Regionalized Sequence of Myocardial Cell Growth and Proliferation Characterizes Early Chamber Formation

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Abstract—Increase in cell size and proliferation of myocytes are key processes in cardiac morphogenesis, yet their regionalization during development of the heart has been described only anecdotally. We have made quantitative reconstructions of embryonic chicken hearts ranging in stage from the fusion of the heart-forming fields to early formation of the chambers. These reconstructions reveal that the early heart tube is recruited from a pool of rapidly proliferating cardiac precursor cells. The proliferation of these small precursor cells ceases as they differentiate into overt cardiomyocytes, producing a slowly proliferating straight heart tube composed of cells increasing in size. The largest cells were found at the ventral side of the heart tube, which corresponds to the site of the forming ventricle, as well as the site where proliferation is reinitiated. The significance of these observations is 2-fold. First, they support a model of early cardiac morphogenesis in 2 stages. Second, they demonstrate that regional increase in size of myocytes contributes significantly to chamber formation. (Circ Res. 2006;99:545-552.)

Key Words: 3D visualization ■ quantitative reconstruction ■ embryology ■ heart development

Growth of the heart is tightly regulated such that the distinct components achieve their appropriate proportions and are appropriately interconnected. Many congenital malformations result from impaired cardiac growth.1,2 In addition, repopulation of the heart by inducing myocytic proliferation has been proposed to be a realistic future option for cardiac repair.3 Despite this great clinical interest, there are major gaps in the understanding of the links between the different features of cardiac growth, such as cellular proliferation and increase in cell size and morphogenesis.

The pioneering studies of Sissman4 and Stalsberg5 in the 1960s have provided a wealth of meticulous observations on cellular proliferation in the early chicken heart. These and other data, however, can only fully be exploited if incorporated into an approachable 3D context. Although regional changes in cell shape have been considered to mediate cardiac looping,6 changes in cellular volume have not been studied. It can be argued, therefore, that the divergent opinions that exist regarding the cellular mechanisms underlying formation of the cardiac chambers take their origin from misinterpretations of the complex 3D architecture of the developing heart. Arguments revolve on whether development of the chambers is achieved by induction of proliferation in 1 region, or by stunting of proliferation in a complementary region of the primary heart tube.4,5,7–10

To gain insight into the contribution of increased volume as opposed to proliferation of the myocytes during formation of the tubular heart, we have developed a method for quantitative reconstruction.11 Using such quantitative reconstructions, we correlated cardiac growth and morphogenesis to the spatiotemporal changes in proliferation and cell size in developing chicken hearts, ranging from Hamburger–Hamilton (HH) stage12 10– through 12. Thus, our study does not go beyond the first phase of the process of looping, in which the ventricle is just formed.13,14 We have demonstrated, first, that the myocardial heart tube takes its origin from a rapidly proliferating precursor pool of small cells that are recruited to the cardiac tube; second, that the tube is a slowly proliferating structure; and, third, that formation of the chambers is initiated by a highly regional activation of increase in cellular volume, followed by an increase in the rate of proliferation of the large cells. As far as we know, our combined morphological and quantitative reconstructions are thus far unique, showing for the first time that coordinated regional changes in both size and proliferation rate of the myocytes contribute to cardiac morphogenesis.

Materials and Methods

5-Bromo-2′-Deoxyuridine Labeling

The embryos were staged according to the system of Hamburger and Hamilton.12 Fertilized eggs were obtained from a local hatchery (Drost, Nieuw Loosdrecht, The Netherlands) and before isolation, the embryos were exposed to 5-Bromo-2′-deoxyuridine (BrdU) for 4 or 10 hours by injection of 100 μL of a 10 mg/mL solution of BrdU.
in a physiological salt solution (0.75 mosmol/L) into the yolk. Cells in the S phase of the cell cycle incorporate BrdU. Because of the linear relationship between the time of exposure and labeling index, a longer exposure duration to BrdU results in a higher labeling index. Therefore, the fraction of BrdU-labeled cells can be regarded as a direct estimate of rate of proliferation for fixed exposure time. At the studied stages, BrdU incorporation attributable to DNA repair is negligible.

**Tissue Processing**

Detailed practical protocols for fixation, paraffin embedding, mounting, and sectioning of embryonic chicken tissues have been described previously. Embryos were isolated, fixed, embedded in paraplast, and cut into 7-μm thick serial sections, which were mounted on to 3-aminopropyltriethoxy-silane–coated slides.

