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# 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 non-proliferating 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 caudo-medial 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.

### Keywords

Cardiovascular development; Proliferation; Heart-fields; *Islet1*; Bromodeoxy-Uridine; quantitative 3D-reconstruction

## Introduction

The heart is sculpted by precisely orchestrated developmental programs[1,2] that are prone to errors, leading to high incidences of congenital malformations.[3] Proliferation, while 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

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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 three-dimensional context of cardiac growth and the scarcity of data of locally involved parameters, such as proliferation. Early heart formation is of perplexing 3D complexity, since 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 firstly determined the cell-cycle length of the myocardium of the primary heart tube. We found that newly formed myocardium is non-proliferating, implying that growth of the primary heart tube can occur solely by differentiation of precursor-cells. Increasing pulses of BrdU-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 observations 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] 100 μL of 10 mg 5'-Bromo-2'-Deoxyuridine (BrdU; Sigma) / mL physiological salt solution was injected into the egg yolk directly under the embryos. After re-incubation, 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 (cTnI) (HyTest Ltd) to detect myocardium, anti-BrdU (Becton Dickinson) to detect BrdU-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 (ISH) was performed as previously described.[18] 12 μm-thick sections of paraformaldehyde fixed embryos were alternately stained for *Islet1* (ChEST 603856748F1, MRC-geneservices), *cTnI*,[19] and a third probe (not included in this paper). 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 picospritzer (General Valve Corp.) and a micromanipulator with a pulled glass capillary tube.[21] To inhibit proliferation, 100 μM of the cyclin-dependent kinase inhibitor Aminopurvalanol A[21] (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 3D-reconstructions of BrdU-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 BrdU-positive nuclei were automatically counted using custom written macros for Image Pro Plus (*Media Cybernetics*). In the resulting 3D-datasets (Figure 1C), the 3D-fraction of BrdU-positive nuclei was spatially determined in sampling cubes of $(105\ \mu m)^3$, which were moved in steps of 21 μm in three dimensions. The sampling cubes contain sufficient nuclei to result in reliable estimates of local BrdU-fractions, while their step size guarantees adequate spatial resolution.[16] The resulting BrdU-positive fraction was projected into the $(21\ \mu m)^3$ center-cube (Figure 1D). The segmented morphological structures were reconstructed with Amira (*Mercury Computer Systems*) (Figure 1B). The reconstructions were overlain with the 3D-BrdU fractions to generate the quantitative 3D-reconstructions of local proliferation (Figure 1E). For this manuscript 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 relation to the presented 3D-quantitative reconstructions are shown in the Supplementary Figures.

**Regional estimation of BrdU-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 in the results-section. Within a selection, all nuclei and BrdU-positive nuclei were pooled to calculate a regional BrdU-positive fraction.

**Distance estimation—**Distances were measured through the reconstructed myocardium, between manually placed waypoints. To this end we implemented an existing length estimator[22] in MatLab (*The MathWorks*). It was adapted for non-isotropic datasets, and has a mean error of 0.4% (SD 1.1%). Details on the software will be published separately.

## Results

### Proliferation rates of the primary heart tube and the embryonic ventricle

To comprehend cardiac growth, we firstly wanted to know the cell-cycle length of cardiomyocytes of the heart tube. To this end we exposed embryos of similar developmental stages to BrdU for an increasing period of time (Figure 2). At the studied stages, BrdU incorporation attributable to DNA repair is negligible and no multinuclear cells occupy the myocardium.[23] The cell-cycle length can be calculated from the linear relationship between BrdU-exposure time and BrdU-positive fraction.[24]

To determine the cell-cycle length of the primary heart tube, we made quantitative reconstructions of myocardium from 2-day-old embryos, exposed to BrdU from 1 to 10 hours (Figure 2A). Irrespective of exposure time, the heart tube consisted mainly of BrdU-negative cardiomyocytes, although with increasing exposure a zone of BrdU-labeled cells broadened at the venous pole. After exclusion of these cells, the BrdU-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,[6] 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 BrdU which will be retained after differentiation into cardiomyocytes and concomitant cessation of proliferation. This explains the observed broadening of the zone of BrdU-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 BrdU-exposure time (Figure 2C). The resulting graph shows that the primary heart tube lengthens at the inflow at a rate of about 70 μm / hour.

