Growth of the developing heart
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Chapter 8

Growth of the Developing Mouse Heart: a quantitative 3D analysis
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Abstract
Much of our current knowledge on cardiac development is derived from the mouse, a mammalian model permitting molecular analyses along with genetic lineage tracing. Important as the results of these analyses are, they can only be fully exploited when supplemented with a clear insight into the growth of the heart, which is currently lacking. In an attempt to fill this gap we assessed, in a quantitative fashion, the patterns of proliferation in the forming mouse heart and in its adjacent splanchnic mesoderm, known to contribute to the forming heart. This, in turn, required 3D insight into early heart formation in the context of the entire cranial region of the forming mouse embryo. The results are presented in an interactive portable document format (pdf) to facilitate communication and understanding. As in human and chicken, we show that the splanchnic mesoderm is highly proliferative and that upon recruitment of cells into the cardiac lineage their proliferation rate drops. Proliferation locally increases at the sites of chamber formation, generating heterogeneous patterns of proliferation. Further quantitative analyses show a gradual decrease in proliferation rate of the ventricular walls with progression of development, and a base-to-top decline in proliferation in the trabecules. Comparison between the left and right sides of the heart tube imply differential addition of cells to the myocardial lineage as a cellular mechanism of rightward looping. Our data offer clear insights into the growth and orphogenesis of the mouse heart and provide a firm basis for future 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 [1]. Insight into these processes is not only crucial for the understanding of cardiac morphogenesis, but is also at the base of cellular lineage analyses [2] and molecular mechanistic data, which have provided a great impetus to the field of cardiac developmental biology [3,4].

Data on cardiac proliferation, however, are scarce and rarely placed within a three-dimensional (3D) context, leading to conflicting conclusions such as either rapid [5] or slow [6] proliferation in the early heart tube. Previously, we analyzed proliferation within the spatiotemporal context of the developing chicken. We observed that proliferation stops when myocardium differentiates from fast proliferating splanchnic mesoderm, to be followed by a new phase of rapid proliferation at the sites of chamber formation [7,8]. A similar pattern was observed during human heart formation [9]. Information on local proliferation rates in the developing mouse heart is, however, entirely lacking.

To generate a general framework of cardiac growth in the mouse we performed a qualitative and quantitative 3D analysis of morphogenesis and proliferation of the developing mouse heart. Whereas in chicken and man the different phases of early heart development, namely formation of the tube, the tubular phase and chamber formation, are clearly separated in time, we show that early heart development in the mouse is very fast and the different growth phases overlap, which interferes with the analysis. The 3D quantification of proliferation shows that, as in chicken and human development, the mouse heart forms by a slow down of proliferation within the splanchnic mesoderm, followed by rapid proliferation at the site of chamber differentiation albeit that these processes overlap in time thus leading to a very heterogeneous proliferation pattern. During further chamber formation the proliferation rate decreases progressively whereas proliferation in the trabecules shows a base-to-top decline at every stage. We supplemented our account with 3D models of the developing mouse heart presented in an interactive format.
**Materials & Methods**

**Animal handling & immunohistochemistry**

Bromodeoxy-Uridine (BrdU) was used to visualize proliferating cells. The more rapid a tissue proliferates, the more cells will incorporate BrdU, making the fraction of BrdU-positive nuclei a marker for proliferation rate \[10\]. Our observed nuclear BrdU-fractions are a direct measure for cellular proliferation, because multi-nucleation and polyploidy does not occur prenatally \[11\].

Exposure to BrdU (Sigma nr. B5002) prior to E9 took place by culturing FVB mouse embryos in 0.05 mg BrdU / ml culture medium (DMEM (Invitrogen), pH 7.4 supplemented with 10% fetal calf serum). Older stages were exposed to BrdU by peritoneal injection of pregnant mice with 50 mg/kg of BrdU (using a 10 mg BrdU / ml 0.9% NaCl solution), as previously described \[12\]. All experimental procedures complied with national and institutional guidelines for animal welfare. Exposure to BrdU in this study was always 1 hour. Immediately after exposure, embryos were fixed in freshly prepared 4% paraformaldehyde. Embryos were staged according to general morphological characteristics \[13\].