**Staining**

**BrdU Triple Staining**

Sections were deparaffinized and rehydrated in a graded alcohol series. Epitope retrieval of the BrdU was achieved by immersing the sections in 0.5 mol/L HCl for 5 minutes, followed by rinsing three times for 5 minutes in PBS. The sections were then incubated overnight with a mixture of a monoclonal mouse antibody against BrdU (Becton Dickinson), diluted 1:100, and a polyclonal rabbit antibody against cardiac troponin I (cTnI) (HyTest Ltd, Turku, Finland) diluted 1:250 in PBST (PBS with 0.05% Tween 20). cTnI was found to be myocardium specific in mice and in chicken. The sections were washed 3 times, for 5 minutes each, followed by an incubation for 4 hours with a mixture of 2 secondary antibodies; a goat anti-mouse antibody coupled to Alexa-568 and a goat anti-rabbit antibody coupled to Alexa-660 (Molecular Probes), both diluted 1:100 in PBST. Subsequently, Sytox Green was used to stain all nuclei (1:30 000 in PBST; Molecular Probes). After incubation, the sections were again washed three times with PBS, for 5 minutes each, and the slides were mounted with Vectashield (Vector Laboratories Inc). All steps of incubation using fluorochromes were shielded from light to prevent photobleaching.

**Phospho-Histone H3 Triple Staining**

The phospho-histone H3 (PH3) staining (and further processing) is similar to the BrdU triple staining, with the exception that the antibody against BrdU was substituted with the PH3 antibody (Phospho-Histone H3 [Ser10] Antibody; Cell Signaling Technology Inc). The presence of phosphorylated Ser10 of histone H3 is tightly correlated with chromosome condensation during both mitosis and meiosis (http://www.cellsignal.com; product no. 9701).

**Acquisition of Images**

The sections were scanned at ×10 magnification using a confocal scanning laser microscope (MRC1024, Bio-Rad Microscopy Division, Helmsdale, UK). The CSLM was used to acquire images from the emission spectra of each of the fluorescent dyes: Sytox Green, Alexa-568, and Alexa-660, which correspond to all nuclei, BrdU-positive nuclei, and myocardium, respectively. To avoid spillover, the fluorochromes in each section were scanned sequentially. To reduce noise, the images were captured using a Kalman modulation (n=4).

**Method of Reconstruction**

The quantitative reconstruction method applied on the resulting sets of images will be published separately. It is important to note that because of the biological variation the development of the heart varies significantly compared with the number of somites. Therefore, multiple reconstructions were made of comparable stages, in which the development of the heart slightly varied but in which the developmental pattern of proliferation and increase in cell size changed gradually. In short, the reconstruction method can be divided into 3 steps: image processing, voxel measurement, and visualization.

**Image Processing**

To obtain a properly stacked series of images, the images were registered (or aligned). Registration was done using the data analysis and geometry reconstruction program Amira (version 3.1; TGS Template Graphics Software, http://www.tgs.com). Then, to enable quantification and visualization, the individual channels contained in the Bio-Rad native pic-file were processed using the image processing program Image-Pro Plus (version 5.0.2.9; Media Cybernetics, http://www.mediacy.com). From the image containing the myocardium signal (Alexa-660), the myocardium was separated from the background and converted into a binary image. Using this binary myocardium image as a mask, the BrdU-positive nuclei (Alexa-568) and all nuclei (Sytox Green) lying within the myocardium were isolated and reduced to individual pixels (centroids). These resulting images were fused with the myocardium mask to obtain an image containing the myocardium area and the BrdU-positive nuclei and all nuclei.
Voxel Measurement
To convey the local cellular data in an interpretable way, a 3D measurement procedure was implemented. The entire stack of preprocessed images was loaded into a matrix and virtually diced into voxels (measurement volumes). Each voxel equals $\frac{21 \times 21 \times 21 \, \mu m^3}{V_{stack}}$, which is, therefore, the spatial resolution of our quantitative data. The myocardial volume ($V$), the total number of nuclei ($T$), and the number of BrdU-positive nuclei ($B$) within each voxel were determined and used to calculate the local BrdU labeling index ($B/T$ and the local cell size ($V/T$). At the stages studied in this article, no epicardium were present; therefore, fibroblasts and endothelial cells have not yet entered the myocardium. Chicken cardiomyocytes become multinuclear only after hatching; thus, the counted nuclei represent single cardiomyocytes. To reduce noise, without reducing the spatial resolution of the reconstruction, the sample size was increased to $105 \times 105 \times 105 \, \mu m^3$ and the corresponding data were mapped into the center voxel of such a sample volume.

