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chapter 3

Formation of the Building Plan of the Human Heart: Morphogenesis, Growth, and Differentiation

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Abstract

Background – Studies in experimental animals have shown that the primary myocardial heart tube does not proliferate, and mainly contains the precursors for the left ventricle. During further development, the heart tube elongates, loops, and gives rise to the chambers through local growth and differentiation. To date, molecular data on heart development in man that allow comparison between human and experimental animals are virtually lacking.

Methods and Results – To explore whether the current concept of cardiac development is applicable to human, we analyzed the 3-dimensional morphology, proliferation, and expression of some key regulatory and functional genes in human embryos. At stage 10, although strong expression of the proliferation markers PCNA and Ki67, and the precursor marker ISL1 was observed in the coelomic wall, the myocardial tube revealed much slower proliferation suggesting, that upon differentiation, cardiomyocytes exit the cell cycle. Growth and differentiation of chamber myocardium was first observed by local expression of PCNA, connexin40 and -43, and ANF at the outer curvature of the looped heart. The myocardium of sinus venosus, atrioventricular canal and outflow tract remained devoid of expression of chamber and proliferation markers, but expressed the transcriptional repressor TBX3.

Conclusions – Patterns of morphogenesis, growth and gene expression in the early and chamber-forming human heart are comparable to those described for experimental animals. Biphasic growth occurs first by addition of cardiomyocytes differentiating from the highly proliferative adjacent coelomic wall to form the heart tube, and then following a phase of quiescence, by local proliferation and differentiation to form the chambers.
Introduction
The intricate process of formation of the heart relies on the spatio-temporal regulation of differentiation and growth of its different parts. Errors in these processes can result in hypoplasia of the chambers, and incorrect alignment of the atria and great arteries with the ventricles, such abnormalities producing the worst forms of congenital heart disease. Although recent advances in molecular embryology have greatly improved our understanding of normal cardiac development, our insights into the pathogenesis of human cardiac malformations remain limited. With the current lack of data on gene expression in man, inferences concerning morphogenesis rely on classic studies of human cardiac development, or on extrapolation of experimental data from animal studies. Most textbooks on human embryology describe the embryonic heart tube as already possessing a linear array of the definitive components, despite this so-called segmental concept being disproved some time ago by fate mapping experiments in chicken, which are now endorsed by recent molecular lineage analyses in mouse.

Proliferation studies in chicken revealed that the primary heart tube hardly proliferates and that its growth occurs through addition of differentiating cells from visceral mesoderm at the venous and arterial poles. These findings underscore previous lineage studies performed in mouse, which have shown that transcription factors Islet1 and Tbx1 play a crucial role in the intricate balance between the cardiac precursor state and differentiation. Subsequent to its formation, the heart tube elongates and loops, producing its inner and outer curvatures, and providing the general building plan for formation of the chambers and conduction system. The chambers themselves form by local proliferation and differentiation at the outer curvature. At the same time, the differentiating chamber myocardium remains flanked by primary myocardium, which is prevented from further differentiation by the transcriptional repressors Tbx2 and Tbx3, thus providing the precursors of the cardiac conduction system.

Because it is impossible to perform experiments in the developing human heart and the scarcity of gene expression data, the descriptions of its development remained controversial. In this study, we report patterns of proliferation and gene expression in tubular and chamber-forming stages of the human heart, supplementing our account with three-dimensional (3D) analyses presented in interactive fashion. Our findings point to the comparability of the mechanisms governing cardiac development in human, mouse, and chicken.
Material and methods

**Human Embryos**

We used human embryos ranging from Carnegie stages 9 till 16 derived from two different collections. Acquisition and preparation of the first group of embryos were performed at the Gynaecology department of Tartu University Hospital, Estonia. To extend the number of embryos in this study we have included a series of previously performed stainings of serial sections of embryos, which were collected from medically induced abortions performed at the Gynaecology department of the First Hospital of Shanxi Medical University, China. As soon as feasible, the aborted tissues were fixed in 4% paraformaldehyde solution. Embryos were then examined under a stereomicroscope, investigated for gross anomalies, and graded according to the Carnegie criteria. After further fixation overnight, embryos were dehydrated in graded ethanol series, immersed in butanol, and embedded in Paraplast. We included only embryos considered normal. Collection and use of the human embryonic material was approved by the Medical Ethical Committees of the Universities of Tartu, Estonia, Amsterdam, the Netherlands, and Shanxi Medical University, China.