Standard procedures were used for dehydration and embedding in paraffin \[14\]. Embryos were serially sectioned at 7µm. Mounted sections were dewaxed and rehydrated. Rehydrated sections were equilibrated in phosphate buffered saline (PBS), after which antigens were retrieved by pressure cooking for 3 minutes in antigen unmasking solution (1:100 Vector laboratories Inc. H-3300). After a PBS wash, sections were blocked with 2% bovine serum albumin. Primary antibodies were then added for overnight exposure; these were rat-monoclonal anti-BrdU (1:600, Immunosource OBT0030CX), rabbit polyclonal anti-cTnI (1:250 HyTest 4T21/2), and goat polyclonal anti-Nkx2.5 (1:200 Santa Cruz 8697). After washing, secondary antibodies were added for 2 hours. Firstly, donkey-anti-goat Alexa-680, and, again after washing, goat-anti-rat Alexa-405 and goat-anti-rabbit Alexa-568 were added (Invitrogen, A-21084, A-31556, A-11077, respectively). After washing, Sytox Green (1:40,000 Molecular Probes S-7020) was added for 15 minutes before a last wash and mounting of a coverslip with vectashield (Vectorlabs H1000).

**3D-reconstruction and morphometry**

Sections were photographed with a fluorescence microscope (Leica DM6000) at 10 times magnification, generating 4 series of images with pixels representing 0.9 x 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 segmented based on either a specific signal (cTnI or Nkx2.5), or by morphological criteria (mostly using the Sytox Green signal). After segmentation, the myocardium and splanchnic mesoderm labels were inspected to ensure that all nuclei were included. This resulted in labels with a voxel-size of 0.9 x 0.9 x 7 µm³. For conversion into a 3D reconstruction, labels were re-sampled to generate near isometric voxels (7.2 x 7.2 x 7 µm³). Smoothing of the data set was done by automatic modules in Amira, and by manual correction of sectioning artefacts. The resulting re-sampled and smoothed data set was then converted into a 3D-surface by triangulation. The number of triangular faces was reduced to approximately 7600, and the resulting surface was smoothened by the Smooth Surface module. For 2D presentation, snapshots were laid out in Illustrator (Adobe, CS4 - 14.0.0). For 3D presentation, 3D pdf-files were generated as previously described [15].

Detection and quantification of nuclei was performed using a program in Matlab (The Mathworks). In short, in the Sytox Green images the local background was subtracted from the nuclear signal. The resulting local maxima were then thresholded into binary nuclei. This image was masked by the segmented label of either the myocardium or the splanchnic mesoderm. For nuclei within this mask, a positive BrdU-signal was defined as a nucleus with a staining intensity of at least a standard deviation above its surrounding background intensity. For embryos from E10 onward, Nkx2.5-positive nuclei were identified in the same manner as was done for the BrdU signal, because from that stage onward non-myocytes gradually invade the heart [16]. BrdU-positive fractions of either all or, for the older stages, of the Nkx2.5-positive nuclei were then determined in cubic sampling volumes of 63³ µm³, and projected onto their 21³ µm³ central volume and mapped onto the surface reconstruction, as described previously [17].

Proliferation in the ventricles was measured relative to the trabecular base. The trabeculations of both ventricles were separately segmented. The number of nuclei and BrdU-positive nuclei were counted, as described above, in the trabecules and in the ventricular wall. For embryos between E10, and E12.5 this occurred in cubic volumes of 7.2 x 7.2 x 7 µm³, and for E14.5 and E17.5 embryos in 13.6x13.6x14 µm³. In addition, for each sample volume the distance to the nearest trabecular base was determined. Subsequently, the counted nuclei and BrdU-positive nuclei were pooled in 15 µm wide bins, which ensured that enough nuclei were counted to reliably determine BrdU-positive fractions for each distance bin [17]. Measurements were done in the left and right ventricular trabeculations and walls.
Results

Morphology

Whereas most knowledge on cardiac morphogenesis comes from classic studies of mostly chicken and human embryos [18,19], the mouse embryo is currently the most important animal model. In order to grasp its complex cardiogenesis, we created a series of 3D reconstructions of the developing mouse heart. The reader is encouraged to read this text along with the interactive 3D-pdf file, available in the online Data Supplement.

