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

Bouke A. de Boer¹, Gert van den Berg¹, Piet A.J. de Boer, Antoon F.M. Moorman, Jan M. Ruijter*

Heart Failure Research Center, Academic Medical Center, Amsterdam, The Netherlands

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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.

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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...
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 salined (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-monomonal 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 × 0.9 μm² 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 μm³) contains enough nuclei to ensure reliable BrdU-positive fractions whereas the target volume (213 μm³) 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 × 7.2 × 7 μm³, and for E14.5 and E17.5 embryos in 13.6 × 13.6 × 14 μm³. 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
volume with developmental time indicates that the heart grows relatively fast in the younger stages and that a growth rate slows down in the oldest stages (Fig. 1). From day 8- in development up to day 11 the number of cardiomyocytes increases from 700 to 68,000, whereas a similar 100-fold increase is observed in cardiac volume (from 0.0014 to 0.150 mm³, Table 1). After E11 both growth rates taper off further, but still show an almost 13-fold increase approaching 1 million cardiomyocytes and a volume of almost 2 mm³ in the E17.5 heart. The parallel development of the number of cells and of total volume suggests that the average volume of cardiomyocytes is constant (approx. 2150 μm³) throughout embryonic and foetal development. Note that the graph gives no information on how the number of cells increase, nor does it imply that the growth rate is equal for the whole heart or that all cardiomyocytes have the same size. The quantitative 3D reconstructions presented below indeed show a highly regionalized pattern of proliferation.

Morphology and proliferation pattern

The reader is encouraged to read this text while referring to the interactive 3D-pdf file, which is available in the online Suppl. PDF.

Initial heart tube formation (Fig. 3)

Mouse development occurs on the bottom of a deeply invaginated yolk sac (Fig. 3a and d). This is in contrast to early chicken and human development, which occurs on an almost flat yolk sac. In chicken, the foregut lengthens more-or-less parallel to the surface of the yolk sac and has a narrow anterior intestinal portal (van den Berg et al., 2009). Due to the invagination of the yolk sac in mouse, the foregut lengthens in a perpendicular orientation to the yolk sac, and the anterior intestinal portal is wide (Kaufman and Navaratnam, 1981).
At E7.75 the cardiogenic part of the splanchnic mesoderm, expressing the transcription factor Nkx2.5, is present as bilateral plates of epithelium (Fig. 3d). The foregut just begins to form and can be identified as a ventral protrusion. No cardiovascular lumen can be discerned yet, which is consistent with previous analyses of the expression of molecular vessel markers (Wood et al., 1997). This flat pre-cardiac mesoderm displays rapid proliferation (Fig. 3g), as was also observed in chicken (van den Berg et al., 2009). Shortly later some endothelial cells are observed between the lateral sides of the splanchnic mesoderm and the endoderm (Suppl. Fig. 1d). The lateral mesoderm forms a gutter (Suppl. Fig. 1d). In line with the progression of development and folding, the foregut elongates. The medial aspects of the splanchnic mesoderm remain in close proximity to the foregut, forming the pericardial back wall with progressive folding (Suppl. Fig. 1e). At the site of vessel formation a drop in proliferation rate is seen in the left splanchnic epithelium (Suppl. Fig. 1f).

At E8- the cardiovascular lumen is formed. Moreover, the gutters, which form in the lateral aspects of the splanchnic mesoderm, have differentiated into Mf20-positive myocardium (Fig. 3e and j). This differentiation is accompanied by a drop in proliferation rate within the myocardium (Fig. 3j). With further elongation of the foregut, the medial aspects of the splanchnic mesoderm grow, forming the pericardial back wall (Suppl. Fig. 2a and d). Within the heart tube, the endocardium shows several finger-like protrusions, adhering to the myocardium (Fig. 3b).

With progressive fusion, the lateral myocardial gutters fuse in the ventral midline (Fig. 3f and k) which can be discriminated by a ventral fusion seam. The medial splanchnic mesoderm now forms a longer pericardial back wall, in line with growth of the foregut. Unlike observations in chicken (van den Berg et al., 2009), but resembling the pattern observed in human development (Sizarov et al., 2011), all splanchnic mesoderm in mouse is fast-proliferating (Fig. 3g–i). The endocardial tubes are entirely suspended in cardiac jelly, as in the human heart (Sizarov et al., 2011). The endocardium within the heart tube is fused and shows multiple ventral protrusions. The adjacent ventral part of the heart tube shows an increase in proliferation rate, especially on the left side (Fig. 3k). The dorsal side of the heart tube is still completely open and displays a low proliferation rate (Suppl. 3D PDF).