**Immunohistochemistry, in situ Hybridization and 3D Reconstructions**

Immunofluorescent staining, in situ hybridization, morphological and quantitative 3D reconstructions were performed on the first group of embryos as detailed in the Data Supplement. Sections of the second group of embryos were processed by indirect immunohistochemistry. For detailed description of the protocols and specification of the antibodies and riboprobe see the Data Supplement.

**Limitations of our study**

Our study has several limitations owing to the use of human embryos. Fixation could not be entirely standardized. The limited number of available embryos did not permit complete optimization of the staining protocol for some antibodies and probes, and prevented assessment of biological variation between specimens at similar developmental stages. Immunohistochemical and in situ hybridization stainings, nonetheless, proved to be reproducible. Since incorporation of bromo-deoxyuridine (BrdU) cannot be performed in human embryos, proliferative status was evaluated indirectly by immunohistochemistry for Ki67 antigen and PCNA (proliferating cell nuclear antigen),\(^{12,13}\) the expression of which correlates well with BrdU incorporation.\(^{14}\) The scarcity of young human embryos allowed only the full quantitative reconstruction of Ki67 expression in one stage 10 embryo, whereas systematic samples of sections were available in three stages to determine the PCNA labeling index of the whole heart. Although these observations are strictly an \(n=1\) observation, a statistical test based on the
number of counted nuclei (Z-test between proportions) showed that the observed differences in labeling indices exceed the methodological variation. The use of immunohistochemically stained serial sections permits 3D molecular analysis of the structures, but the corollary is that reconstructions have to be corrected for distortions of the surface because of uneven stretching and occasional damage of the sections. While very fine details could not be reproduced in the 3D models, no spurious structures were gained or important details lost during the correction procedure.

Results
The reader is encouraged to read the results along with the interactive 3D-PDF file in the online Data Supplement (accessible at http://circ.ahajournals.org).

Formation and Growth of the Primary Heart Tube
First we analyzed the formation and growth of the primary heart tube in one stage 9, four stage 10 and three stage 11 embryos, which correspond to approximately 20-25 days of human development, and embryonic days (E) 8.0 through 9.0 in the mouse. Figure 1 shows a 3D overview of the formation and looping of the myocardial tube. In the stage 9 embryo, the youngest of our series, the heart tube is not yet formed. At the cranial end of the embryo, the myocardialized part of the walls of the bilateral intra-embryonic coelomic cavities are already fused, with the fusion line recognizable by a characteristic groove in the midline of the myocardial trough (Figure 1A,A*). The borders of the myocardium are contiguous with the coelomic wall. The two cell-thick myocardial layer of the forming heart tube is separated by acellular jelly from the endothelial cells lining two narrow lumens. The cardiac jelly extends outside the cardiac trough dorsally, where the ventral wall of the foregut borders the cardiac trough (Figure 1A**). In late stage 10 embryos the heart tube is closed and the tubes show various stages of looping. At this stage the heart tube contains a single and wider lumen surrounded by jelly, which still extends outside the myocardial tube (Figure 1B,B*,B**). In stage 11 embryos the heart tube is almost completely looped (Figure 1C,C*), and the first signs of formation of the atrial and ventricular chambers were seen at the dorsal and ventral sides of the tube, respectively (Figure 1C**). Regionally, the heart tube has expanded and the myocardial layer thickened, albeit that trabecules have yet to form. Interiorly, the heart tube is still filled by acellular jelly, which separates the myocardial and endothelial layers. As yet, no epicardial layer covers the outer surface of the myocardial heart tube.