Initial heart tube formation

Within one day, from embryonic day (E) 6.75 through E7.75 the straight heart tube is formed from the cardiogenic mesoderm, which occurs on the bottom of a deeply invaginated yolk sac (Figure 1). This is in contrast to early chicken and human development, which occurs on an almost flat yolk sac [18]. In chicken, the foregut lengthens more-or-less parallel to the surface of the yolk sac and has a narrow anterior intestinal portal [7]. 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 [20]. Albeit not principally, due to this distinct orientation, heart formation in the mouse differs architecturally from the “text-book view” of heart formation in several aspects, as shown and discussed below.

At E6.75 the cardiogenic splanchnic mesoderm, defined as expressing the transcription factor Nkx2.5 and facing the endoderm, is present as bilateral plates of flat epithelium (Figure 1 a-c). The foregut has just begun to form and can be identified as a ventral protrusion in the yolk sac. No cardiovascular lumen can yet be discerned, which is in line with previous analyses of the expression of vascular markers [21]. Note that for the sake of clarity only part of the splanchnic mesoderm was included in the reconstructions. Shortly later, at E7 some endothelial cells were observed between the lateral sides of the splanchnic mesoderm and the endoderm. The lateral mesoderm formed a gutter, which is more prominent on the left side (Figure 1-f). The left lateral view (Figure 1-e) shows that, in line with the progression of development and folding, the foregut is more elongated. The medial aspects of the splanchnic mesoderm remained in close proximity to the foregut, forming the pericardial back wall with progressive folding (Figure 1-g).

At E7.5 the cardiovascular lumen was clearly present. Moreover, the lateral aspects of the splanchnic mesoderm had not only formed gutters, but these gutters had also differentiated into cardiac troponin I (cTnI) positive myocardium (Figure 1-h,j). With further elongation of the foregut, the medial aspects of the splanchnic mesoderm
Figure 1: Morphological overview of initial mouse heart-tube formation. Different views of four reconstructions of mouse heart development, between embryonic day 6.75 and 7.75. Panel a, d, h & l show the relation of the forming heart with the endoderm of the yolk sac. Panel b, e, i & m show a right lateral view of the cardiogenic mesoderm and/or the developing myocardium with the foregut. Other views and structures as depicted. All reconstructions are also interactively available in the supplements. (Abbreviations: AIP: anterior intestinal portal; myo: myocardium.)
grew, forming the pericardial back wall (Figure 1-k) The lumina at the cardiac inflow now also connect to the vessels that return to the blood islands covering the yolk sac (Figure 1-j). Within the heart tube, the endocardium showed several finger-like protrusions (obscured by myocardium - see supplemental interactive 3D-pdf). With progressive fusion, at E7.75, the lateral myocardial gutters had fused in the midline (Figure 1-l,n). The site of this ventral fusion could be discriminated by a ventral seam. The medial splanchnic mesoderm had formed a longer pericardial back wall, in line with growth of the foregut. The endocardium within the heart tube showed fusion, and multiple ventral protrusions (see interactive 3D supplements for individual structures). At the venous pole, the inflowing vessels were growing and discernable as the vitelline veins, which were connected to the blood islands (Figure 1-n).

In chicken, the differentiation of mesoderm into myocardium occurs concomitantly with midline fusion of the left and right lateral mesoderm [22,23]. In mouse, due to its wide intestinal portal, the lateral aspects of the splanchnic mesoderm cannot easily adjoin, by which myocardial differentiation occurs prior to midline fusion while they are still laterally extending [20]. Although this phase of cardiac development in the mouse is generally known as the “cardiac crescent”, this stage in reality represents a laterally orientated, not yet fused, straight heart tube, if compared to chicken development. 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 midline [14], which can still be appreciated by the ventral seam seen at E7.75.