Myocardium of the looped tubular heart will re-initiate proliferation at its outer curvature at approximately stage 12, forming the primitive ventricle.[6] To determine the proliferation rate of the forming ventricle we conducted similar experiments as explained above. We made quantitative reconstructions of hearts of three-day-old embryos that were treated with BrdU for 1 to 6 hours (Figure 2B). At these stages, the primitive ventricle is morphologically recognizable by the presence of trabeculae. We observed a transmural gradient of proliferation within this ventricular myocardium (data not shown), since the rapidly proliferating compact myocardium gives rise to the slow proliferating trabeculae.[7,25] Analysis of the BrdU-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 non-proliferating 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 BrdU for 1 hour only, to minimize the BrdU-labeling due 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,[26,27] 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. Presented reconstructions are also interactively approachable via a supplementary 3D-pdf.

**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,[28,29] whereas mRNA-expression of sarcomeric markers is more abundant.[30] Although myocardial differentiation is gradually commencing, we designated all reconstructed mesoderm at stage 8 to be “non-myocardial”. 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 in 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 BrdU 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 exposure to BrdU. 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 two 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 intra-embryonic mesoderm that was located between the coelomic cavity and the endoderm was included. Similar to the previous stages, no mesenchyme occupied these sites. Presented reconstructions are also interactively approachable via a supplementary 3D-pdf.

**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 approximately 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.[29] 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; ▲ and △, respectively). The vitelline veins were 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 BrdU-incorporation, with the exception of some BrdU-positive cells in the left inflow and the dorsal mesocardium. The non-myocardial 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 BrdU-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 three-dimensional 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 co-localizes 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 upon differentiation of myocardium, expression becomes confined to the mesoderm adjacent to the anterior intestinal portal and the dorsal mesocardium, as assessed by whole-embryo ISH.[34] In order 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, caudo-dorsally 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 BrdU 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.[35] Nonetheless, the outflow is flanked by slow proliferating mesoderm. Cardiogenic mesoderm is reported to be a cohesive epithelial sheet,[36] 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).[37]

In line with the observations after long exposure to BrdU, 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.[37]

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,[21] dissolved with DiI. Exposure to Aminopurvanol disrupted proliferation within 3 hours, as reflected in an absence of BrdU-incorporation (Supplementary Data). Due to 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 altered. The embryos, however, did have a consistent cardiac phenotype. 9 out of 16 embryos displayed malformations at both poles of the heart: on the side of proliferation-inhibition, the atrium did not develop, while also a shortened outflow tract with a hypoplastic right ventricle was observed. showing that the cranial movement of progenitors towards 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 one growth-center, which is located in the caudomedial pericardial wall. This process will be discussed below and is summarized in Figure 7 (also interactively approachable via the Supplementary Data).

### 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 non-proliferating heart tube (compare △ in Figure 7), as shown by our current cell traces and also indicated previously.[13,29] In the inner caudal mesoderm a center of rapid proliferation remains, from where 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 non-proliferating 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 BrdU-labeled myocardium after 12 hours of exposure. No heart is present 12 hours prior to such early stages, meaning that the observed heart consisted exclusively of cells that incorporated BrdU as cardiac precursors, and differentiated into cardiomyocytes during the time-frame of BrdU-exposure. As shown in Figure 2, this differentiation lengthens the early heart tube at a rate of 70 μm/hr. The non-proliferating heart tube is still capable of reinitiating proliferation, which occurs at about 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 time of 8.5 hours.

### 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 cranialwards via the coelomic wall, to the myocardium of the outflow tract (Figure 6; arrows in Figure 7).[35,37] Unlike the inflow, after 10 hours of BrdU-exposure only a narrow rim of labeled cells can be seen at the arterial pole (Figure 2A). This delayed arrival of BrdU-labeled cells can be explained by the fact that the cells from the caudal growth-center need to move approximately 700 μm along the pericardial back wall, before reaching the arterial pole. This suggests that the cranial dispatchment through the pericardial back wall occurs at a rate similar to the lengthening of the heart tube at its venous pole (70 μm / hour - Figure 2A'). This bi-directional 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 co-localizes with the expression of *Islet1*, which has been used to mark precursors belonging to a second heart-forming field.[11,12] Examination of the expression of *Islet1* in multiple species showed initial expression in all cardiogenic mesoderm, which disappeared upon differentiation into myocardium.[34,38,39] This suggests that *Islet1*-expression does not discriminate between distinct fields of cardiac precursors. We observed co-localization of *Islet1*-expression with fast proliferation in the coelomic wall, but also in mesoderm that will not contribute to the heart. Co-localization 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.[11] 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 one 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.