To provide insight into the precursor, differentiation and proliferative status of the primary heart tube and adjacent mesoderm, we stained sections with ISL1, NKX2-5, troponin-I and the proliferation markers PCNA and Ki67 (Figures 2,3). In the stage 9 embryo the domains
Figure 1. Formation and looping of the human embryonic heart. At stage 9 (panels A-A**), the heart tube is just forming. At stage 10 (panels B-B**) the heart tube begins to loop and at the right side of the venous pole the forming atrium is already discernable. At stage 11 (panels C-C**) the heart tube is elongated and looped, and it is possible to distinguish the morphological landmarks of the primordia of the future cardiac chambers, as well as the inner and outer curvatures (white and black dotted lines in panel C**). Note that at shown stages myocardium at the venous and arterial poles is directly contiguous with the coelomic wall (black arrows). Abbreviations: aip, anterior intestinal portal; ao, (dorsal) aorta; avc, atrioventricular canal; cc, coelomic cavity; ht, heart tube; fg, foregut; PV, primitive ventricle; v, vein.

The scale bars apply to the dorsal and ventral views.
of the NKX2-5 and ISL1 expression in the forming heart, adjacent mesoderm and foregut are largely overlapping. Weak ISL1 expression in the myocardial trough (Figure 3A,B) indicates the recent differentiation of the ISL1-positive precursors toward cardiomyocytes. At stages 10-11 the non-differentiated (i.e., troponin I-negative) mesodermal cells at the venous and arterial poles, as well as cells of the dorsal coelomic wall and ruptured mesocardium, co-express NKX2-5 and ISL1, which suggests that these cells are the progenitors to be added to the heart tube. In contrast, most of the myocardial heart tube is negative for ISL1, with the exception of the

Figure 2. Growth of the primary heart tube at the venous and arterial poles. Sections of embryos at stages 10 through 12 were incubated with antibodies as indicated; no proliferation data are available for the stage 9 embryo. Note the sharp border of myocardial (troponin I positive) to non-myocardial coelomic wall at both poles of the heart tube. At these borders the expression of ISL1, PCNA and Ki67 is almost overlapping, being high in the non-myocardial cells and decreasing along the myocardial wall of the inflow and outflow tracts (arrows). Also note that the expression patterns of Ki67 and PCNA are similar (panels B,E,D,G) and that the number of phosphohistone H3 (PH3) expressing cells is very low, even in highly proliferating regions (panel H). Abbreviations: as, aortic sac; L/RV, left/right ventricle; OFT, outflow tract; SV, sinus venosus; for other abbreviations see Figure 1. Scale bars, 100 μm.
Figure 3. Formation and growth of the primary heart tube during stages 9 through 11. Transverse sections, which correspond to the cross-sections of the 3D models shown in Figure 1, were incubated with antibodies as indicated. Note that in the stage 9 embryo (panels A-C) the domains of expression of NKX2-5 and ISL1 in the forming heart and foregut are largely overlapping, albeit expression of ISL1 in the myocardium is very weak. Compared to the expression of troponin I the expression of NKX2-5 extends into the adjacent coelomic wall. At the next stages most of the cardiomyocytes do not express ISL1 (panels E,K), irrespective of their proliferative status. Importantly, the linear myocardial tube contains very few cells positive for Ki67 or PCNA (panels F,G). The quantitative reconstruction of Ki67 expression in a late stage 10 embryo (panels H,I) shows a striking discrepancy of the percentage of the Ki67-positive cells in the myocardium as compared to the coelomic wall, indicating that the vast majority of the cardiomyocytes at this stage is quiescent. Note the local increase in proliferative activity of the myocardial tube of this particular embryo (arrowheads in H). Panel L shows the section through the looped heart of the stage 11 embryo, where the inner curvature (dotted line) is negative for PCNA, while the left ventricular and atrial walls show re-initiation of proliferation (green arrowheads). Note that cells making up the foregut and neuro-ectoderm, are strongly positive for PCNA and Ki67. Yellow arrows in panel L point to the developing epicardium. For abbreviations see previous Figures. Scale bars, 100 μm.
venous and arterial poles, where strong myocardial expression of ISL1 is present (Figure 2). All myocardial cells express NKX2-5, indicating that the sinus venosus, characterized by absence of this factor,\textsuperscript{15} is not yet formed at these stages. The level of expression of the proliferation markers PCNA and Ki67 in the myocardial heart tube at stage 10 was much lower when compared to surrounding tissues (Figures 2,3). The coelomic wall contiguous with the myocardium at the venous and arterial poles highly co-expresses proliferation and progenitor cell markers, expression of which tapered off in the heart.