**Lengthening and looping of the heart tube**

Figure 2 shows 3D reconstructions of mouse hearts ranging from E8 to E9. The other embryonic structures that provide an anatomical context and indicate developmental progression, such as the forming neural tube and coelomic cavity, are shown in the interactive supplemental 3D-pdf. At E8 the heart had grown and the ventral fusion seam was no longer discernable (Figure 2-b). At the inflow, however, the left and right components could still be discriminated by the un-fused endocardial tubes. Outside the heart tube these vessels extended laterally (Figure 2-a). Cranially, the endocardium still showed multiple protrusions adhering to the myocardium, best seen in the left lateral view (Figure 2-d). Compared to E7.75 (Figure 1-m), the foregut was further elongated. During the further folding of the embryo, the pericardial back wall extended by growth of the medial splanchnic mesoderm (Figure 2-c). Moreover, the left and right components of the back wall were fusing, indicating the closure of the dorsal mesocardium.
**Figure 2:** Morphological overview of looping of heart tube. Panels a - p show different views of four reconstructions of mouse heart development, between embryonic day 8 and 9. Panels a, e, i & m show the development of the cardiovascular lumen in relation to the myocardium (transparent). Panels b, f, j & n show the myocardium and the splanchnic mesoderm. Panels c, g, k & o show the splanchnic mesoderm (myocardium is shown in transparent grey). Panels d, h, l & p show a left lateral view of the relation of the lumina and the myocardium (transparent) with the developing foregut and anterior intestinal portal. Note that the images of the lumina were taken at a slightly different angle than those of the myocardium. The bottom panel illustrates closure of the dorsal mesocardium by showing dorsal views of 5 hearts, ranging from embryonic day 7.75 to 9. * indicates the connection of the heart tube with the dorsal mesocardium. All shown reconstructions are interactively available in the supplements (Abbreviations: cardiovasc.: cardiovascular; E: embryonic day)
A few hours later, at E8.25, the lengthening and closure of the pericardial back wall continued and fusion of the endocardial tubes progressed. The inflow vessels were now identifiable as the vitelline veins and were in contact with the blood islands covering the yolk sac (Figure 2-e). These inflow vessels bended caudally by the progressive folding of the anterior intestinal portal. Inside the myocardial heart tube, ventral endocardial protrusions were present (Figure 2-e,h). The heart tube itself had clearly looped, and its elongation at the arterial pole had become apparent by a clearly distinguishable outflow tract (Figure 2-f). Caudally from the outflow tract 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 was completed and the resulting fused intra-cardiac lumen had slightly displaced to the left of the embryonic midline (Figure 2-i). Like the previous stage, the vitelline veins bended caudally and there were multiple protrusions of endocardium, attached to the ventral ventricular region of the myocardium (Figure 2-i,l). The ventricular region bulged ventrally and caudally, and the embryonic outflow tract was further elongated (Figure 2-j).

At E9, the orientation of the inflow vasculature had changed significantly. The inflowing vitelline veins had grown and clearly bended towards caudal and twisted towards the right following the general embryonic rotation [13]. The intracardiac portion of the inflowing vasculature was located left from the midline (Figure 2-m). The endocardium at the inflow showed a constriction (Figure 2-p) that we designated as the future atrioventricular canal, because the atria were not yet present. The lengthening of the heart tube progressed, as depicted by the grown outflow tract and the further bulging of the prospective ventricle and the caudal displacement of its apex (Figure 2-n). This can best be observed in the left lateral image, which also shows the foregut with its forming pharyngeal pouches (Figure 2-p). The dorsal mesocardium had ruptured completely, thus closing the pericardial back wall and leaving the heart tube suspended only by its arterial and venous poles (Figure 2-o).

The closure of the dorsal mesocardium is also shown in the bottom panel of Figure 2 in a series of dorsal views of six hearts, ranging from E7.75 to E9. At E7.75 it can be seen that the dorsal side of the heart tube is still completely open. At this stage the endocardial tubes are entirely suspended in cardiac jelly, as in the human heart [9]. At E8 the dorsal aspects of the heart tube approached each other and closure followed a few hours later at stage E8.5. As described in chicken [24], closure of the dorsal mesocardium starts in the middle of the heart tube and proceeds in cranial and caudal direction to “zipper up” the dorsal heart tube. Interestingly, unlike chicken development, elongation of the outflow tract, which occurs by addition of cells from the pericardial back wall [25]
was initiated prior to rupture of the dorsal mesocardium. The distance between the arterial and venous poles remained constant while the heart rapidly lengthened. In mouse this distance was approximately 200 µm; in chicken at comparable stages this distance measured about 700 µm [7,26]. It is this small region that is called the second heart field and provides cells that are added to both poles of the heart [25,27-31].

