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Growth of the developing mouse heart: An interactive qualitative and quantitative 3D atlas

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ABSTRACT

Analysis of experiments aimed at understanding the genetic mechanisms of differentiation and growth of the heart, calls for detailed insights into cardiac growth and proliferation rate of myocytes and their precursors. Such insights in mouse heart development are currently lacking. We quantitatively assessed the 3D patterns of proliferation in the forming mouse heart and in the adjacent splanchnic mesoderm, from the onset of heart formation till the developed heart at late gestation. These results are presented in an interactive portable document format (Suppl. PDF) to facilitate communication and understanding. We show that the mouse splanchnic mesoderm is highly proliferative, and that the proliferation rate drops upon recruitment of cells into the cardiac lineage. Concomitantly, the proliferation rate locally increases at the sites of chamber formation, generating a regionalized proliferation pattern. Quantitative analysis shows a gradual decrease in proliferation rate of the ventricular walls with progression of development, and a base-to-top decline in proliferation rate in the trabecules. Our data offers clear insights into the growth and morphogenesis of the mouse heart and shows that in early development the phases of tube formation and chamber formation overlap. The resulting interactive quantitative 3D atlas of cardiac growth and morphogenesis provides a resource for interpretation of mechanistic studies.

Introduction

A central challenge in cardiac developmental biology is the understanding of the mechanisms that effectuate the growth of the heart. Proper formation of the four-chambered heart depends on a delicate balance between growth and differentiation, which is highly prone to errors, witness the high incidence of congenital cardiac malformations (Hoffman and Kaplan, 2002). Insight into these processes is crucial for the understanding of cardiac morphogenesis, and the interpretation of cellular lineage analyses (Buckingham et al., 2005) and molecular mechanistic data that provide a great momentum to the field of cardiac developmental biology (Bruneau, 2008; Evans et al., 2010; Vincent and Buckingham, 2010).

The mouse embryo is currently the most important animal model for molecular genetics (Rosenthal and Harvey, 2010). However, most knowledge on cardiac morphogenesis comes from classic studies of mostly chicken and human embryos (His, 1885; Romanoff, 1960). Also, data on cardiac proliferation are scarce and rarely placed within a three-dimensional (3D) context, leading to conflicting conclusions such as either rapid (Thompson et al., 1990) or slow (Sissman, 1966) proliferation in the early heart tube. Previously, we analyzed local proliferation rates within the developing chicken heart and observed that proliferation stops when myocardium differentiates from rapidly proliferating splanchnic mesoderm, to be followed by a new phase of rapid proliferation at the sites of chamber formation (Soufan et al., 2006; van den Berg et al., 2009). A similar pattern is observed during human heart formation (Sizarov et al., 2011).

Information on local proliferation rates in the developing mouse heart is, however, entirely lacking. To generate a general framework of the 3D architecture and growth of the developing mouse heart, we performed a 3D analysis of morphogenesis and proliferation of the developing mouse heart. Our analysis spans the entire gestational period and the results are presented in an interactive fashion. In contrast to chicken and human development, where clearly separated phases of early heart development can be distinguished, we show that in early mouse heart development the phases of tube formation and chamber formation overlap. The mouse heart, nevertheless, forms by differentiation of splanchnic mesoderm into myocardium accompanied by a slowdown of proliferation, which is followed by rapid proliferation at the site of chamber differentiation. During later chamber

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development the proliferation rate decreases progressively, while proliferation in the trabecules shows a base-to-top decline at every stage. All reconstructions are supplemented with interactive 3D models, and thus form a comprehensive 3D atlas of the growth and morphogenesis of the developing mouse heart.

Materials and methods

Animal handling and immunohistochemistry

To visualize proliferating cells, FVB mouse embryos were exposed to Bromodeoxy-Uridine (BrdU) (Sigma nr. B5002) for 1 h. BrdU is a thymidine analogue which is incorporated into the nucleus during the S-phase of the cell cycle (during DNA synthesis). Prior to embryonic day (*E*) 9 exposure was by culturing in 0.05 mg BrdU/ml medium (DMEM (Invitrogen), pH 7.4, supplemented with 10% foetal calf serum). Older stages were exposed by peritoneal injection of pregnant mice with 50 mg BrdU/ kg (using a 10 mg BrdU/ ml 0.9% NaCl solution), as previously described (Aanhaanen et al., 2009). Immediately after exposure, embryos were fixed in freshly prepared 4% paraformaldehyde. Embryos were staged in days of development according to their general and their cardiac morphological characteristics (Bard et al., 1998; Kaufman, 1994; Theiler, 1972). It is important to appreciate that early heart development progresses very fast, especially in a few hours around day 8 of embryonic development, which means that the developmental variation within a single mouse litter can range from cardiac crescent to tubular heart stages. All experimental procedures were in line with institutional and national regulations for animal welfare.