Visualization
Using Amira, surface reconstructions of the myocardium were made (Figure 1) onto which the local quantitative data (BrdU labeling index and cell size) were mapped in pseudocolors (Figures 3 and 4, respectively).

Results
Myocardial Reconstructions
We set the stage by describing the morphological context in which we have mapped our quantitative data of cellular proliferation and increase in cell size (Figure 1). The myocardium was reconstructed as assessed by the expression of cardiac troponin I protein, which is a marker for cardiomyocytes. The obtained series of morphological reconstructions of the developing heart range from the stages 10 through 12, which correspond with the stages before looping and the c-shaped looped heart. The use of cardiac troponin I as our myocardial marker prohibits the representation of other cardiac components, such as cardiac jelly and endocardium.

Each reconstruction shown is based on an individual embryo. As has been meticulously documented previously by Patten, cardiac morphology was observed to show considerable variation when related to the time of incubation and the number of somites. Accurate estimates of the elapsed time between stages, therefore, cannot be given. Because of this, we do not show reconstructions in duplicate. Instead, a selection of 4 reconstructions was chosen to represent the overall pattern observed in a series of eight reconstructions, sorted according to morphology. Even though we illustrate individual embryos, they collectively represent a temporal pattern, which spans approximately half a day of cardiac development. The appearance of the embryonic heart provided by our reconstructions is remarkably similar to the images resulting from light and electron microscopic studies, validating the method of reconstruction.

Proliferation and Size of Cells in the Forming Heart Tube
Figure 2 shows an example of the series of sections on which we have based our quantitative reconstructions. In these sections, cells incorporating BrdU, and hence proliferating, can clearly be seen in the mesenchyme and dorsal mesocardium, whereas the myocardium of the heart tube itself is made up mainly of nonproliferating cells. The local labeling index and the local cell size data were mapped onto the myocardial reconstructions shown in Figure 1, resulting in quantitative reconstructions (Movies 1 through 8 in the online data supplement, available at http://circres.ahajournals.org). Such reconstructions were made from embryos of stages 10, 10, and 12, which have been exposed to BrdU for 4 hours, and from a stage 12 embryo, which has been exposed to BrdU for 10 hours, to glean additional information.
about recruitment of cells. Starting with the stage before looping, which was the youngest stage studied, very slow proliferation is observed in the region of fusing myocardium. This area at stage 10− has a labeling index of \(0.05\) (Figure 3). Caudal from this region, however, the separate right and left heart-forming regions display a very high rate of proliferation. In these regions, a decreasing caudal to cranial gradient of incorporation of BrdU is clearly present, with a labeling index up to \(0.6\). This pattern is also present in the stages of 10 and 12−. At both stages, the primary tube displays very low proliferation, in contrast to the caudal edge of the myocardium and the separate caudal heart-forming regions. The pattern of cell size at these stages is reciprocal to this pattern of proliferation (Figures 3 and 4). Large cells are proliferating slowly, whereas small cells have a high rate of proliferation. Interestingly, this reciprocal pattern is not seen in the ventral region of the heart tube, where the site of development of the morphologically identifiable primitive ventricle coincides with a focus of cells increasing in size as well as in proliferation rate (Figure 4, arrowheads). This pattern of a high-proliferation rate and increase in cell size is even more pronounced in the forming ventricle at stage 12. Thus, formation of the ventricle becomes evident by an increase in the size of its cardiomyocytes and is then followed by an increase in cellular proliferation, leading to a focus of large proliferating cells in the ventral myocardial wall. The remainder of the heart tube maintains its relative low index of labeling, most notably in the inner curvature, which represents the shortest path from the venous to the arterial poles. The rapidly proliferating caudal edge of the myocardium is still present. This myocardial edge is directly contiguous with visceral mesenchyme surrounding the foregut, which, at stage 10, displays a similar or higher incorporation of BrdU (Figure 2d). The regions that show the highest labeling index, also display the highest intensity of cells in M phase, as assessed by staining with antibodies specific to PH3 (Figure 5), further underscoring the fact that this region consists of rapidly proliferating cells.

By using the quantitative information contained in the reconstructions, we were able to calculate the volume, and the total number of cells, in each heart. Within half a day, the volume of the myocardium increases approximately 6-fold, with two-thirds of this increase accounted for by the number of cardiomyocytes and the remainder by their increased size (Figure 6). It is, however, important to appreciate that, in some regions, like at the inner curvature, the size of the cells hardly increases, whereas in the forming ventricular wall the cells increase 2- to 3-fold in size.