Formation of the four-chambered heart

Figure 3 shows morphological reconstructions of the embryonic mouse hearts from E10 through E17.5. At embryonic day 10 (Figure 3-a,b), both atria had clearly formed, when compared to E9 (Figure 2, E9 – bottom panel). The outflow tract had elongated, and showed a bend. In the proximal part of the outflow tract the first trabecules appeared, indicating differentiation of outflow tract myocardium into right ventricle [25]. A clear ventricular septum could not be discerned. At the inflow, a left and right atrium had clearly formed. Like at E8.5 and E9 (Figure 2-I,m) the blood flow entered the left ventricle. The confluence of the systemic veins, however, shifted towards the right. One day later, at E11 (Figure 3-c,d), the blood flow from the atria entered both ventricles, as the atrioventricular canal became suspended over the interventricular...
septum, which was becoming discernable. During further development, the overall morphology in the ventricles and outflow tract remained largely similar, although there was progressive volume growth (also see Figure 4). From E12.5 onwards, the ventricular septum progressively formed and the outflow tract gradually differentiated into the right ventricle (Figure 3-f), finally generating a fully septated four chambered heart.

**Morphometry**

*Volume and number of cells*

To quantify growth during the formation of the mouse heart we counted nuclei in, and measured the volume of the developing heart at stages ranging from E7.25 to E17.5, thus ranging from the initially formed “cardiac crescent” to the prenatal four-chambered heart (Figure 4). Because the cardiomyocytes are mono-nuclear until post-natal development,[11] the number of nuclei represents the number of cardiomyocytes.

![Figure 4: Quantification of number of cells and volume](image)

The number of cardiomyocytes in the developing mouse heart was derived from the number of nuclei observed within the tissue labelled by the specific staining with a cardiac marker. The number of nuclei per tissue area was converted into number per volume according to Abercrombie [65], including the section thickness and the average nuclear diameter. Myocardial volume was calculated according to the Cavalieri principle [66]. Note that the data are displayed on a logarithmic Y-axis. Accompanying the data are reconstructions of the myocardium ranging from embryonic day 7.5 to 17.5, displayed at the same scale.
The approximate linear relation of the logarithm of the number of cells, as well as total myocardial volume, to the days of development indicated that the increase in cell number and volume was exponential until E11. During this period, the number of cardiomyocytes increased from 680 to 44000, whereas a similar 65-fold increase was observed in cardiac volume (from 0.002 to 0.128 mm$^3$). After E11 these relations tapered off, pointing to a slower but still almost 20-fold increase until approximately 1 million cardiomyocytes and a volume of 2 mm$^3$ in the E17.5 heart. The parallel development

**Figure 5:** Local proliferation rates in the early mouse heart. The figure shows the patterns of BrdU-incorporation (after one hour exposure) in the reconstructions that are shown in Figure 2 and 3. Note that for esthetical reasons the reconstruction of the mouse heart at embryonic day 8.5 was not included. The patterns of proliferation rate were highly similar to those at embryonic day 8.75, and available in the interactive supplements. (Abbreviations: BrdU: BromodeoxyUridine)
of the number of cells and total volume graphs showed that the average volume of individual cardiomyocytes is constant (approx. 2300 μm$^3$) throughout embryonic development. This average volume is in agreement with earlier observations [8]. Note that this graph gives no information on how the number of cells increases, nor does it imply an equal growth rate for the whole heart, as often implied. Indeed, volume measurements on the developing atria [32] showed that different parts of the atria grew at different and changing rates during development. The quantitative 3D reconstructions presented below clearly show a very heterogeneous pattern of proliferation laying at the basis of early cardiac growth and morphogenesis.