Lengthening and looping of the heart tube (Fig. 4)

Fig. 4 shows 3D reconstructions of mouse hearts ranging from E8- to E8.5. Other embryonic structures that provide a spatial context and indicate developmental progression are shown in the interactive Suppl. 3D-pdf. At E8 the ventral fusion seam of the myocardium is no longer discernable (Fig. 4d). Compared to E7.75 the endocardium shows more protrusions adhering to the

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### Table 1

Numerical summary of the analyzed individual mouse hearts. For each heart the total number of cells (from E9.5 onwards only nkx2.5 positive cells) within the heart, the total myocardial volume and the percentage of BrdU labelled cells are given.

<table>
<thead>
<tr>
<th>Embryonic day</th>
<th>Total #cells</th>
<th>Volume (mm$^3$)</th>
<th>% BrdU</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-</td>
<td>648</td>
<td>0.0014</td>
<td>33</td>
</tr>
<tr>
<td>8-</td>
<td>721</td>
<td>0.0013</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>1069</td>
<td>0.0025</td>
<td>32</td>
</tr>
<tr>
<td>8</td>
<td>1240</td>
<td>0.0024</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>1549</td>
<td>0.0031</td>
<td>34</td>
</tr>
<tr>
<td>8</td>
<td>1321</td>
<td>0.0022</td>
<td>37</td>
</tr>
<tr>
<td>8.25</td>
<td>1751</td>
<td>0.0033</td>
<td>36</td>
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<tr>
<td>8.25</td>
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<td>0.0043</td>
<td>39</td>
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<td>2332</td>
<td>0.0064</td>
<td>37</td>
</tr>
<tr>
<td>9.5</td>
<td>16745</td>
<td>0.0285</td>
<td>33</td>
</tr>
<tr>
<td>9.5</td>
<td>21319</td>
<td>0.0487</td>
<td>33</td>
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<tr>
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</tr>
<tr>
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<td>0.1480</td>
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<td>0.2941</td>
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</tr>
<tr>
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<td>23</td>
</tr>
<tr>
<td>17.5</td>
<td>919598</td>
<td>1.8611</td>
<td>21</td>
</tr>
</tbody>
</table>

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At E7.75 the cardiogenic part of the splanchnic mesoderm, expressing the transcription factor Nkx2.5, is present as bilateral plates of epithelium (Fig. 3d). The foregut just begins to form and can be identified as a ventral protrusion. No cardiovascular lumen can be discerned yet, which is consistent with previous analyses of the expression of molecular vessel markers (Wood et al., 1997). This flat pre-cardiac mesoderm displays rapid proliferation (Fig. 3g), as was also observed in chicken (van den Berg et al., 2009). Shortly later some endothelial cells are observed between the lateral sides of the splanchnic mesoderm and the endoderm (Suppl. Fig. 1d–f). The lateral mesoderm forms a gutter (Suppl. Fig. 1d). In line with the progression of development and folding, the foregut elongates. The medial aspects of the splanchnic mesoderm remain in close proximity to the foregut, forming the pericardial back wall with progressive folding (Suppl. Fig. 1e). At the site of vessel formation a drop in proliferation rate is seen in the left splanchnic epithelium (Suppl. Fig. 1f).
myocardium (Fig. 4a). The foregut (Suppl. Fig. 2c) elongates further and the pericardial back wall extends by the concomitant growth of the medial splanchnic mesoderm (Fig. 4g). Moreover, closure of the dorsal mesocardium starts by fusion of the left and right components of the pericardial back wall (Fig. 4g and m). The pattern of proliferation resembles that of the previous stage, displaying rapid proliferation in the ventral heart tube (Fig. 4j) and the splanchnic mesoderm (Fig. 4g) and slower proliferation where the myocardium is connected to the splanchnic mesoderm (Fig. 4m).

A few hours later, at E8.25, the lengthening of the heart tube and closure of the pericardial back wall continues and fusion of the endocardial tubes progresses. The heart tube loops, and its elongation at the arterial pole becomes apparent by a clearly distinguishable outflow tract (Suppl. Fig. 3g). Upstream from the outflow tract, relative to the blood flow, the future left ventricle forms at the outer curvature of the looped heart. At E8.5, fusion of the endocardial tubes at the inflow of the heart tube is completed and the resulting fused intra-cardiac lumen is slightly displaced to the left of the embryonic midline (Fig. 4b). The ventricular region bulges ventrally and caudally accompanied by rapid proliferation at the outer curvature of the outflow tract and the forming ventricle (Fig. 4k); foci with rapid proliferation appear to be associated with endocardial protrusions. The inner curvature still shows slower proliferation and is now completely closed (Fig. 4n).

