Differentiation 89 (2015) 117–127

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

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

Gianfranco Matrone *[,1], Kathryn S. Wilson, John J. Mullins, Carl S. Tucker, Martin A. Denvir

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

ARTICLE INFO

Article history:
Received 19 October 2014
Received in revised form
6 April 2015
Accepted 10 May 2015
Available online 19 June 2015

Keywords:
Zebrafish
Heart
Mechanisms
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. While early cardiac development in the zebrafish has been well characterized, functional changes during development and how these relate to architectural, cellular and molecular aspects of development have not been well described previously. 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. 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 hpf, 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 particularly 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 structure and function within the first few days of development. These changes are associated with changes in expression of developmental and maturational genes. This study provides important insights into the complex temporal relationship between structure and function of the developing zebrafish heart.

1. 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 VonBaer (1828) (as cited by Moorman and Christoffels (2003)), it is the embryonic rather than the adult heart that should be compared since the common features of a vertebrate group appear early in development. In the last twenty years the zebrafish (*Danio rerio*) has emerged as a powerful and increasingly popular model to study cardiac development (Hu et al., 2000; Bakkers, 2011) and cardiac defects (Antkiewicz et al., 2005; Tu and Chi, 2012). Forward genetic screens have identified many novel regulatory mechanisms with essential roles in cardiogenic specification and

Abbreviations: hpf, hours post-fertilization; *myl7*, myosin light chain 7; GFP, green fluorescent protein; VCt, total ventricular cardiomyocytes; VCm, mitotic ventricular cardiomyocytes; PHH3, Phospho-histoneH3; Endo, endocardial; Myo, myocardial; Epi, epicardial; WMA, wall motion amplitude; CV, ventricle contraction velocity; RV, ventricle relaxation velocity; EF, ejection fraction; VDA, ventricular diastolic area; AV, atrioventricular; TBX, T-Box; MEF, myocyte enhancer factor; NKX, NK-Homeobox

* Correspondence to: Center for Cardiovascular Regeneration, Department of Cardiovascular Sciences, Methodist Hospital Research Institute, 6670 Bertner Avenue, Houston, TX 77030, USA.
E-mail address: gmatrone@HoustonMethodist.org (G. Matrone).
[1] Present address: Methodist Hospital Research Institute (R6-South), 6670 Bertner Avenue, Houston, TX 77030, USA.

http://dx.doi.org/10.1016/j.diff.2015.05.001
Join the International Society for Differentiation (www.isdifferentiation.org)
0301-4681/

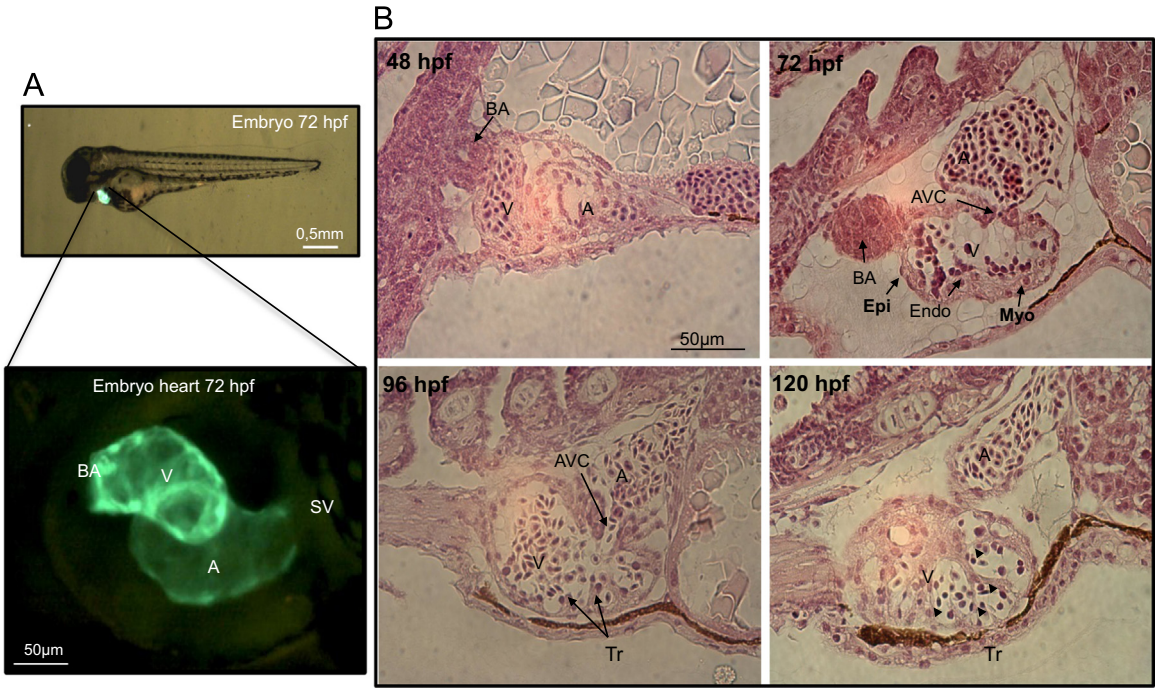

Fig. 1. Histology of developing zebrafish heart (48–120 hpf). (A) Transgenic *myl7:GFP* zebrafish embryo (72 hpf). The GFP positive two chambered heart is shown in the lower panel. (B) Longitudinal sections of wild-type (Wik) zebrafish embryonic heart ventricle between 48 h post-fertilization (hpf) and 120 hpf (Haematoxylin & Eosin), showing the significant morphological changes during this time period. Key: V – ventricle; A – atrium, BA – Bulbus arteriosus, SV – Sinus Venosus, Tr – trabeculae.

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 (Fig. 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 h 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 a few studies that have specifically detailed the relationship between cardiac structure and function, cardiomyocyte proliferation and molecular markers of cardiac development over the first 5 days of development.

The study reported here has examined the changes in ventricle size, structure, function and cardiomyocyte proliferation over this time period. We have also analyzed the changing expression pattern of cardiac specific growth factors in isolated embryonic hearts over the same time period.

2. Material and methods

2.1. 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.

2.2. Zebrafish maintenance

Zebrafish husbandry, embryo collection and maintenance were performed according to accepted standard operating procedures (Nüsslein-Volhard and Dahm, 2002). The *myl7:GFP* transgenic line (Tg(*myl7:GFP*)^{f1}) (Burns et al., 2005) was used for all experiments, unless stated. GFP expression is driven by a cardiac-specific promoter of *myl7* (myosin light chain 7, also known as cardiac myosin light chain 2). Germ-line transmission of the transgene was achieved on the AB strain background. Embryos were maintained at 28.5 °C on a 14 h light/10 h 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).

2.3. 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 analyzed by observing the GFP signal. In order to visualize the shape of in-situ single cardiomyocytes, hearts analyzed 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