**Figure 6: Local proliferation rate in older embryonic mouse hearts.** Panels a, c, e, g & i show frontal views of quantitative 3D reconstructions of the myocardium of hearts ranging from embryonic day 10 to 17.5. Panels b, d, f, h, j show the same quantitative reconstructions with a frontal surface cut. Note that the hearts are not depicted on the same scale. The bottom panels show detailed quantifications of the ventricular walls and trabeculations. The y-axes indicate the BrdU-labelled fractions. The x-axes indicate the distance from the trabecular base.

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Proliferation pattern during early stages of heart development

To gain insight in the patterns of proliferation in the myocardium and its precursors, we measured the proliferation index after one hour of BrdU exposure and projected the proliferation patterns onto the 3D-reconstructions [17]. In chicken, such experiments showed withdrawal of myocytes from the cell-cycle upon myocardial differentiation, and a decrease in proliferation rate was observed when vessels formed within the splanchnic mesoderm. Growth of the early heart tube occurred by addition of cells from a rapidly proliferating group of cells at the inflow of the heart, which were shown to contribute to both the inflow and outflow of the heart. Where primary myocardium differentiated into chamber myocardium, proliferation was locally reinitiated [7,8]. In human embryos, similar observations were recently made [33].

At the youngest mouse stage studied, E6.75, no myocardium was observed. In line with observations in chicken, the flat pre-cardiac mesoderm displayed rapid proliferation (Figure 5-a). A little later, at E7, a drop in proliferation was seen in the left splanchnic epithelium, at the site of vessel formation (Figure 5-b). Later differentiation into myocardium at stage 7.5, showed a drop in proliferation rate within the myocardial “cardiac crescent”. In contrast to chicken development [7], proliferation was never absent, but a rather scattered pattern of proliferation could be observed in the initially formed myocardium (Figure 5-c).

At E7.75 there was still a heterogeneous pattern of proliferation, with less BrdU-incorporation at the dorsal connection with the splanchnic mesoderm, and an increase in proliferation rate in the ventral heart tube (Figure 5-e,f). Unlike observations in chicken, but resembling the pattern observed in human development [9], no clear regionalization of proliferation could be observed within the splanchnic mesoderm (Figure 5-d,g,j,m,p). At embryonic day 8 the pattern of proliferation resembled that of the previous stage, displaying slow proliferation in the myocardium near the dorsal mesocardium and rapid proliferation in the ventral heart tube (Figure 5-h,i). At stage E8.5, with clear differentiation of the outflow tract, a focus of rapid proliferation occurred at the outer curvature of the outflow tract and forming ventricle, contrasting the slow proliferation at the inner curvature of the heart (Figure 5-k,l). This pattern of proliferation still existed at E9 (Figure 5-n,o). Proliferation was also slow within the myocardium of the future atrioventricular canal, consistent with previous studies [12].

Proliferation during formation of the four-chambered heart

Between E9 (Figure 5-n) and E10 (Figure 6-a,b) the BrdU-positive fraction in the distal outflow tract decreased. At E11 the outflow tract was almost devoid of BrdU-incorporation (Figure 6-c) indicating that cells of the outflow tract display very slow
proliferation and growth occurs almost exclusively by addition of cells [25]. At later stages (Figure 6-e,g,i) the proliferation rate in the outflow tract remained slow. From E10 through E12.5 (Figure 6-a,c,e), both ventricles showed BrdU-positive fractions that were similar to those at E9 (Figure 5-n). At these stages, the forming ventricular septum showed a similar high proliferation rate except at its top where proliferation is slow (Figure 6-f), as reported previously [34]. The proliferation rate in the ventricular wall decreased steadily in later stages. The BrdU-positive fractions in both ventricles, were measured in relation to the base of the trabecules (Fig 6, bottom panel). The youngest stages showed the highest BrdU incorporation in the ventricular wall close to the trabecular base, which diminished with increasing distance. In later stages the BrdU-positive fractions were lower and this gradient became less obvious, coinciding with the disappearance of the patchy pattern on the surface of the ventricle. Although at all stages the BrdU-fractions in the trabecules were about 10 percent lower than in the outer wall, the observed decrease with increasing distance from the trabecular base remained significant at all stages and corresponding trabecular length. This persistent base-to-top gradient, in both ventricles, showed that the focus of proliferation of cardiomyocytes in the forming ventricles was at the base of the trabecules.