At E8.5 the intra-cardiac portion of the inflow vasculature is located left from the midline (Fig. 4c). The endocardium at the inflow shows a constriction (Fig. 4c) that we designate as the future atrioventricular canal (AVC). Note that this does not imply a definite indication of lineage, since these cells will eventually contribute to the left ventricular free wall (Aanhaanen et al., 2009). Within the myocardium of the future AVC slower proliferation is observed (Fig. 4o), consistent with previous studies (Aanhaanen et al., 2009). The lengthening of the heart tube progresses, as is revealed by the grown outflow tract and the further bulging of the prospective ventricle and the caudal displacement of its apex (Fig. 4f). The dorsal mesocardium is ruptured completely, leaving the heart tube suspended only by its arterial and venous poles (Fig. 4f and i).

**Formation of the four-chambered heart (Figs. 5 and 6)**

Figs. 5 and 6 show reconstructions of the embryonic mouse hearts from E9.5 through E12.5 and from E14.5 through E17.5, respectively. At E9.5 the outflow tract has elongated, and shows the characteristic bend (Fig. 5d). Only the distal part of the OFT (downstream from the bend) shows slow proliferation. In contrast, closer to the left ventricle the outflow tract shows higher proliferation rates along with formation of trabecules (Fig. 5g), indicating differentiation into right ventricular myocardium (Zaffran et al., 2004). A clear ventricular septum cannot be discerned, although an indentation between both ventricles is present. The AVC is still clearly recognizable between the left ventricle and the atria. At the inflow, the left and right atria are formed, only proliferating at the lateral sides (Fig. 5d and j). The
confluence of the systemic veins is shifted towards the right. At E9.5 the highest rates of proliferation are found in the developing chambers. One day later, at E11 (Fig. 5h), the indentation between the ventricles has become more prominent and a clear trabecular ridge has developed. The AVC is now suspended over this future interventricular septum enabling a direct connection of the atria to their respective ventricles (Fig. 5h). At this stage the distal outflow tract still is almost devoid of BrdU-incorporation (Fig. 4e) indicating that its growth occurs almost exclusively by addition of cells (Zaffran et al., 2004).

**Fig. 4. Morphology and proliferation in early looping mouse heart.** Panels (a)–(o) show different views of three reconstructions of mouse heart development, between embryonic day 8 and 8.5. Panels (a)–(c) show the intra cardiac vasculature (myocardium shown in transparent grey). Panels (d)–(f) show the myocardium (grey) and the splanchnic mesoderm (yellow). Proliferation rates in the splanchnic mesoderm and myocardium are shown in panels (g)–(i) and (j)–(o), respectively.
Fig. 5. Morphology and proliferation of mid-gestational embryonic mouse hearts. Reconstructions of the myocardium and lumen of mouse hearts ranging from embryonic day 9.5–12.5 are shown. Panels (a)–(c) show the development of the cardiovascular lumen in relation to the myocardium (shown transparently). Panels (d)–(l) show proliferation in the myocardium. Panels (a)–(f) show a ventral views, panels (g)–(i) show a ventral surface cut of the same hearts, and panels (j) and (k) show dorsal views. All reconstructions are also interactively available in the supplements. (Abbreviations: BrdU: BromodeoxyUridine, AVC: atrioventricular canal)
During further development, the overall morphology in the ventricles and outflow tract remains largely similar, although volume growth continues. From E12.5 onwards, the ventricular septum progressively forms and shows a high proliferation rate except at its top where proliferation is slower (Fig. 5i) as reported previously (Bakker et al., 2008). The trabeculated part of the outflow tract gradually differentiates into the right ventricle (Fig. 5d–f, Fig. 6c and d), finally generating a fully septated four chambered heart. At E11 and E12.5 proliferation in the outflow tract remained slow but both ventricles still show BrdU-positive fractions (Fig. 5e and f) that are similar to those at E8.5 (Fig. 4i and l). The proliferation rate in the ventricular wall decreases steadily in later stages (Fig. 6c–h).

