A caudal proliferating growth center contributes to both poles of the forming heart tube


Published in:
Circulation Research

DOI:
10.1161/CIRCRESAHA.108.185843

Citation for published version (APA):
A Caudal Proliferating Growth Center Contributes to Both Poles of the Forming Heart Tube

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Abstract—Recent studies have shown that the primary heart tube continues to grow by addition of cells from the coelomic wall. This growth occurs concomitantly with embryonic folding and formation of the coelomic cavity, making early heart formation morphologically complex. A scarcity of data on localized growth parameters further hampers the understanding of cardiac growth. Therefore, we investigated local proliferation during early heart formation. Firstly, we determined the cell cycle length of primary myocardium of the early heart tube to be 5.5 days, showing that this myocardium is nonproliferating and implying that initial heart formation occurs solely by addition of cells. In line with this, we show that the heart tube rapidly lengthens at its inflow by differentiation of recently divided precursor cells. To track the origin of these cells, we made quantitative 3D reconstructions of proliferation in the forming heart tube and the mesoderm of its flanking coelomic walls. These reconstructions show a single, albeit bilateral, center of rapid proliferation in the caudomedial pericardial back wall. This center expresses Islet1. Cell tracing showed that cells from this caudal growth center, besides feeding into the venous pole of the heart, also move cranially via the dorsal pericardial mesoderm and differentiate into myocardium at the arterial pole. Inhibition of caudal proliferation impairs the formation of both the atria and the right ventricle. These data show how a proliferating growth center in the caudal coelomic wall elongates the heart tube at both its venous and arterial pole, providing a morphological mechanism for early heart formation. (Circ Res. 2009;104:179-188.)

Key Words: cardiovascular development ■ proliferation ■ heart fields ■ Islet1 ■ bromodeoxy uridine ■ quantitative 3D reconstruction

The heart is sculpted by precisely orchestrated developmental programs1,2 that are prone to errors, leading to high incidences of congenital malformations.3 Proliferation, although not the only mechanism, is an important parameter for the formation of the heart.4–8 Research on heart formation was recently revolutionized by the understanding that the initially formed myocardial heart tube continues to grow by recruitment of cells that originate from flanking mesoderm, dubbed the second heart field.9–11 This second heart field was originally reserved for cells feeding into the outflow of the primary heart tube to form the right ventricle.9 Shortly after these findings, cells were also shown to be added to the inflow,11 and a debate developed regarding the existence of multiple fields of cardiac precursor cells.12–15

Limiting factors in this debate are the virtual lack of a 3D context of cardiac growth and the scarcity of data of locally involved parameters, such as proliferation. Early heart formation is of perplexing 3D complexity, because it occurs concomitantly with folding of the embryonic disc and formation of the coelomic cavity. Most likely, this complexity has contributed to the diversity of opinions, because of differences in interpretation rather than observation.13 Also, comprehensive studies of proliferation parameters of the myocardium and the heart-forming mesoderm do not exist.

To improve insight into heart formation, we first determined the cell cycle length of the myocardium of the primary heart tube. We found that newly formed myocardium is nonproliferating, implying that growth of the primary heart tube can occur solely by differentiation of precursor cells. Increasing pulses of 5-bromo-2-deoxyuridine (BrdUrd) exposure showed that dividing cells are rapidly incorporated into the inflow of the heart tube. To track the origin of these cardiac precursors, we mapped proliferation in a 3D context using a novel method for generating quantitative reconstructions.6,16 Observations started at the fusion of the coelomic walls and ended at the looped heart-tube stage. These obser-
vations revealed a proliferating growth center within the caudal coelomic wall, which expressed Islet1. Tracing experiments showed that cells from this center move to both poles of the forming heart. Inhibition of proliferation within this caudal growth center resulted in impaired development of both poles of the heart.