Discussion
Because the mouse embryo is currently the most used experimental model we prepared a comprehensive series of 3D reconstructions that give insights into both the morphology as the proliferation patterns in the developing mouse heart. This information can be used to interpret the growing body of scientific data harvested in the mouse. Whereas in chicken and human the different phases of early heart development, namely formation of the tube, the tubular phase and chamber formation, are clearly separated in time, our 3D reconstructions show that in the mouse early heart development is very fast and that the different growth phases overlap. Some aspects of mouse heart development will be discussed in the context of our current findings.
Cardiac looping

The looping of the heart is the first morphological sign of asymmetry in the embryo. The heart tube loops by both bending and rightward rotation [35] where the outer curvature of the looped heart tube forms the site of future chamber differentiation [36]. Directional movement has been observed in the myocardium of the early zebrafish heart [37], and has been shown to be subject to left/right signalling [37,38]. Although a multitude of genes, such as Pitx2c, is differentially expressed between the left and right sides during the early stages of cardiac development [39], the precise driving forces of cardiac looping remain unclear (review: [40]). It has since long been recognized in chicken [26], that because the heart tube lengthens faster than the distance between its arterial and venous poles, looping occurs of necessity. Our reconstructions (Figure 2) show that this principle may also be valid during mouse heart formation. During the lengthening of the heart tube, the distance between the arterial and venous poles remains approximately 200 µm. Differential addition of precursor cells to the left and right components of the heart tube might then offer an explanation for the rightward looping. Discrimination and quantification of the left and right components of the myocardium is possible by the site of adherence of the endocardium to the myocardium [41]. However, this endo-/myocardium connection in the mouse is not as clear as in the chicken, which prevented the unambiguous distinction of the left and right parts of the mouse heart after looping. Before looping, in the cardiac crescent and initial tube stages, such a distinction could be made on the basis of the ventral fusion seam and no left-right differences in the number of cells were observed (data not shown).

Therefore, we analysed our library of quantitative reconstructions of chicken development [7,8]. At the studied stages the chicken myocardium does not show proliferation [7], therefore any difference in the number of cells between the left and the right side should result from a differential addition of precursors. Figure 7 shows

**Myocardium - left/right difference in chicken**

![Graph showing the left-right difference in number of cells in chicken heart.](image)

*Figure 7: Left - right difference in number of cells in chicken heart.*

The number of cardiomyocytes in the left and right side of the developing chicken heart was determined. The ventral and dorsal connection between endocardium and myocardium was used to distinguish left and right [41]. The difference in number of cells is displayed; each point represents one heart. Regression analysis was performed and revealed a significant increase of the left-right difference with age (p=0.0001); the 95% confidence shows that after 36 hours of development the difference is significantly higher than 0.
the difference between the number of cells within the left and right sides of the heart tube. Regression analysis showed that prior to looping, there was no statistical difference between the left and the right side. From the onset of looping onwards, after around 36 hours of development, the number of cells in the left side became progressively higher. These data show that a difference in addition of precursor cells may provide an important driving force of cardiac looping in chicken and possibly mammals.

**Proliferation and differentiation**

The discontinuous and localized patterns of proliferation within the myocardium show that myocardium, unlike skeletal muscle [42], has the capacity to both differentiate and proliferate. The differentiation of chamber myocardium is known to be controlled by several Tbx-transcription factors. For instance, Tbx2 is expressed in the inner curvature and atrioventricular canal of the looped heart tube [43], and functional disruption of this gene not only expands the differentiation of chamber myocardium into the atrioventricular canal, but also increases the proliferation rate within this otherwise slowly proliferating region [12].