The Spatial Relationship Between Proliferation and Size of Cells Reveals Two Phases of Early Cardiac Growth

The novel approach we have used for reconstruction permits the mapping of the spatial correlation between rate of proliferation and size of the cells. To this end, we created bivariate scatterplots of the labeling indices and cell size of each voxel for each reconstructed stage (Figure 7A). The original spatial information, however, was not used to create these scatterplots, and is thus completely lost at this step.
These scatterplots already show that, at each of the stages studied, there is a notable relation between proliferation and size of the cells. The scatterplot reveals cells of small volume which have varying rates of proliferation at stage 10 (Figure 7A). During further development, cells increase in size, with a concomitant decrease in their rate of proliferation. At stage 10, cells have developed of medium size which exhibit only very slow rates of proliferation. Interestingly, in the subsequent stages, a further increase in size is also accompanied by an increase in rate of proliferation. Clearly, these temporal changes, and the remarkable “c”-shaped distribution observed in the scatterplot at stage 12 and 12 (Figure 7A), indicates the presence of a process of cellular transition during this period of heart development. This transition can be divided into 2 phases. In the first phase, small and rapidly dividing cells become dormant cells of medium size (Figure 7A, red arrow). In the second phase, these medium sized cells give rise to large rapidly dividing cells (Figure 7A, blue arrow).

The scatterplots were segmented by placing lines perpendicular to the c-shaped distribution (Figure 7A), permitting segregation of the process of transition into several classes. Each class was color-coded and mapped back onto the corresponding voxel in the reconstruction, producing a 3D view of the process of cellular transition (Figure 7B). Astonishingly, despite the loss of 3D information in the scatterplots, and their division into an arbitrary number of classes, the spatial distribution of the classes of data points when viewed in 3 dimensions shows a discrete and highly organized pattern at every developmental stage (Figure 7B and supplemental Movie 9 for the stage 12 reconstruction). Not only do the data points of every class cluster together, they are also border only by their neighboring classes and the order of the classes in the reconstructions and the scatterplots is the same. It is thus apparent that the process of cellular transition derived from the scatterplots is directly correlated to the process of cardiogenesis. If we translate the 2 phases observed in the scatterplots into morphogenetic terms, the developmental process can be divided into caudal recruitment, resulting in the elongation of the heart tube (Figure 7A,
formation of the increase in cell size to cardiac growth (black).

Based on the mean volume of cardiomyocytes at stage 10 through 12. Based on the mean volume of cardiomyocytes at stage 10 through 12, and the number of cells per stage, we were able to calculate the fraction of the cardiac volume resulting from the increase in number of cells (gray). Note the important contribution of the increase in cell size to cardiac growth (black).

red arrow) and into a ventral expansion, leading to the formation of the ventricle (Figure 7A, blue arrow).

**Discussion**

The combined sets of data for proliferation and size of myocytes demonstrate that, from stage 10 through 12, there is a transformation from a highly proliferative pool of small cells, to a nonproliferating population of medium-sized cells, which form the heart tube. These medium sized cells, in turn, give rise to a population of large dividing cells, which form the primitive ventricle. These various pools can be distinguished at the venous pole, the myocardium of the straight heart tube, and the developing ventricle, respectively. The process of quantitative reconstruction clearly shows that these populations of cells form distinct regions, which are nonetheless morphologically and temporally contiguous.

**Formation of the Primary Heart Tube by Recruitment**

The highest proliferative activity was observed at the extreme cranial end of the tube (Figure 3, stages 10 and 12–), indicating that the primary heart tube itself takes origin from an actively proliferating pool of precursor cells. This conclusion is compatible with classic experiments, which showed that particles of iron oxide placed into the nonfused heart-forming regions were located in the fused heart tube at later stages. Recent molecular studies of lineage tracing of isll-expressing progenitors have shown that these conclusions also hold true for the mouse.

Together, these observations suggest that there is proliferation of a mesenchymal precursor pool for cardiomyocytes, before its overt differentiation into cardiac muscle. The highest incidence of M-phase nuclei is present in the same caudal proliferating area and indicates that the cells residing in this area complete their cell cycle in this part of the heart. Using myocardial specific antibodies, we have now demonstrated that this mitotic area is present at the caudal myocardial border and is composed of very small cardiac precursor cells.

Although it has been shown that cardiac precursor cells are added to the heart at both its cranial and caudal ends, such addition was not seen at the cranial end of the heart in the stages examined in our study. This observation is consistent with marking studies that revealed that cells are not added to the arterial pole until after stage 12. In our reconstruction of stage 12, the first signs of this recruitment might just be visible as a narrow rim with a high labeling index (Figure 3).