Materials and Methods

Embryo Processing, Staining Procedures, and Image Acquisition

Triple Staining Immunohistochemistry

This study made use of chicken embryos, which were staged according to Hamburger and Hamilton.17 One hundred microliters of 10 mg BrdUrd (Sigma) per milliliter physiological salt solution were injected into the egg yolk directly under the embryos. After reincubation, the embryos were fixed in methanol/acetone/water (40:40:20 vol/vol), dehydrated, embedded in paraplast, and serially sectioned at 7 μm. Each section was fluorescently triple-stained with anti–cardiac troponin I (anti-cTnI) (HyTest Ltd) to detect myocardium, anti-BrdUrd (Becton Dickinson) to detect BrdUrd-positive nuclei, and SYTOX Green (Molecular Probes) to detect all nuclei. Images were recorded sequentially using a laser scanning microscope.6

In Situ Hybridization

In situ hybridization was performed as previously described.18 Sections of paraformaldehyde-fixed embryos (12 μm thick) were alternately stained for Islet1 (ChEST 603856748F1, MRC Genservices), cTnI,19 and a third probe (not included in this article). Sections were photographed using bright-field illumination. For the resulting reconstructions, signals were masked and interpolated over 36 μm.

Cell Tracing and Inhibition of Proliferation

For cell-tracing and inhibition of proliferation chicken embryos were either cultured,20 or manipulated in ovo via an opening in the shell, which was closed with cellophane tape during further incubation. As previously described, embryos were exposed to DiI and DiO (Molecular Probes) using a Picoprinter (General Valve Corp) and a micromanipulator with a pulled glass capillary tube.21 To inhibit proliferation, 100 μmol/L the cyclin-dependent kinase inhibitor aminopurvalanol A21 (Alexis Biochemicals) was dissolved with the DiI and administered locally. Bright-field and fluorescent photos were made immediately after labeling and during further culturing.

Reconstructions and Morphometry

Quantitative Three-Dimensional Reconstructions of BrdUrd-Positive Fractions

A detailed protocol for the generation of quantitative reconstructions was published previously.16 From the triple-stained sections, the myocardium was automatically segmented using the cTnI signal. Other structures were segmented manually (Figure 1A). Limits for inclusion of splanchnic mesoderm differ per reconstruction and are mentioned in the results section.

Within the myocardium and the splanchnic mesoderm, all nuclei and the BrdUrd-positive nuclei were automatically identified within the myocardium and selected splanchnic mesoderm. C. Nuclei of the myocardium in their 3D context. To facilitate interpretation, and allow reliable estimations, nuclei were counted and BrdUrd+ fractions were determined in sample cubes, as shown in D. This information was then projected on the morphological reconstruction (B) to give a quantitative 3D reconstruction of local proliferation (E). (Displayed reconstruction is the myocardium of a stage 12 chicken embryo, after 4 hours of BrdUrd exposure.6)
tions were overlain with the 3D BrdUrd fractions to generate the quantitative 3D reconstructions of local proliferation (Figure 1E). For this work, we selected 4 reconstructions from our library of 3D quantitative reconstructions, which contains more than 30 embryos and spans the developmental period between stage 8 and stage 21. Examples of original triple stained sections in relationship to the presented 3D quantitative reconstructions are shown in the figures in the online data supplement, available http://circres.ahajournals.org.

Regional Estimation of BrdUrd-Positive Fraction
Within the datasets, as shown in Figure 1C, regions of interest were selected using the VolumeEdit module of Amira. The selection criteria are defined under Results. Within a selection, all nuclei and BrdUrd-positive nuclei were pooled to calculate a regional BrdUrd-positive fraction.

Distance Estimation
Distances were measured through the reconstructed myocardium, between manually placed waypoints. To this end, we exposed embryos of similar developmental stages to BrdUrd for an increasing period of time (Figure 2). At the studied stages, BrdUrd incorporation as a result of DNA repair is negligible and no multinuclear cells occupy the myocardium. The cell cycle length can be calculated from the linear relationship between BrdUrd exposure time and BrdUrd-positive fraction. For calculations of ventricular myocardium, we counted BrdUrd fractions in the compact layer at the outer curvature of the reconstructions in B, between the distal ventricular groove (left arrow) and the atrioventricular canal (right arrow). D shows these linear relations of both the ventricular and the primary myocardium (hatched line).

Results
Proliferation Rates of the Primary Heart Tube and the Embryonic Ventricle
To comprehend cardiac growth, we first wanted to know the cell cycle length of cardiomyocytes of the heart tube. To this end, we exposed embryos of similar developmental stages to BrdUrd for an increasing period of time (Figure 2). At the studied stages, BrdUrd incorporation as a result of DNA repair is negligible and no multinuclear cells occupy the myocardium. The cell cycle length can be calculated from the linear relationship between BrdUrd exposure time and BrdUrd-positive fraction.