Because Tbx2 is expressed within the myocardium from E8.5 onwards [12], its inhibitory effect on proliferation does not yet explain the regionalization of proliferation at earlier stages. This early pattern might by due to an interaction via the endo- and myocardial connections, which are known sites where, via Notch signalling, proliferation and trabecular differentiation are initiated [44]. Such endo- and myocardial connections are only present in the ventral part of the heart tube, which might explain why there is more proliferation there than in the dorsal side. Evidence for such early myocardial differentiation was also demonstrated, using electron microscopy, to already occur at the 3-4 somites stage (approximately E7.75 [13]), indicated by formation of gap-junctions at the ventral side of the heart tube [45]. This study also showed mitotic divisions within the region of formation of gap-junctions and formation of a multilayered and trabeculated wall of the early heart tube [45].

**Proliferation and morphogenesis**

Recent lineage analyses showed that, after formation of the primary heart tube, cells from a second heart field are added to the cardiac lineage at the anterior pole of the heart [28,30,46,46]. The primary heart tube would then originate from a first heart-forming field. In our reconstructions we were unable to observe a clear distinction between a primary heart tube stage and further growth, but rather observed a
gradual growth of the embryonic heart. Therefore, we find it difficult to envision when the contribution of the first heart field would stop and the second field would begin. Subsequent to the discovery of the second heart field, it was demonstrated that this field also contributed cells to the inflow of the heart [27,29,31]. At this stage, the rupture of the dorsal mesocardium limits the contact between the forming heart tube and the pericardial back wall, and cells can only be added to the forming heart at the arterial and venous poles. This can easily be envisioned when one appreciates that the distance between the arterial and venous poles is only about 200 µm, or a line of about 15-20 precursor cells. In addition, transplantation studies and other lineage analyses, did not reveal distinct fields of cardiac precursors [47-50] (review: [51]). Recent observations show that marker genes of the second heart-field are already expressed in the first heart-field [52-54]. Therefore, we rather propose a gradual temporal formation of the heart.

In chicken, we showed that the cells added to both the in- and outflow of the heart tube take origin from a focus of rapidly proliferating precursor cells, located in the caudal pericardial back wall [7]. As in the human [9], our current observations of proliferation did not reveal clear regionalization of proliferation in the pre-cardiac mesoderm in the mouse, which may result from the rapid development of the mouse compared to chicken. However, as in the human and the chicken, proliferation in the mouse myocardium is highly regionalized, with slow proliferation in the atrioventricular canal, outflow tract and inner curvature. The patterns of clonal growth within the myocardium observed by Meilhac and coworkers [29,55,56], are similar to this proliferation pattern. For instance, in the slow proliferating outflow tract and atrioventricular canal (Figure 6a,b,c,d) dispersed clones, orientated along the veno-arterial axis were seen, whereas coherent clones, larger in older embryos, were observed in the fast proliferating regions of chamber formation. The lack of local proliferation data made earlier analyses of clonal data to assume a homogeneous cell cycle length [29].

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. Multiple genes are preferentially expressed in either the trabeculated myocardium or in the outer myocardial wall, reflecting both change in function, and differential modes of growth. For instance, the trabecules display a high expression of Nppa [57,58] and of the fast-conducting gap-junctional protein connexion 40 [32,59], which are not expressed in the ventricular compact
layer. There also is preferential expression of signalling molecules, among which Tbx5 [60] and genes that function in the TGF-beta pathway, such as BMP10 [61], the Notch-pathway [44], and Neuregulin, signalling via the Erb receptor pathway [62]; the latter pathways signalling occurs from the endocardium. Our analyses of proliferation within the myocardial walls and the trabecules show the most rapid proliferation at the junction between the outer wall and the base of the trabecules, while proliferation tapered off toward the top of the trabecules. Retroviral labelling of pre-cardiac mesoderm in chicken showed elongated clusters of labelled cells in the myocardial wall, spanning into the trabecules [63]. Above mentioned genetic lineage analyses in mouse, using randomly generated clones, also showed such orientation of growth in the transmural axis [55]. 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. 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 [44], causing an outward expansion of the ventricular chambers [36]. Finally, regression of proliferation in the trabecules has been suggested to imply terminal differentiation into the conduction system [64]. Our study shows, however, that both compartments continue to proliferate at different rates. Furthermore, the proliferating outer-layer also differentiates, albeit in a contractile phenotype. Therefore an inverse relationship between differentiation and proliferation cannot be strictly assumed.

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

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