Standard procedures were used for dehydration and embedding in paraffin (Mommersteeg et al., 2010). Embryos were serially sectioned at 7 μm. Mounted sections were dewaxed, rehydrated and equilibrated in phosphate-buffered saline (PBS), after which antigens were retrieved by pressure cooking for 3 min in antigen unmasking solution (1:100 Vector laboratories Inc. H-3300). After a PBS wash, sections were blocked with 2% bovine serum albumin and exposed overnight to a mixture of primary antibodies; rat-monoclonal anti-BrdU (1:600, Immunosource OBT0030CX), rabbit polyclonal anti-cTnI (1:250 HyTest 4T21/2), monoclonal anti-MHC (Mf20, Hybridomabank, Iowa City, IA, USA) and goat polyclonal anti-Nkx2.5 (1:200 Santa Cruz 8697). After washing, secondary antibodies were added for 2 h periods. Firstly, donkey–anti-goat Alexa-680, and, again after washing, goat–anti-rat Alexa-405 and goat–anti-rabbit Alexa-568 (Invitrogen, A-21084, A-31556, A-11077, respectively). After washing, Sytox Green (1:40,000 Molecular Probes S-7020) was added for 15 min before a last wash and mounting of a cover slip with either PBS/glycerol or Vectashield (Vectorlabs H1000).

3D-reconstruction and morphometry

Images of each channel were acquired with a fluorescence microscope (Leica DM6000) at 10 times magnification, generating image series with pixels representing $0.9 \times 0.9\ \mu m^2$ tissue. Upon visual inspection, damaged sections were replaced by neighbouring sections. Images were loaded into Amira (Mercury Computer Systems) and, with an automatic module, aligned by translation and rotation. This procedure was visually inspected and corrected where necessary. In the aligned stacks of images, anatomical structures were manually segmented based on either a specific signal (Mf20, cTnI or Nkx2.5), or on morphological criteria (mostly using the Sytox Green signal). In E7.75 embryos, Nkx2.5 could be used as an early cardiogenic marker to identify the heart-forming region in the splanchnic mesoderm. After segmentation, the myocardium and splanchnic mesoderm labels were inspected to ensure that all nuclei were included. 3D surface reconstructions were generated as previously described (Fig. 1, top row) (van den Berg and Moorman, 2011). For interactive 3D presentation, 3D-pdf files were generated as previously described (de Boer et al., 2011).

Detection and quantification of nuclei was performed using a program in Matlab (The Mathworks). The complete procedure is illustrated in Fig. 1. In short, background staining was removed from the Sytox Green image by subtracting the local average from the image. The resulting local maxima were then thresholded into binary nuclei. This binary image was masked by the segmented label of either the myocardium or the splanchnic mesoderm. For nuclei within these masks, a positive BrdU-signal was defined as a nucleus with a staining intensity of at least a standard deviation above its local background intensity (Fig. 1, middle row) resulting in a 3D representation of the BrdU-positive and BrdU-negative cardiomyocyte nuclei (Fig. 1, bottom row). From E10 onwards non-myocytes gradually invade the heart (Zhou et al., 2008) and, therefore, Nkx2.5-staining was used to identify cardiomyocyte nuclei from stage E9.5 onwards. These Nkx2.5-positive nuclei were identified similar to the BrdU-positive nuclei. The BrdU-positive fraction at each location was then determined by counting the total number of nuclei and the number of BrdU-positive nuclei in a cubic sampling volume surrounding the location and by plotting the resulting fraction into the target volume at the location (Fig. 1, bottom row) (Soufan et al., 2007). The sampling volume ($105^3\ \mu m^3$) contains enough nuclei to ensure reliable BrdU-positive fractions whereas the target volume ($21^3\ \mu m^3$) is small enough to avoid loss of biological resolution. This measurement procedure serves to recover the continuous proliferation information (Fig. 1, bottom right) from the binary information (Fig. 1, bottom left). Based on the total number of nuclei in the sample volume the maximum width of the 95% confidence interval of the BrdU-positive fraction at each location is 0.50 ± 0.07; for higher and lower fractions the confidence range is even narrower (Soufan et al., 2007).

Proliferation rate in the ventricles was also measured relative to the trabecular base which is defined as the junction of the trabecules and the compact myocardium. The trabeculations of both ventricles were separately segmented. For each position in the trabecules and the compact myocardium, the BrdU-positive nuclei were counted and the Euclidean distance to the nearest trabecular base was determined. For embryos between E10, and E12.5 this was done in cubic volumes of $7.2 \times 7.2 \times 7\ \mu m^3$, and for E14.5 and E17.5 embryos in $13.6 \times 13.6 \times 14\ \mu m^3$. Subsequently, the counted nuclei and BrdU-positive nuclei were pooled in 15 μm wide distance categories, which ensured that enough nuclei were counted in each distance zone to reliably determine the BrdU-positive fraction.

The original image data on which the 3D reconstructions are based are available from the authors on request.

Results

Morphometry: number of cardiomyocytes and cardiac volume

To quantify growth during the formation of the mouse heart we counted nuclei in, and measured the volume of, developing hearts between E8- and E17.5, thus ranging from the initially formed cardiac crescent to the prenatal four-chambered heart (Fig. 2). Because the cardiomyocytes are mono-nuclear until postnatal development (Liu et al., 2010) the number of nuclei represents the number of cardiomyocytes. The non-linear logarithmic relation of the number of cells and of the total myocardial