To quantify and visualize the proliferation of the primary heart tube and its adjacent mesoderm, a quantitative reconstruction of Ki67 expression in one single embryo of late stage 10 was made (Figure 3H,I). From this reconstruction one can appreciate the significant contrast in proliferative status of the myocardium and adjacent mesoderm, on average, only 33% of the cardiomyocytes versus 84% of the cells within the coelomic wall are positive for Ki67. At the end of stage 10 about half of the cardiomyocytes within the outer layer of the forming outer curvature of the cardiac tube is positive for Ki67 and PCNA (Figure 3H). At stage 11 expression of proliferation markers is confined to the myocardium of the forming ventricular and atrial chambers (arrowheads in Figure 3L). The PCNA labeling index displays an increase in overall proliferation rate from stage 10 to 12 (Figure 4). Ki67- and PCNA-stained sections of other embryos confirm these quantitative observations. Because peak level of PCNA is reached around the early S-phase of the cell cycle,\textsuperscript{14} the observed PCNA labeling is lower than the average Ki67 labelling index. Phosphohistone H3-[Ser10] expression\textsuperscript{16} showed a low number of M-phase nuclei; although maybe slightly more at the venous pole than at the arterial pole (Figure 2F,H).

![Figure 4. Quantitative assessment of the overall increase of the PCNA labelling index of the myocardial heart tube at stages 10 through 12 (one embryo per stage; the error bars indicate the standard error due to binomial sampling).]
Growth and Differentiation of the Cardiac Chambers

Next we analyzed the 3D morphology, proliferation and expression of molecular markers in chamber-forming hearts of eight embryos of stage 12-13, ten embryos of stage 14-15 and six embryos of stage 16, which corresponds to approximately 26 to 38 days of human development, and E9.5 through E12.5 in the mouse. The inner and outer curvatures of the chamber-forming heart display significant differences in the patterns of proliferation and gene expression. At the
outer or greater curvature, atrial and ventricular chambers differentiate and balloon out from the cardiac tube dorsally and ventrally, respectively. The inner curvature is the point of continuity between the primary myocardium of the floor of the common atrium, the atrioventricular canal and the proximal outflow tract (Figure 5A,B,C). Overt signs of differentiation toward chamber myocardium were first observed at stage 12 at the outer curvature of the completely looped heart tube. Now tiny trabecules, covered by a markedly thinner layer of acellular jelly than at the smooth-walled inner curvature, are formed in the left ventricular wall (Figure 5A*). From stage 14, when septation has started, the expansion of the chambers is significantly progressed.

Figure 6. Growth of the chamber-forming and septating heart analyzed by PCNA expression. Panels A to B show PCNA expression in transverse sections of embryos at stages 12 through 16. Note that the wall of the ballooning atrial and ventricular chambers are highly positive for PCNA, indicating their intensive growth, while the walls of atrioventricular canal, inner curvature (asterisk in panel A*) and outflow tract are virtually negative for this proliferation marker. Note also, that the ventricular trabecules at stage 16 are devoid of PCNA (panel C*). Another interesting observation is the absence of proliferation in the vestibular spine (panel B). For abbreviations see previous Figures. Scale bars, 200 μm.
(Figure 5B) and ventricular trabecules become extensive (Figure 5B*). Subsequent to the local re-initiation of proliferation of the myocardial walls of the future atrial and ventricular chambers in the looping heart at late stage 10 (Figure 3H,L), the proportion of proliferating cardiomyocytes at stage 12 increased substantially (Figure 4). Later on, atrial and ventricular chamber myocardium of stages 12 through 16 showed high PCNA expression (Figure 6), albeit that proliferation in the ventricular trabecules declined by stage 16 (Figure 6C*). Similar to the previous stages, the expression of phosphohistone H3 was observed only in a few cells throughout the sections (not shown).

