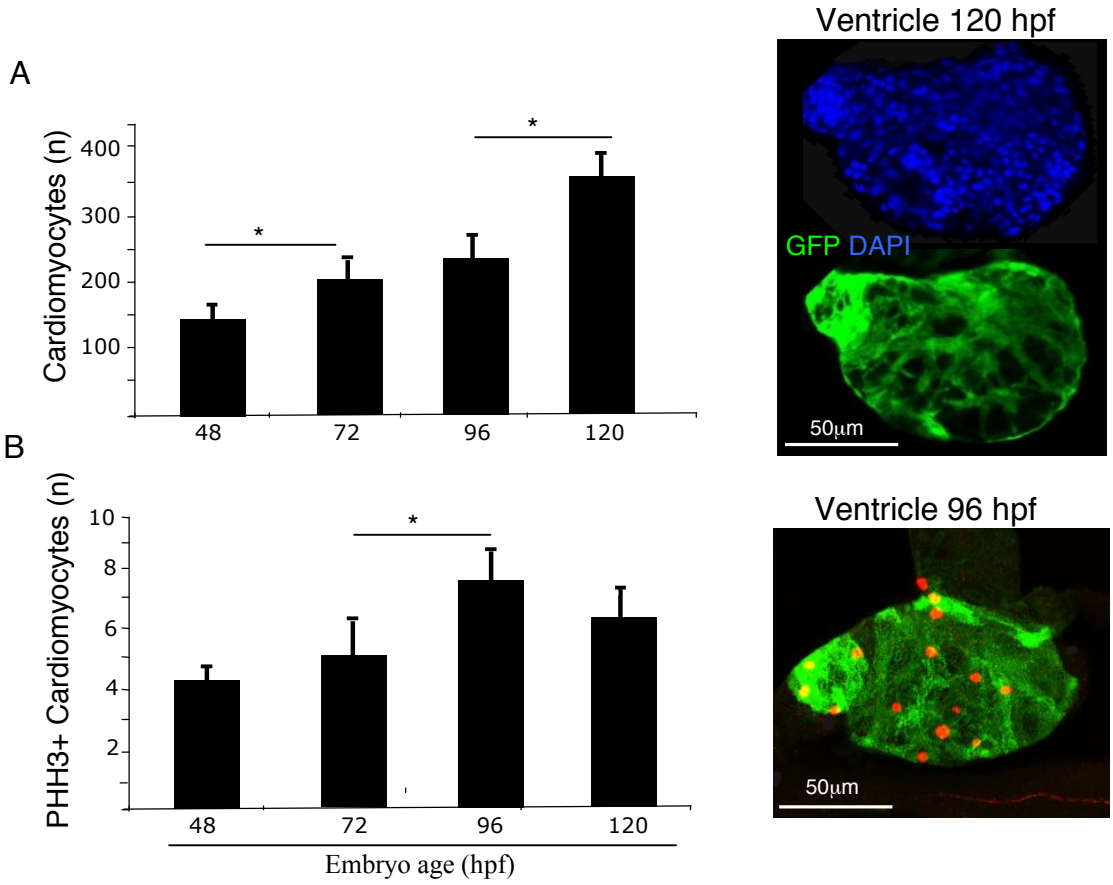

Figure 4 – Analysis of ventricle structure and function during the first 120hpf in the zebrafish embryo

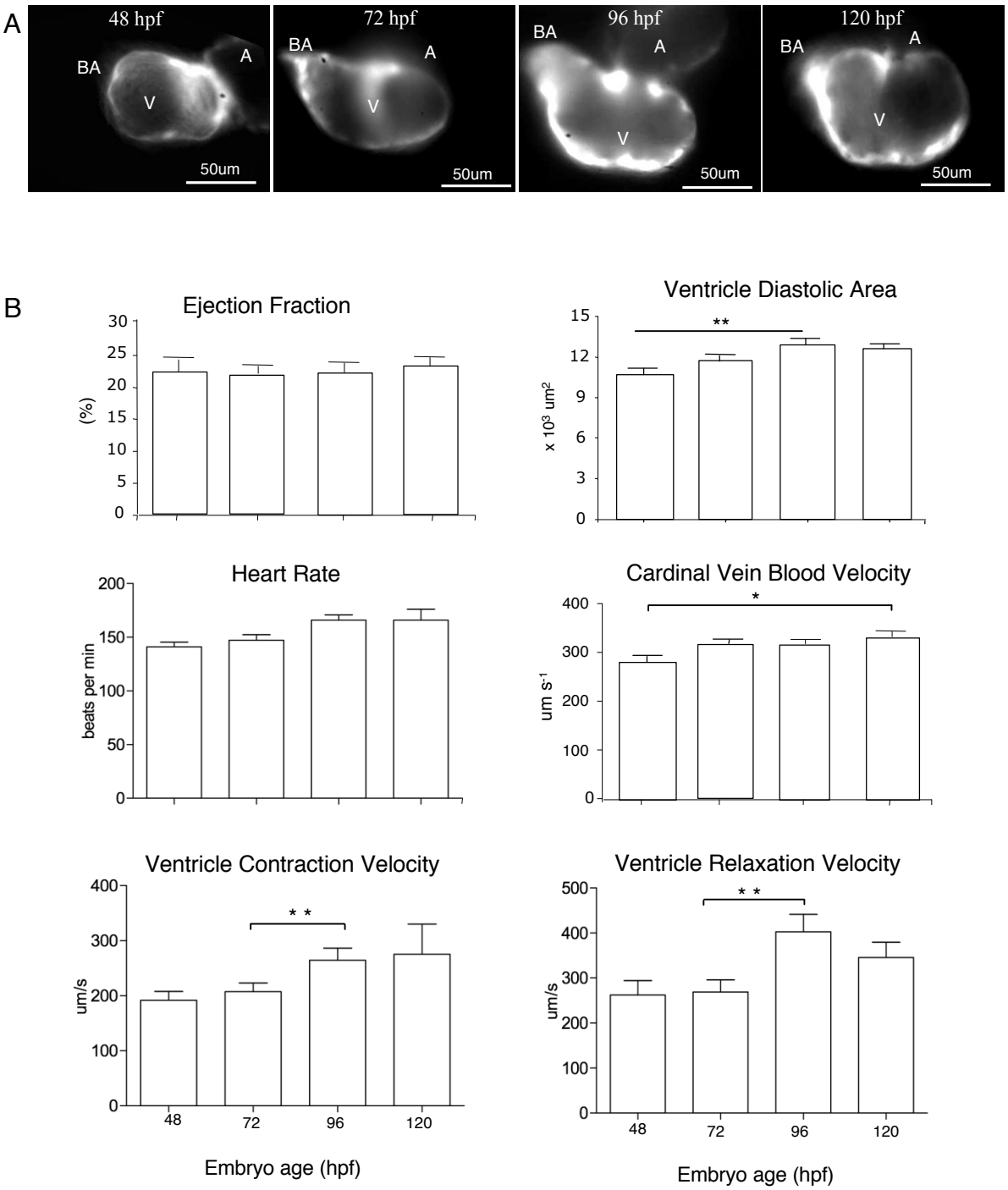

Figure 5 – Cardiac genes expression pattern during development in isolated zebrafish embryonic and adult hearts