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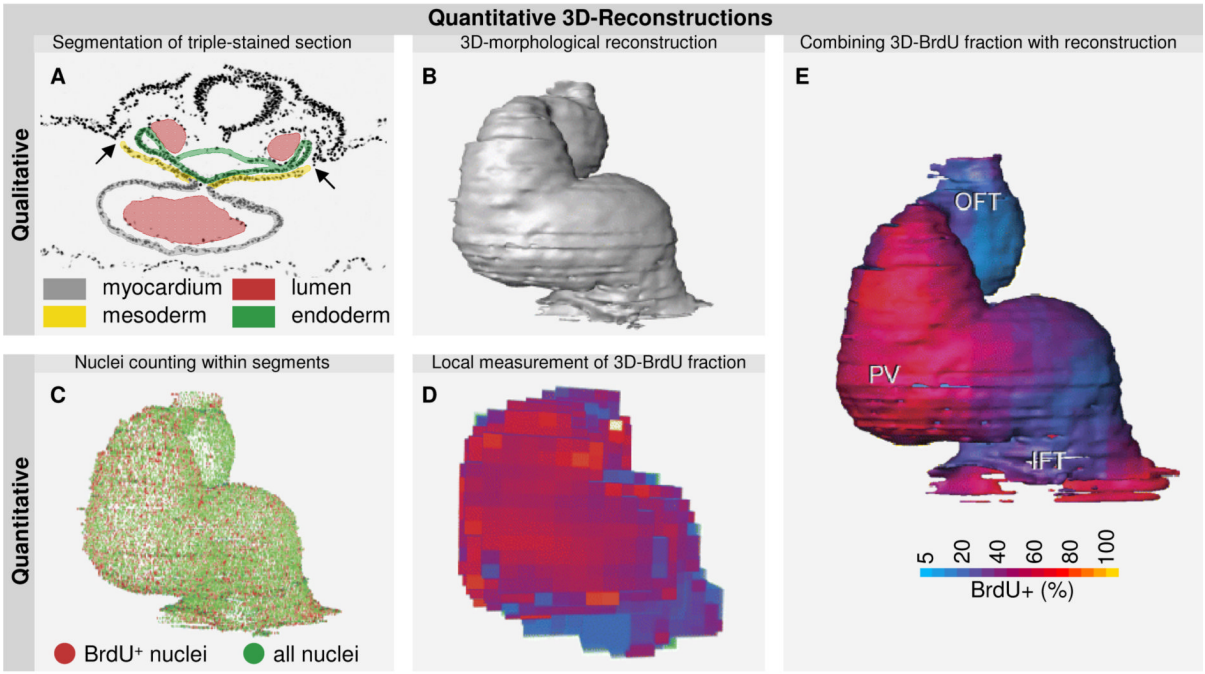


**Figure 1.**

Panel A shows a representative image of the Sytox-green channel of a triple-stained section. Overlaying the image are the segmented myocardium (grey), splanchnic mesoderm (yellow), the endoderm (green) and the cardiovascular lumen (red). Arrows indicate the borders between intra and extra-embryonic mesoderm. BrdU-positive and all nuclei were automatically identified within the myocardium and selected splanchnic mesoderm. Panel C shows nuclei of the myocardium in their 3D-context. To facilitate interpretation, and allow reliable estimations, nuclei were counted and BrdU-fractions were determined in sample-cubes, as shown in panel D. This information is then projected on the morphological reconstruction (panel B) to give a quantitative 3D-reconstruction of local proliferation (panel E). (Displayed reconstruction is the myocardium of a stage 12 chicken embryo, after 4 hours of BrdU-exposure.[6])