To determine the cell cycle length of the primary heart tube, we made quantitative reconstructions of myocardium from 2-day-old embryos, exposed to BrdUrd from 1 to 10 hours (Figure 2A). Irrespective of exposure time, the heart tube consisted mainly of BrdUrd-negative cardiomyocytes, although with increasing exposure a zone of BrdUrd-labeled cells broadened at the venous pole. After exclusion of these cells, the BrdUrd-positive fractions in the primary heart tube showed a linear increase from 2% to 8% (hatched line in Figure 2D), corresponding to a cell cycle length of the myocardium of the primary heart tube of 5.5 days.

Irrespective of this lack of inherent proliferation, the heart tube rapidly increases its number of cardiomyocytes, implying that early myocardial growth occurs fully by addition of differentiating cells from flanking mesoderm. If such precursor cells are rapidly proliferating, they will incorporate BrdUrd, which will be retained after differentiation into...
cardiomyocytes and concomitant cessation of proliferation. This explains the observed broadening of the zone of BrdUrd-labeled cells with increasing exposure time. We measured the width of this cranially directed broadening along the line of contact of the heart with the coelomic wall (white lines in Figure 2A) and plotted it against BrdUrd exposure time (Figure 2C). The resulting graph shows that the primary heart tube lengthens at the inflow at a rate of $\frac{70}{h}$. Myocardium of the looped tubular heart will reinitiate proliferation at its outer curvature at approximately stage 12, forming the primitive ventricle. To determine the proliferation rate of the forming ventricle, we conducted similar experiments as explained above. We made quantitative reconstructions of hearts of 3-day-old embryos that were treated with BrdUrd for 1 to 6 hours (Figure 2B). At these stages, the primitive ventricle is morphologically recognizable by the presence of trabeculae. Analysis of the BrdUrd-positive fractions in the compact layer (Figure 2D) revealed a cell cycle length of 8.5 hours.

These experiments reveal that proliferation within the looped heart tube is highly heterogeneous. This tube, however, originates as a nonproliferating structure, which lengthens rapidly at its inflow by addition of recently divided progenitors. To gain insight into the origin of these cells, we made quantitative 3D reconstructions of proliferation within the forming coelomic wall and heart tube. Examined embryos were exposed to BrdUrd for 1 hour only, to minimize the BrdUrd labeling attributable to cell migration.

Formation of the Primary Heart Tube and Pericardial Mesoderm

Figure 3 shows reconstructions of embryos at stages 8 and 9. Morphological reconstructions (rows A and C) show the endoderm, the myocardium, the thickened precardiac splanchnic mesoderm, classically attributed to the heart-forming regions, and the mesoderm of the forming pericardial back wall. Quantitative 3D reconstructions of proliferation were made of the myocardium and the coelomic mesoderm (rows B and D). At these stages, no mesenchyme occupied the space between the coelomic walls and the endoderm. Interactive 3D reconstructions are presented in the online data supplement.
Morphology

At stage 8, we observed a heterogeneous expression of cTnI protein at low levels. This is in line with the reported patterns of expression of other sarcomeric proteins,\textsuperscript{28,29} whereas mRNA expression of sarcomeric markers is more abundant.\textsuperscript{30} Although myocardial differentiation is gradually commencing, we designated all reconstructed mesoderm at stage 8 to be “nonmyocardial.” Cranially, the splanchnic coelomic walls fused in the embryonic midline, indicating the cardiac anlage. Lateral from the cardiac anlage, coelomic mesoderm faced the floor of the foregut, forming the anlage of the pericardial back wall. Caudally, the coelomic mesoderm followed the anterior intestinal portal and spread laterally and ventrally.

At stage 9, a few hours further into development, the ventral fusion of the coelomic walls proceeded and could be seen as a seam along the longitudinal axis of the overtly cTnI-expressing heart tube. At the venous pole, the forming heart tube was medially contiguous with the coelomic wall and laterally contiguous with the forming vitelline veins.