Along with the morphological changes marking the formation of the chambers, there are several molecular markers, such as atrial natriuretic factor (ANF) and fast conducting connexins 40 and 43, which become expressed in the developing working myocardium in the mouse. In the early chamber-forming heart of the stage 12 embryo, the atrial wall and ventricular myocardium expressed ANF and connexin40 (Figures 5A*,7E). At later stages the expression of ANF and connexin40, while remaining very strong in the atrial myocardium, became confined to the trabecular myocardium in the ventricles, as reported previously for the human fetal heart (Figures 5,7). As the antibody staining for connexin43 was unsuccessful, we investigated the expression of this gene at the mRNA level by in situ hybridization. The expression of connexin43 was observed in the ventricular trabecules, being absent from the atrial and ventricular walls at least till stage 16 (Figure 5), as reported previously. In contrast, the non-working myocardium of the atrioventricular canal and outflow tract was devoid of ANF, connexin40 and 43, and PCNA expression (Figures 5-7). Remarkably, the mesenchyme of the vestibular spine, a structure supposed to play major role in completing atrial septation, does not proliferate, whereas the contiguous extra-cardiac mesenchyme displays high PCNA expression (Figure 6C).

**Expression of T-box Factors 2 and 3 in the Non-chamber Forming Myocardium**

As in mouse and chicken the myocardium of the atrioventricular canal and outflow tract flanking the ballooning atrial and ventricular chambers, does not proliferate as assessed by PCNA expression (Figure 6) and is devoid of chamber markers (Figures 5,7), underscoring the hypothesis of the persistence of primary myocardium at these specific locations. Expression of the transcriptional repressor and conduction system marker TBX3 was first observed in the floor of the common atrium and in the atrioventricular canal myocardium at stage 12 (Figure 7D), when chamber formation is well underway. Subsequent to the initiation of septation at stage 14, TBX3 expression in the atrioventricular canal becomes asymmetric, being stronger at the right side (Figure 7J). Weak expression of TBX3 was observed also in parts of the myocardial systemic venous sinus and the primary atrial septum.
Figure 7. Molecular analysis of the non-chamber forming parts of the heart. At stage 12 the myocardium of the inner curvature (dotted line in panel E) and atrioventricular canal expresses TBX3, but not connexin40 (arrowheads in panels D,E). In contrast, the walls of the developing chambers express connexin40 and no TBX3. In the stage 14 embryonic heart the chambers have been substantially expanded. Like in the previous stages, the connexin40-negative regions of venous sinus and atrioventricular canal flanking the chamber myocardium, express TBX3 (panels G-J), marking the regions were the pacemaker and conduction system develops. When assessed by routine histological staining (hematoxylin-azophloxin, H&A) the cells of the atrioventricular canal and ventricular wall look similar (panels F,L). Abbreviations: pas, primary atrial septum; pr.f, primary foramen; rvv, right venous valve. For other abbreviations see previous Figures. Scale bars, 200 μm.
Expression of TBX2, which also represses chamber formation, is similar to that of TBX3, albeit with some differences. Unlike TBX3, TBX2 is expressed in the outflow tract. In the atrioventricular canal the expression of TBX2 observed at stage 12, has gradually disappeared at stage 16 (Figure 8). As in the mouse, expression of TBX2 in the atrioventricular canal tapers off into left ventricular direction (arrowheads in Figure 8A). The expression pattern of connexin40 (Figure 7E,K) is complementary to that of TBX3, except for the atrial septum and vestibular spine, indicating conservation of the repressive action of TBX3 in mouse and human. The general histology and cellular morphology of the myocardium in the atrioventricular canal and ventricular chamber wall at stages 12 through 14 (Figure 7F,L) are similar despite their distinct molecular signature.

Discussion

The mechanisms of development of the four-chambered heart from a primary linear heart tube have remained a fertile topic for research and debate for more than a century. Classic studies, which were to date the best original works describing early human heart development, relied on the evaluation of histologically stained serial sections and 3D reconstructions. Lacking the availability of molecular markers and genetic lineage tools, classic embryologists working on human heart development had to draw their conclusions on morphogenetic mechanisms underlying growth and differentiation, only based on changes in visible anatomical landmarks and cellular morphology. Constrictions visible on the external surface of the early heart tube were interpreted as the borders of segments representing the compartments of the septating heart. However, fate mapping and lineage studies in chicken and mouse have demonstrated that the linear heart tube contains little more than the precursors for the definitive left ventricle. In human, molecular data allowing comparison with animal studies on cardiogenesis,
are practically lacking. We now report an analysis of morphogenesis, growth and differentiation of the human embryonic heart at key stages of its development, allowing such a comparison. The significance of our observations is two-fold. First, they support a biphasic model of early cardiac morphogenesis, with the myocardial tube initially recruited from the highly proliferative adjacent coelomic wall and then, subsequent to a phase of quiescence, the chambers forming by local re-initiation of proliferation and differentiation. Second, they show that the mechanisms underlying the formation, growth and differentiation of the primary heart tube and the chambers as seen in mouse and chicken are highly conserved in human.