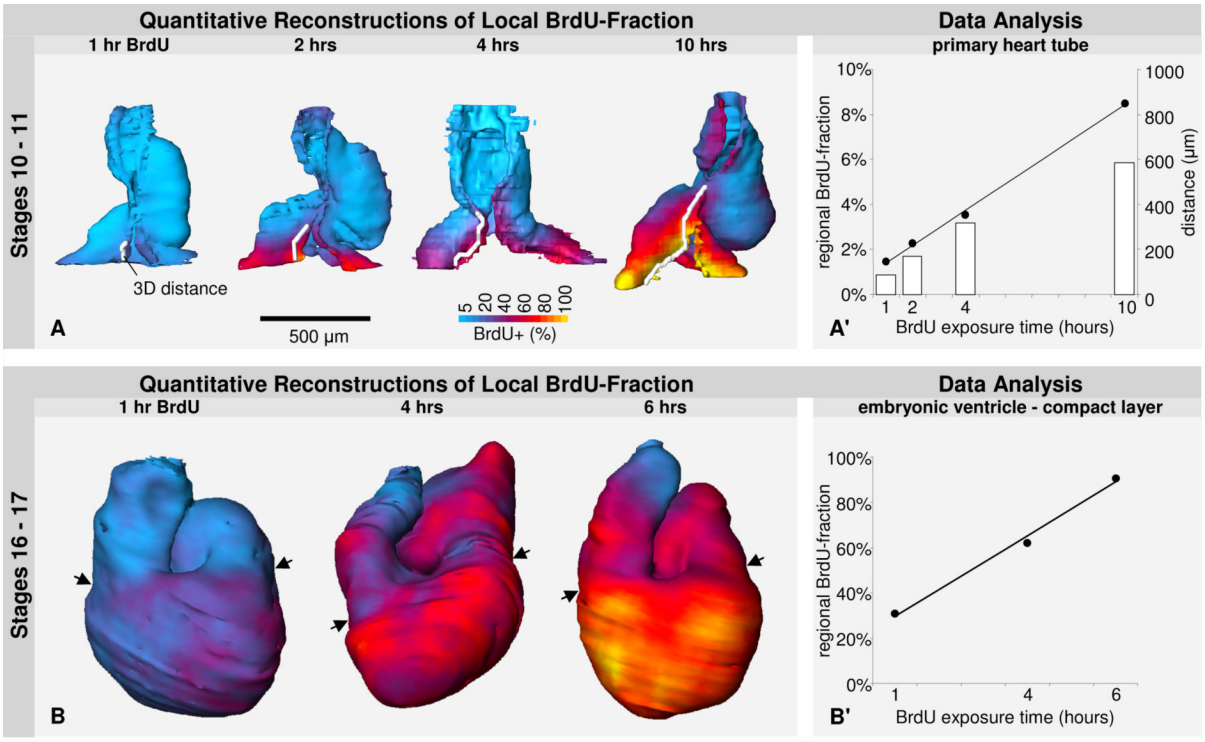


**Figure 2.**

Panels A and B show quantitative 3D-reconstructions of local $BrdU^+$-fractions of embryos that were increasingly exposed to BrdU. The nuclear BrdU-fractions are presented using a color-bar, ranging from blue (0%) to yellow (100%). The length of expanding BrdU-positive zone along the left dorsal mesocardium was measured and indicated in white. The relation of this expansion to the BrdU exposure-time is shown in panel C. To calculate cell-cycle times we exploited the linear relation between BrdU-fractions ($F_B$) and BrdU-exposure time ($T_B$). This relation follows the equation: $F_B = T_S/T_G + 1/T_G \times T_B$, with $T_S$ and $T_G$ representing the length of the S-phase and the cell cycle, respectively. The inverse of the slope of this linear relation equals the cell-cycle length.[24] For calculations of the primary heart tube we counted $BrdU^+$-fractions of the reconstructions in panel B, with exclusion of the expanding zone of BrdU-labeled cells. For calculations of ventricular myocardium, we counted $BrdU^+$-fractions in the compact layer at the outer curvature of the reconstructions in panel B, between the distal ventricular groove (left arrow) and the atrioventricular canal (right arrow). Panel D shows these linear relations of both the ventricular and the primary myocardium (hatched line).