Proliferation

At stage 8, the cranial mesoderm of the anlagen of both the myocardium and the pericardial back wall, proliferated slowly. Contiguous mesoderm underlying the anterior intestinal portal, however, showed rapid proliferation. This pattern of proliferation was similar at stage 9, with slow proliferation in the pericardial back wall and the forming heart tube. Consistent with the calculated cell cycle time of 5.5 days, the forming heart tube showed a virtual lack of BrdUrd incorporation. There was, however, a small focus of labeled cells at the left inflow, presumably caused by recruitment of proliferating precursors occurring within the 1 hour of exposure to BrdUrd. At the level of the anterior intestinal portal, the medial mesoderm of the coelomic wall proliferated rapidly.
At the outer edges, however, the luminizing mesoderm displayed a low proliferation rate.

**Growth of the Tubular Heart**

Figure 4 shows the morphology and proliferation of early looping hearts at stages 10 and 14. A clear cardiovascular lumen was observed and reconstructed. Reconstruction of the coelomic mesoderm was cranially restricted to the pericardial reflection. The coelomic cavity could be divided into a pericardial part surrounding the heart and 2 pericardioperitoneal canals that extended deep into the embryo. Therefore, reconstruction of the mesoderm covering the pericardioperitoneal canals was restricted to approximately 300 μm below the level of the anterior intestinal portal. Only intraembryonic mesoderm that was located between the coelomic cavity and the endoderm was included. Similar to the previous stages, no mesenchyme occupied these sites. Interactive 3D reconstructions are presented in the online data supplement.

**Morphology**

The scaling grids in Figures 3 and 4 show that the pericardial back wall, between the anterior intestinal portal and the pericardial reflection, remained ∼700 μm during the stages 9, 10, and 14. The heart tube, however, had elongated and looped. This is in line with previous observations.31 Looping started at the midlevel of the heart tube at the initial rupture of the dorsal mesocardium.32 With this rupture, the heart tube closes dorsally and the left and right pericardial back walls fuse.39 Caudal to the stage 10 heart, there was overt separation of the coelomic mesoderm into an inner part, covering the anterior intestinal portal and an outer part, covering the vitelline veins (Figure 4A; A and Δ, respectively). The vitelline veins are in direct contact with the plexus that covered the yolk sac, as can be seen by the extensive lumina near the inflow at stage 10.

At stage 14 the dorsal mesocardium had broken completely, connecting the bilateral coelomic cavities (hatched arrows in Figure 4C). Initially, only the vitelline veins drained into the heart, but at stage 14, the cardinal veins were also incorporated.

**Proliferation**

Similar to stage 9, the myocardium of the stage 10 heart tube showed virtually no BrdUrd incorporation, with the exception of some BrdUrd-positive cells in the left inflow and the dorsal mesocardium. The nonmyocardial mesoderm also showed a pattern of proliferation similar to stage 9. The pericardial back wall cranially displayed slow proliferation. Caudally, the inner coelomic mesoderm covering the anterior intestinal portal proliferated rapidly, whereas the outer mesoderm covering the vitelline veins showed a low proliferation rate.

At stage 14, the differentiating embryonic ventricle showed an increased proliferation rate but remained flanked by slow-proliferating primary myocardium. At the venous pole, there was increased BrdUrd incorporation at the contact with the pericardial wall. In contrast to earlier stages, proliferation within the pericardial back wall had expanded cranially and approached the arterial pole of the heart. Mesoderm covering the vitelline veins now also showed an increased proliferation rate, with faster proliferation on the right, where the proepicardium differentiates.33

Taken together, our 3D analyses of local proliferation within the developing coelomic walls and heart tube show a bilateral center of fast proliferation. This growth center lies in the inner coelomic wall, dorsal to the inflow of the heart. After rupture of the dorsal mesocardium, this growth center expands cranially.

**Islet1 Expression Colocalizes With Proliferation**

The expression of the transcription factor *Islet1* has been used to mark cells that belong to the second heart field.12 In chicken, the entire heart-forming region initially expresses *Islet1*, and on differentiation of myocardium, expression becomes confined to the mesoderm adjacent to the anterior intestinal portal and the dorsal mesocardium, as assessed by
whole-embryo in situ hybridization. To assess how expression of this gene relates to the observed pattern of proliferation, we reconstructed its 3D pattern of expression from sections (Figure 5).