The BrdU-positive fractions in both ventricles at E9.5 till E17.5 were measured in relation to the base of the trabecules (Fig. 7). The youngest stages show the highest proliferation rate in the ventricular wall close to the trabecular base. This proliferation rate decreases with increasing distance to the base. In later stages the BrdU-positive fractions are lower and this gradient becomes less obvious, coinciding with the disappearance of the fast proliferating foci on the surface of the ventricle (Fig. 6d and h). Although at all stages the BrdU-positive fractions in the trabecules are about 10% lower than in the outer wall, the observed decrease in proliferation rate with increasing distance from the trabecular base remained significant at all stages.

Discussion

The comprehensive series of 3D reconstructions that we prepared provides insights into both morphology and proliferation pattern in the developing mouse heart and can thus be used to interpret the growing body of scientific data harvested in the mouse.

Mouse heart development

Whereas in chicken and human the different phases of early heart development, namely the formation of the tube, the tube elongation phase and the chamber formation, are clearly separated in time, our 3D reconstructions show that during early mouse heart development these different growth phases overlap.

Initial tube formation

In chicken, the differentiation of mesoderm into myocardium occurs concomitantly with midline fusion of the left and right lateral mesoderm (O’Connor and Runquist, 2008; Stalsberg and de Haan, 1969). In mouse, due to its wide intestinal portal, the lateral aspects of the splanchnic mesoderm cannot easily adjoin, and myocardial differentiation occurs prior to midline fusion (Kaufman and Navaratnam, 1981). Although this phase of cardiac development in the mouse is generally known as the cardiac crescent, if compared to chicken development this stage represents a laterally orientated, not yet fused, straight heart tube. Progressive folding, as seen by lengthening of the foregut, causes the left and right myocardial components of this early heart tube to finally join in the midline (Mommersteeg et al., 2010) which can still be appreciated by the ventral seam seen at E8- (Fig. 3k). As in chicken (Soufan et al., 2006; van den Berg et al., 2009) the proliferation rate drops when mesoderm differentiates into cardiomyocytes. However, the re-initiation of proliferation that was observed in chicken after looping at the site of chamber formation (Soufan et al., 2006), already occurs at this stage, most prominently at the left side. The different growth phases observed in chicken (van den Berg et al., 2009) and human heart development (Sizarov et al., 2011) are thus represented in this one stage of mouse heart development. During further development a slowing down of proliferation is observed when cells pass through the transition of mesoderm to myocardium, whereas the foci of fast proliferation at the outer curvature are a sign of the start of chamber development.
Lengthening and looping of the heart tube

As described in chicken (Moreno-Rodriguez et al., 2006), closure of the dorsal wall of the heart tube starts in the middle and proceeds in the cranial and the caudal direction to “zipper up” the heart tube. In contrast to chicken development, elongation of the outflow tract in mouse is initiated prior to rupture of the dorsal mesocardium. However, in both species the distance between the arterial and venous poles remains constant while the heart tube rapidly lengthens. In mouse this distance is approximately 200 μm (Patten, 1922); in chicken at comparable stages this distance measures about 700 μm (van den Berg et al., 2009).

In chicken the caudal part of the splanchnic mesoderm harbours a fast proliferating growth centre (van den Berg et al., 2009); in mice, however, the whole dorsal region displays the highest proliferation rate and the cranial, slower proliferating region that is present in chicken does not exist. This overall high proliferation rate is probably a requirement because of the small size of the region that needs to provide the cells that are added to both poles of the heart (Cai et al., 2003; Kelly et al., 2001; Meilhac et al., 2004; Snarr et al., 2007; Waldo et al., 2001; Zaffran et al., 2004). The precise driving forces of cardiac looping remain unclear (review: Taber, 2006), although it has long been recognized in chicken (Patten, 1922) that ventral looping occurs of necessity because the heart tube lengthens faster than the distance between its arterial and venous poles. Our reconstructions show that this principle may also be valid during mouse heart formation.

Chamber formation

The regionalization of proliferation, which can already be seen in the developing ventricular wall in early stages might be due to a genetic interaction at the endo- and myo-cardiac connections, which are known sites where Notch signalling mediates, proliferation and trabecular differentiation (Grego-Bessa et al., 2007). Such endo- and myo-cardiac connections are present in the ventral, fastest proliferating, part of the heart tube. Evidence for chamber differentiation was previously demonstrated to occur already at the 3–4 somites stage; using electron microscopy, formation of gap-junctions was observed in the ventral region of the developing heart, which also showed mitoses and formation of a multilayered and trabeculated wall (Navaratnam et al., 1986).