From the morphological stance, it is highly intriguing that the primary heart tube increases almost 4-fold in length in its linear stage, whereas the tube hardly displays proliferative activity (labeling index, =0.05). This increase in length has also been observed by other researchers. Particles of iron oxide placed in the developing tube were observed to move apart, demonstrating intrinsic growth of the tube, whereas particles placed outside the heart moved into the tube. We now show that one-third of the growth of the tube is attributable to the increase in size of its cardiac cells, whereas the other two-thirds is attributable to recruitment from a caudal precursor pool.

**Cardiac Morphogenesis Occurs in Multiple Phases**

The segmented scatterplots, and subsequent mapping of the classed data back onto the reconstructions, showed strikingly that each combination of cell size and labeling index has its own unique location within the heart. Furthermore, this procedure revealed 2 developmental axes. The quantitative reconstructions showed that, after having been recruited along the cranio-caudal axis (Figure 7a, red arrow), the cardiomyocytes enter a second phase of increase in cell size along the dorsoventral axis (Figure 7a, blue arrow), with the largest cells positioned at the ventral side of the forming cardiac tube. Cells at the outer curvature are 2- to 3-fold larger than those at the inner curvature, thus underscoring the initial formation of the primitive ventricle and the asymmetry of the primary tube. This is then followed by a proliferative phase of growth. Interestingly, in Drosophila, it has been demonstrated that large cells have a unique cycle of progression and are able to differentiate according to environmental cues. For example, patterning and regulation of cellular proliferation in the Drosophila wing responds to a
gradient of the Decapentaplegic morphogen. We speculate that formation of the ventricle can be considered as a process of differentiation of the primary myocardium of the linear heart tube into working ventricular myocardium governed by such environmental cues. The fact that the initiation of the increase in cell size, and subsequent proliferation of cells, occurs exclusively at the ventral side of the tube indicates the existence of a dorsoventral developmental axis. This observation is at odds with the representation of the tube as a transversely segmented structure. A similar conclusion was drawn from functional, expressional, and clonal analyses during development of the mouse heart. The factors controlling the dorsoventral axis are largely unknown, but Hand1 may play an important role, as it is specifically expressed at the outer curvature. It is noteworthy, therefore, that mice deficient for Hand1 display hypoplastic ventricles. The manner in which proliferation of cardiomyocytes contributes to development of the heart tube, and subsequent formation of the chambers, is currently a subject of debate. One view is that the myocardial cells of the cardiac tube have an inherently slow mode of proliferation and that the chambers develop by a local increase in proliferation. This mode of formation of the chambers has been characterized by us as the ballooning model. Our quantitative reconstructions show that on formation of the cardiac tube, proliferation decreases significantly, concomitant with overt differentiation into cardiac muscle culminating in the first heart beat at stage 10. This order of events is remarkably reminiscent to the differentiation of skeletal muscle. Thompson and coworkers inferred from the commonly accepted inverse relationship between cellular proliferation and

Figure 7. Spatial relationship between cellular proliferation and size. A, Scatterplots of the local labeling index and size measured in reconstructions of chicken hearts ranging from stage 10 through 12. The scatterplots have been segmented perpendicular to the c-shaped distribution apparent in the stage 12 embryo. B, Dorsal and ventral views of reconstructions displaying the spatial distribution of the color-coded segments in the scatterplots. See the online data supplement for a video file of the stage 12 embryo (A). Although the original 3D information is lost in the scatterplots, the spatial distribution of the segmented scatterplot data shows a discrete and highly organized pattern at every developmental stage. Initially, the cells are small in size and proliferate rapidly (green segment). These cells become dormant and increase in size to give rise to the majority of the primary heart tube (cream/yellow segment). Within the primary tube, a region (arrowheads) appears in which the cells increase even further in size and in which proliferation is reinitiated (yellow/orange/red regions).
differentiation that those regions of the developing heart that proliferated slowly were more specialized than the ones giving rise to the myocardium of the chambers. The former regions would constitute the so-called “cardiac specialized tissues” that develop into the conduction system.8,9,41,42 Our current study, however, demonstrates unequivocally that the entire straight heart tube is initially a slowly proliferating structure. It is, nevertheless, still capable to locally reinitiate proliferation. It is of major interest, therefore, to discover the cues that govern this reinitiation of proliferation, with concomitant differentiation of the myocardium of the primary heart tube into the myocardium of the forming ventricle.

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Disclosures
None.

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