The Early Embryonic Heart Grows through Proliferation and Differentiation of Cardiac Precursor Cells

The primary linear heart tube is formed through myocardialization and subsequent fusion of parts of the bilateral walls of the intra-embryonic coelom. Although already in 1933 Patten and Kramer had demonstrated experimentally that the early embryonic chicken heart does not contain all its definitive parts, embryologists studying human development continued to assume that the primary myocardial heart tube elongated by growth of its initially present segments. Later Sissman demonstrated that the early myocardial heart tube in chicken embryos had very low proliferative activity, and more recently, lineage studies in chicken and mouse provided unequivocal evidence of addition of cardiomyocytes from the second heart field to both poles of the heart tube. The transcription factors Islet1 and Tbx1 were shown to be crucial for the maintenance of cells in the dorsal coelomic wall in the precursor state, prior to their differentiation into cardiomyocytes and addition to the growing heart tube.

Our current observations, that ISL1 and the proliferation markers PCNA and Ki67 are co-expressed in the cells of the coelomic wall contiguous to the myocardium at the venous and arterial poles, suggest a similar role of ISL1 in the growth of the heart tube in human and mouse. The myocardium of the forming heart tube in the youngest embryo of our series still expressed ISL1, supporting a role of ISL1 as cardiac progenitor cell regulator within both the primary and secondary heart fields. The proliferating myocardium of the expanding chambers was negative for ISL1, as reported previously, indicating that ISL1 is not essential for the proliferation of cardiomyocytes.

Chambers Form through Strictly Regional Re-initiation of Proliferation and Differentiation

Although the notion of chamber formation in the human heart was appreciated previously, the process was described as expansion of pre-existing segments of the primary heart tube, rather than as local differentiation and growth of the myocardium at the outer curvature of the looped heart. Based on differences in conduction velocities between the fast-conducting chamber myocardium and the slow-conducting primary myocardium of inflow tract,
atrioventricular canal and outflow tract along with molecular differences between the different compartments we developed the so-called “ballooning model” of chamber formation. In essence, this concept distinguishes two phases in the formation of the vertebrate heart. Initially, the so-called primary myocardial tube forms, which loops, by which obvious inner and outer curvatures are formed. This then sets the scene for formation of the chambers at the outer curvature. These chambers are walled by so-called working myocardium, which permits fast conduction of the impulse and efficacious contraction. We now provide molecular evidence for evolutionary conservation of this concept in human.

Studies in experimental animals showed that the developing chambers display high proliferative activity and remain flanked by slowly proliferating primary myocardium. As in chicken, we observed that in human re-initiation of proliferation in the myocardium of the outer curvature is a first sign of chamber formation. Slightly later small trabecules were observed within the forming ventricles, which were positive for proliferation and working myocardium markers. The myocardium of the inner curvature does not express these markers, similar to the patterns reported for the developing mouse heart. These observations fully support the concept of chamber formation, which we developed based on animal studies.

At first glance, the high percentage of proliferating cardiomyocytes in the walls of the atrial and ventricular chambers we observed, seems in discrepancy with the counts of mitotic figures, which are the only available data on cell multiplication in the human developing heart. Similar to the reported mitotic indices, we observed very low percentages of phosphohistone H3-positive cells within the growing chambers. Both findings can be explained by the short duration of the mitotic phase of the cell cycle underscoring the shortcoming of the use of mitotic indices for the assessment of embryonic growth.