At stage 11, *Islet1* mRNA was observed in the proliferating part of the coelomic wall, caudodorsally to the inflow of the heart, extending into the dorsal mesocardium. *Islet1* expression was not detected in the dorsal pericardial wall, although the endoderm facing this mesoderm did show expression. Later in development, at stage 16, both the pericardioperitoneal canals and the now also proliferating pericardial back wall displayed expression of *Islet1*. These experiments show expression of *Islet1* in regions that display rapid proliferation.

Caudal Proliferation Is Necessary for Development of Both Poles of the Heart Tube

By increasing the exposure time of BrdUrd, we showed that proliferating cells are added to the cardiac inflow (Figure 2A). During the time frame of our observations, there is also addition of cells to the cardiac outflow. Nonetheless, the outflow is flanked by slow-proliferating mesoderm. Cardiogetic mesoderm is reported to be a cohesive epithelial sheet, suggesting that the precursors added to the arterial pole could also originate from the caudal growth center. To investigate this hypothesis, we used fluorescent vital dye to mark and trace cells from the proliferative center, as described previously (Figure 6).

In line with the observations after long exposure to BrdUrd, the caudal and outer mesoderm (red dye) was taken up into the venous pole of the heart. Cells from the caudal and inner mesoderm (green dye), however, were observed to move along the pericardial back wall into the outflow of the heart tube, where they will differentiate into cardiomyocytes.

To examine whether normal development of the heart depends on proliferation in the caudal mesoderm, we locally inhibited cell division in the caudal and inner mesoderm by exposure to aminopurvalanol, dissolved with DiI. Exposure to aminopurvalanol locally disrupted proliferation within 3 hours, reflected in absence of BrdUrd incorporation (online data supplement). Because of embryonic lethality, we were unable to inhibit proliferation in the entire growth center (data not shown). The location of the inhibition is shown in Figure 6B. We started injection at straight heart tube stages and developed the embryos for 3 more days in ovo. After harvesting, general embryonic morphology did not seem to be

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**Figure 6.** A, Movement of fluorescently labeled cells from the caudal splanchnic mesoderm of a stage 9 embryo. The inner mesoderm is labeled with Dio (green), and the outer mesoderm is labeled with Dil (red). For a spatial appreciation of the inner and outer mesoderm, refer to Figure 4 (indicated with ▲ and △, respectively). With culturing, the outer mesoderm can be seen to be incorporated into the inflow of the heart, whereas the inner mesoderm moves, via the dorsal pericardial wall, into the outflow of the heart. B, Effect of local inhibition of proliferation. At the right side of the embryo, a focus in the caudal and inner mesoderm was exposed to aminopurvalanol, dissolved with Dil. After reincubation, 9 of 16 treated embryos underwent hypoplasia of both the right ventricle and the right atrium. Of the other embryos, 2 died before analysis, 1 showed only outflow malformations, and 4 seemed to be unaffected.
altered. The embryos, however, did have a consistent cardiac phenotype. Nine of 16 embryos displayed malformations at both poles of the heart: on the side of proliferation/inhibition, the atrium did not develop, whereas a shortened outflow tract with a hypoplastic right ventricle was also observed, showing that the cranial movement of progenitors toward the arterial pole was impaired. Taken together, these data demonstrate that growth of the heart occurs at both poles and depends on a caudal source of proliferating progenitors.

Discussion

Key processes in embryonic growth, such as proliferation and cell movement, are of crucial importance for the conceptualization of organ formation. Our studies are the first to accurately quantify and visualize proliferation in both the forming heart and coelomic walls during early cardiogenesis. Our results indicate that the entire heart gradually forms at both poles from a single growth center, which is located in the caudomedial pericardial wall. This process will be discussed below and is summarized in Figure 7 and in the online data supplement.

Formation of the Heart Tube at the Venous Pole

When the coelomic walls fuse to form both the heart tube and the pericardial back wall, proliferation ceases. Reduction of proliferation is also observed in the outer luminizing mesoderm. By progressive fusion, this outer mesoderm will form the nonproliferating heart tube (compare △ in Figure 7), as shown by our present cell traces and also indicated previously.13,29 In the inner caudal mesoderm, a center of rapid proliferation remains, from which cardiomyocytes are added to the venous pole. Not surprisingly, if cell division is inhibited at this site, the atrium fails to develop.