Proliferation and morphogenesis

Evidence has accumulated that, after formation of the primary heart tube, cells from the second heart field are added to the heart tube at the arterial pole of the heart (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). The primary heart tube would then originate from a first heart-forming field. It was later demonstrated that the second heart field also contributed cells to the venous pole of the heart (Cai et al., 2003; Snarr et al., 2007) and that two lineages of precursor cells contribute to the heart (Meilhac et al., 2004).

A separation of the heart-forming region does occur with the rupture of the dorsal mesocardium, limiting the contact between the forming heart tube and the pericardial back wall to the arterial and venous poles. The distance between the arterial and venous poles is then still only about 200 μm, or a column of about 15–20 precursor cells. Within this small region distinct anterior and posterior domains of gene expression have been proposed and prior to rupture of the dorsal mesocardium the medial part of this region contained the precursors for the left ventricle, or first heart field (Kelly and Buckingham, 2002). Our analyses of morphogenesis and proliferation cannot address these issues, but reveal a continuing temporal formation of the heart from precursors in the dorsal mesoderm, which in all stages shows the overall highest proliferation rate.

As in human and chicken, proliferation in the mouse myocardium is highly regionalized, with slower proliferation in the atrioventricular canal, outflow tract and inner curvature, and rapid proliferation in the forming chambers. The patterns of clonal growth within the myocardium observed by Meilhac and coworkers (Meilhac et al., 2004; Meilhac et al., 2004; Meilhac et al., 2003) are in agreement with the observed proliferation patterns. Meilhac and co-workers observe a dispersive growth phase, generating cells that intermingle during recruitment into the heart, followed by a coherent growth phase leading to clusters of daughter cells (Meilhac et al., 2003). Our current observations show that the highly proliferative cardiac precursors in...
the dorsal mesocardium are separated from the proliferating chamber-forming myocytes by a zone of non-proliferative newly differentiated cardiomyocytes. This indicates that both growth phases occur simultaneously. The start of the coherent growth phase, at E8.5 (Meilhac et al., 2003), coincides with the observed re-initiation of proliferation at the site of chamber formation and can thus explain the occurrence of most and largest clones in the developing chambers (Meilhac et al., 2004). The observed variation in cluster size in different cardiac compartments (Meilhac et al., 2004a; Meilhac et al., 2004b) may result from the local differences in proliferation rate thus leading to remodelling of the heart tube.

Formation of the trabecules

During chamber differentiation, the walls of the left and right ventricles form trabeculated myocardium on the luminal side, while the remaining outer lining later differentiates into compact myocardium. The trabeculated myocardium and the outer myocardial wall adopt distinct programs of gene expression, reflecting both changes in function and differential modes of growth.

Our analysis of proliferation within the myocardial walls and the trabecules shows the most rapid proliferation at the junction between the outer wall and the base of the trabecules, while proliferation rate tapered off towards the top of the trabecules. This persistent base-to-top gradient, in both ventricles, showed that the focus of proliferation of cardiomyocytes in the forming ventricles is at or near the base of the trabecules. Similarly, retroviral labelling of pre-cardiac mesoderm in chicken showed elongated clusters of labelled cells in the myocardial wall, extending into the trabecules (Mikawa et al., 1992). Genetic lineage analyses in mouse, using randomly generated clones, also showed orientation of growth along the transmural axis (Meilhac et al., 2003). In combination with the fast proliferation observed at the base of the ventricular trabecules, these data suggest growth of the ventricles by apposition, thereby elongating the trabecules and causing an outward expansion of the ventricular chambers (Moorman and Christoffels, 2003). This conclusion is further supported by the observation that activated Notch signalling, which regulates the proliferation and differentiation of the trabecules, is most abundant at the base of the trabecules (Grego-Bessa et al., 2007). Regression of proliferation in the trabecules has been suggested to imply terminal differentiation into the conductive system (Sedmera et al., 2003). Our study shows, however, that both compartments continue to proliferate, albeit at different rates. Furthermore, both compartments differentiate into distinct phenotypes. Therefore, a strict inverse relationship between differentiation and proliferation cannot be assumed.

Taken together, our analyses of proliferation rate in the developing mouse heart from the initial formation of the heart up to the late prenatal four-chambered heart forms a valuable resource for future mechanistic studies. The interactive 3D reconstructions allow for an independent inspection of the 3D-architecture in relation to this important parameter of growth.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.05.001.

References