The suppression of differentiation of the primary myocardium toward working myocardium in parts of the sinus venosus, atrioventricular canal and outflow tract is regulated by two transcriptional repressors, T-box factors 2 and 3. The expression patterns of TBX3 and TBX2 we have found in the human embryonic hearts are very similar to those reported in mouse, suggesting conservation of the mechanisms underlying the formation of the chambers and the conduction system. Expression of TBX2 decreased into ventricular direction (Figure 8A), which is very reminiscent to the expression in the mouse, where the Tbx2-lineage has been shown to contribute to the left ventricular free wall. It is tempting to speculate that impairment of this contribution would result in left ventricular hypoplasia.

In summary, the morphological and molecular description of growth and differentiation of the developing human heart reported in this study harmonizes well with our current knowledge of cardiac development in experimental animals and provides the base required for subsequent investigations of the pathogenesis of congenital cardiac malformations.
Acknowledgments

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Disclosures

None

Reference List


Supplemental Methods

**Immunofluorescence Staining**

Paraffin-embedded embryos were sectioned at 7 μm. Sections were mounted onto silane-coated slides, deparaffinized in xylene, rehydrated in graded ethanol series and washed in phosphate-buffered saline (PBS, pH 7.4).

Triple immunofluorescent staining was performed as described previously. Here we report the results of staining with the following primary antibodies: rabbit polyclonal for ANF, goat polyclonal for connexin40, rabbit polyclonal for NKX2-5, goat polyclonal for TBX3, mouse monoclonal for PCNA (all from Santa Cruz Biotechnology; diluted 1:250), mouse monoclonal for TBX2 (1:100; non-commercial; generous gift by Dr. Colin R. Goding, Marie Curie Research Institute, Oxted, UK), goat polyclonal for Islet-1 (1:250; Neuromics), rabbit polyclonal for Ki67 (1:250; Monosan) and rabbit polyclonal for phosphohistone H3 [Ser10] (1:250; Cell Signaling Technology Inc). Every combination contained mouse monoclonal for troponin I (1:250; Chemicon), mouse monoclonal for SERCa2a (1:500; Abcam), or rabbit polyclonal for cardiac troponin I (1:500; HyTest Ltd.) antibodies as myocardial marker. After washing in TNT (100 mM Tris, pH 7.4; 150 mM NaCl; 0.05% Tween-20, Sigma) sections were incubated at room temperature for 1.5 - 2 hr in the dark with a mix of fluorochrome-coupled secondary antibodies containing donkey-anti-goat Alexa568, chicken-anti-rabbit Alexa488 and donkey-anti-mouse Alexa680 (all diluted 1:250; Molecular Probes, Invitrogen). For TBX3 biotinylated anti-goat antibody (1:250; Jackson ImmunoResearch) and a Tyramide Signal Amplification kit (Perkins & Palmer) were used according to the manufacturer’s instructions. The sections of the embryo used to prepare the quantitative reconstruction were incubated additionally with DAPI (Sigma) to stain all nuclei.

For indirect immunohistochemical staining sections were pre-treated for 15 min with TENG-T solution (10 mM Tris, pH 8.0; 5 mM EDTA; 150 mM NaCl; 0.25% gelatine, 0.05% Tween-20) to reduce non-specific antibody binding. Then consecutive sections were incubated overnight at room temperature with different primary antibodies in repetition through the whole series. Here we report the results of staining with the mouse monoclonal antibody for PCNA (1:500; Santa Cruz Biotechnology). The next morning, after washing the sections for 3 times in phosphate-buffered saline (PBS, pH 7.4), they were incubated with appropriate alkaline phosphatase-coupled secondary antibodies for 4 hr at room temperature and washed again 3 times in PBS. For PCNA visualization a goat-anti-mouse antibody, Sigma, was used at a dilution of 1:100 in PBS. Then NBT/BCIP (Roche) diluted 1:50 in TNM (100 mM Tris, pH 9.5; 100 mM NaCl; 50 mM MgCl2) was added to the sections to develop the colour at the binding site of the primary antibody. Reaction was stopped by rinsing the sections in distilled water, after which they were dehydrated in a graded ethanol series, washed in xylene and covered with Entellan (Merck).