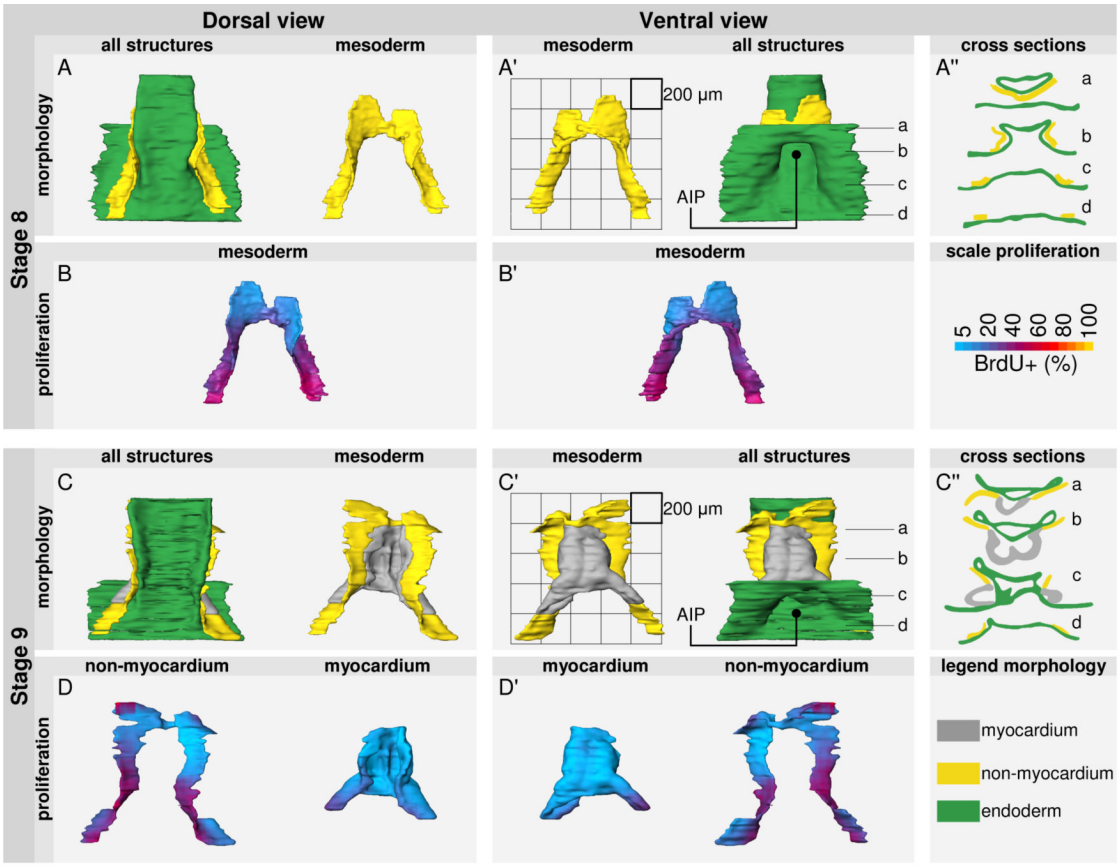


**Figure 3.**

shows morphology and proliferation (as defined by the $BrdU^{+}$-fraction after 1 hour of exposure) of the heart region of stage 8 (rows A and B) and stage 9 (rows C and D) chicken embryos. Myocardium is shown in grey, non-myocardial mesoderm in yellow and endoderm in green. In A' and C' a scale grid of $(1000\ \mu m)^2$ is shown. Also, in A' and C' locations are indicated of cross-sections that are shown in A" and C", respectively. (AIP: anterior intestinal portal)