At first glance, a nonproliferating early heart tube seems to be at odds with previous publications, stating that this structure is rapidly proliferating.8 This conclusion was, however, based on the presence of BrdUrd-labeled myocardium after 12 hours of exposure. No heart is present 12 hours before such early stages, meaning that the observed heart consisted exclusively of cells that incorporated BrdUrd as cardiac precursors and differentiated into cardiomyocytes during the time frame of BrdUrd exposure. As shown in Figure 2, this differentiation lengthens the early heart tube at a rate of 70 μm/h. The nonproliferating heart tube is still capable of reinitiating proliferation, which occurs at approximately stage 11, to form the embryonic ventricle at the outer curvature of the looped heart tube.6

We also show that the compact myocardium of this forming ventricle proliferates rapidly with a cell cycle length of 8.5 hours.

Figure 7. Heart formation from the proliferating growth center in the dorsal pericardial wall. The left column shows transverse sections, ranging from cranial in an old embryo to caudal in a young embryo. Firstly, outer mesoderm luminizes and stops to proliferate. Next, it bends inwards and fuses to form the ventral wall of the heart tube (2 to 4). The inner mesoderm keeps proliferating and forms the pericardial back wall and its connection with the heart tube (5). These sections were transformed into the model that is shown on the right. The model shows that expansion from the caudal growth center leads to a radial addition of medially located mesoderm to the heart tube. After regression of the dorsal mesocardium, addition to the arterial pole occurs via the pericardial back wall.
Formation of the Heart Tube at the Arterial Pole

Fluorescent tracing shows that cells from the caudal growth center do not only feed into the inflow of the forming heart but also move cranialward via the coelomic wall, to the myocardium of the outflow tract (Figure 6 and arrows in Figure 7). Unlike the inflow, after 10 hours of BrdUrd exposure, only a narrow rim of labeled cells can be seen at the arterial pole (Figure 2A). This delayed arrival of BrdUrd-labeled cells can be explained by the fact that the cells from the caudal growth center need to move ~700 μm along the pericardial back wall, before reaching the arterial pole, suggesting that the cranial dispatch through the pericardial back wall occurs at a rate comparable to the lengthening of the heart tube at its venous pole (70 μm/h; Figure 2C). This bidirectional movement is also reflected in the effect of local inhibition of proliferation in the caudal growth center. Besides hypoplasia of myocardium at the inflow, the formation of the right ventricle is also impaired.

The Heart-Forming Fields

Proliferation in the coelomic walls colocalizes with the expression of *Islet1*, which has been used to mark precursors belonging to a second heart-forming field. Examination of the expression of *Islet1* in multiple species showed initial expression in all cardiogenic mesoderm, which disappeared on differentiation into myocardium. This suggests that *Islet1* expression does not discriminate between distinct fields of cardiac precursors. We observed colocalization of *Islet1* expression with fast proliferation in the entire coelomic wall, also in those regions of the coelomic wall that will not contribute to the heart. Colocalization of *Islet1* expression with proliferation has also been described outside the mesoderm, and disruption of *Islet1* expression resulted in a loss of proliferation in all these structures. This suggests that *Islet1* plays a general role in the control of proliferation, rather than having a specific function in delineating fields of cardiac progenitors.

The fact that *Islet1* does not specifically mark cardiac precursor lineages does not undermine the concept that the heart is formed by differentiation of precursor cells. Our experiments show that this differentiation occurs gradually and directionally from 1 pool of precursors. When and where these precursors become determined to specific cardiac components cannot be deduced from our experiments. Our data do show how caudal proliferation is a driving force of cardiac morphogenesis. A single, bilateral growth center contributes cells to both poles of the heart during the time frame that encompasses the developmental period in which both the first and the second heart-forming fields have been described.

Acknowledgments

We thank Jaco Hagoort, Bram van Wijk, and Rob van der Ven for technical assistance.

Sources of Funding

This work was funded by Netherlands Heart Foundation grant NIS1996M002, NIH grant R01HL070140, a grant from the Duke Children’s Miracle Network, and a seed grant from University of Michigan—Dearborn.

Disclosures

None.

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