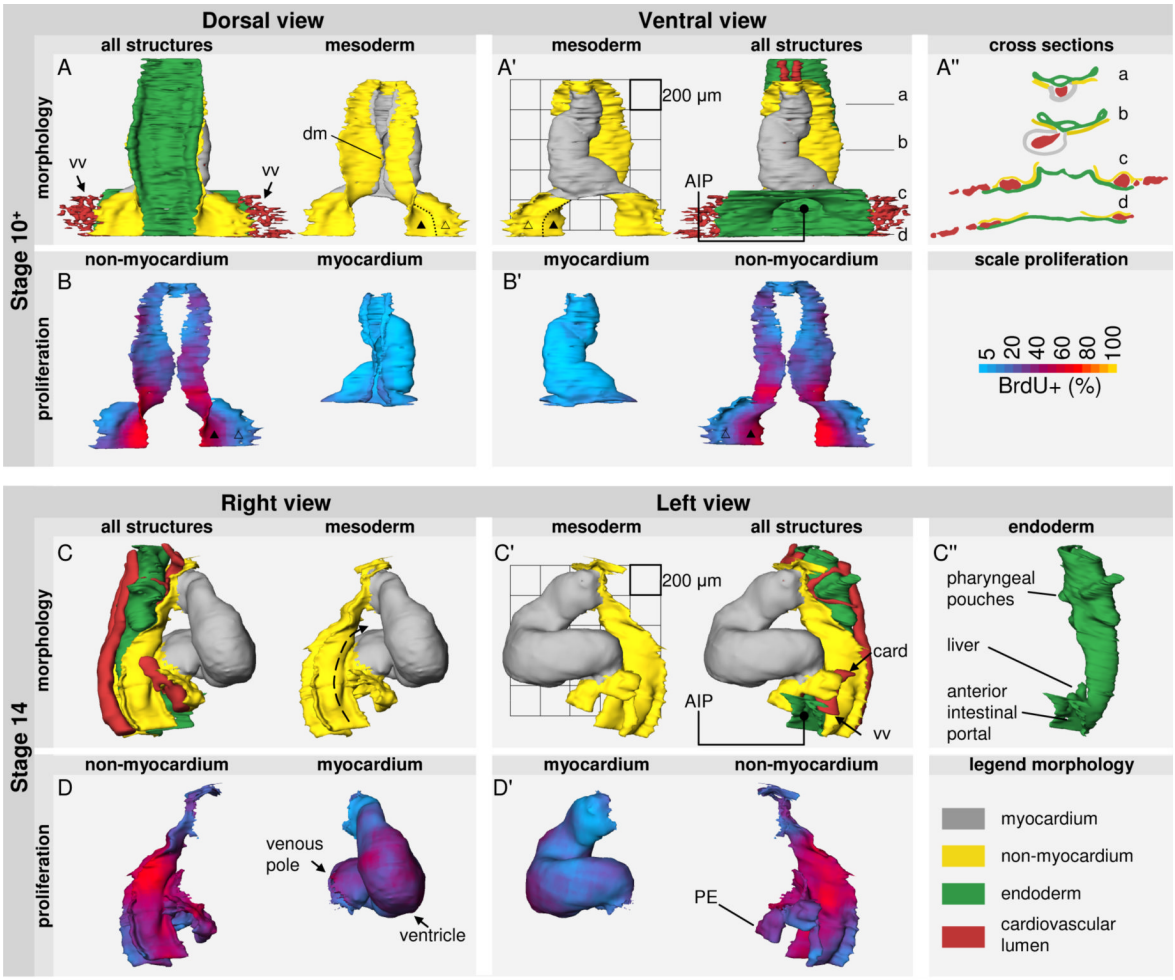


**Figure 4.**

shows morphology and proliferation (as defined by the BrdU$^+$-fraction after 1 hour of exposure) of the heart region of stage 10$^+$ (rows A and B) and stage 14 (rows C and D) chicken embryos. The myocardium is shown in grey, non-myocardial mesoderm in yellow, endoderm in green, and cardiovascular lumen in red. In A' and C' a scale grid of (1000 µm)$^2$ is shown. Also, in A' locations are indicated of cross-sections that are shown in A". Panel C" shows the reconstructed endoderm. (AIP: anterior intestinal portal, card: cardinal vein, dm: dorsal mesocardium, PE: proepicardium, vv: vitelline veins, △: outer mesoderm that covers the vitelline veins, ▲: inner mesoderm that covers the anterior intestinal portal, hatched arrows: pericardioperitoneal canals)

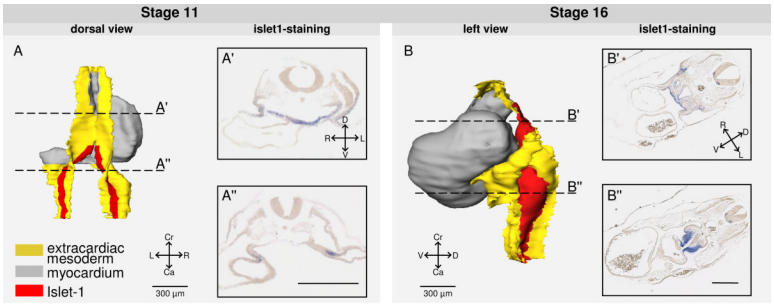


**Figure 5.**

shows reconstructions of extension of the expression of *Islet1*-mRNA in red. Reconstructed are the myocardium (grey) and non-myocardial mesoderm (yellow). Panel A shows a dorsal view of a stage 11 embryo. Section A' shows expression of *Islet1* in the endoderm. Section A" shows expression in the pericardioperitoneal canals. Panel B shows a left view of a stage 16 embryo. Section B' shows expression in the pericardial back wall. Section B" shows expression in the pericardioperitoneal canals. (Ca: caudal, Cr: cranial, D: dorsal, L: left, R: right, V: ventral) Scale bars: 300 µm.

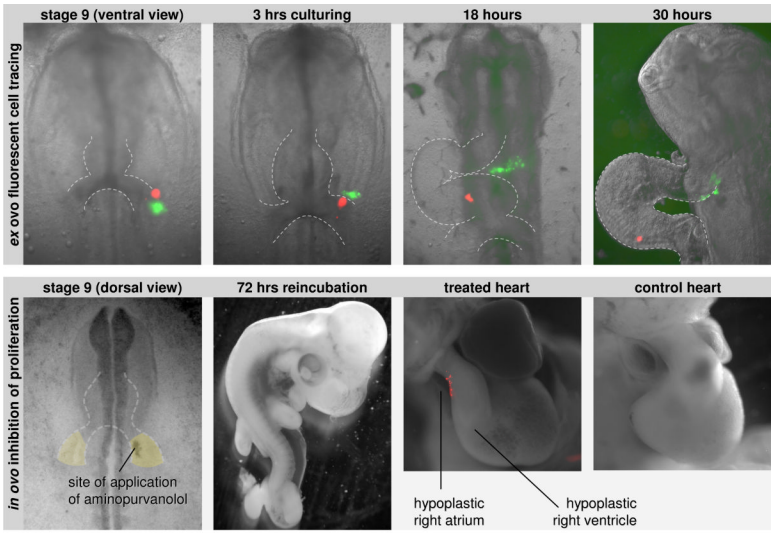


**Figure 6.**

Panel A shows the 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 DiI (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, while the inner mesoderm moves, via the dorsal pericardial wall, into the outflow of the heart. Panel B shows the effect of local inhibition of proliferation. At the right side of the embryo, a focus in the caudal and inner mesoderm was exposed to Aminopurvanolol, dissolved with DiI. After reincubation 9 out of 16 treated embryos suffered from hypoplasia of both the right ventricle and the right atrium. Of the other embryos, 2 deceased before analysis, 1 showed only outflow-malformations, and 4 seemed to be unaffected.

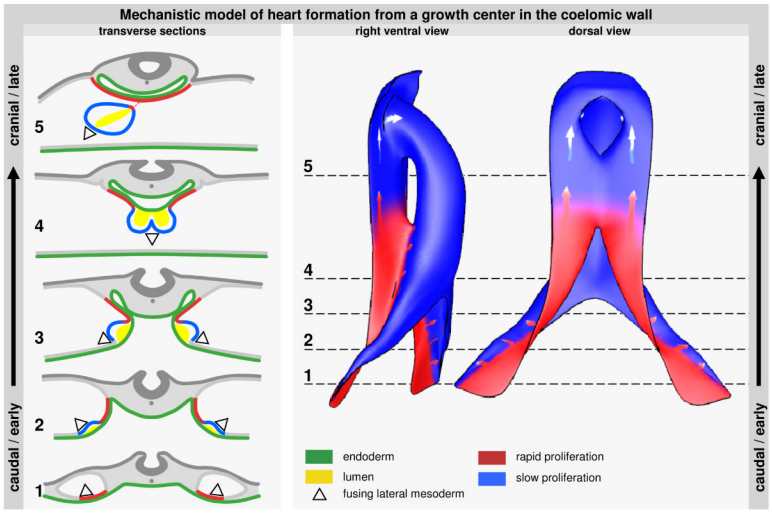


**Figure 7.**

illustrates 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 (2-4). 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.