**Staining for general histology**

Subsequent to immunofluorescent staining and photography, sections were washed in PBS and stained with hematoxylin and azophloxin according to a standard protocol. Then sections were dehydrated in a graded ethanol series, washed in xylene and covered with Entellan (Merck).

**In situ Hybridization on Sections**

In situ hybridization was performed as described previously with small changes. Embryos were serially sectioned at 10 μm. Sections were mounted onto silane-coated slides, deparaffinized in xylene, rehydrated in a graded ethanol series and washed in PBS. Then sections were incubated for various time, depending on the developmental stage, in 20 μg/mL proteinase K (Invitrogen) solution in PBS, and in 0.2% glycine solution in PBS. Then sections were washed in PBS and submersed into 4% paraformaldehyde, containing 0.2% glutaraldehyde solution in PBS for 10 min for post-fixation and washed once again in PBS. After the sections were pre-treated for 1.5-2 hr at 70 °C with hybridization mix (50% formamide; 300 mM NaCl; 30 mM Na-citrate, pH 7.0;
500 mM EDTA; 1% Blocking reagent, Roche; 10% CHAPS, Sigma; 0.05% heparine, Sigma; and 0.1% yeast RNA, Roche), they were incubated overnight at 70 °C with different digoxigenin-labeled riboprobes diluted in hybridization mix. Here we report the results of staining with a connexin43 probe, transcribed from nucleotides 399-1183 of the human connexin43 gene (GenBank nr X52947) subcloned into pT7/T3-α18 plasmid (kindly provided by Dr. Emmanuel Dupont, National Heart and Lung Institute, London, UK). The next morning sections were washed 2 times at 65 °C in 50% formamide with SSC (300 mM NaCl; 30 mM Na-citrate, pH 7.0) for 30 and 60 min and 3 times at RT in TNT for 10 min each. Then sections were incubated at RT with 2% Blocking Powder (Roche) diluted in MAT buffer (100 mM maleic acid, pH 7.4; 150 mM NaCl; 0.1% Tween-20, Sigma) for 30-45 min to block unspecific binding of the Fab-fragments. After blocking sections were incubated at room temperature with anti-digoxigenin Fab-fragments coupled to alkaline phosphatase (Roche, diluted 1:1500 in MAT buffer) for 2 hr and washed with TNT and TNM solutions. Then NBT/BCIP (Roche, diluted 1:50 in TNM) was added to the sections to develop the colour at the binding site of the probes. Reaction was stopped by rinsing the sections in distilled water. Then sections were dehydrated in a graded ethanol series, washed in xylene and covered with Entellan (Merck).

Three-dimensional (3D) morphological and quantitative reconstructions

Fluorescently stained serial sections were photographed with a microscope, Leica DM6000, driven by ImagePro Plus 6.2 software (Media Cybernemetics, www.mediacy.com) using filters corresponding to each individual fluorochrome, yielding three images per section, representing the patterns of expression of 3 different proteins. Images with myocardial staining were used to prepare the morphological reconstructions (i.e, the entire heart and related structures) in Amira 4.1 software (Visage Imaging, www.amiravis.com) as described previously. In short, digital images were checked and were loaded into Amira, subsequent to replacing grossly damaged ones with their neighbours. They were then aligned and segmented into labels of myocardium and different other structures either related to the heart or needed to appreciate the general anatomy of the embryo. Some structures (e.g, coelomic wall, jelly) were not labelled in their entirety for the sake of clarity. For two embryos at stages 12 and 14 the myocardial expression domain of TBX3 from every third section of the series was projected upon the myocardium label. For technical reasons the areas containing scattered or graded patterns of expression were similarly labelled as the areas containing strong gene expression. The extent of the labels for other structures, which have been not specifically stained on the sections, was defined according to general embryology knowledge. After completion of the segmentation the label-set was corrected manually in two other planes for distortions introduced through imperfect alignment of images due to uneven stretching of the sections. Such corrections only were performed subsequent to control of the original images.

Quantitative 3D reconstruction of Ki67 expression was performed using MatLab 2009a (Mathworks, www.mathworks.com) and Amira software based on the previously described protocol. In brief, after alignment the images with TnI staining were segmented into labels of myocardium, coelomic wall and lumen. Then the stacks of serial images with Ki67 and DAPI were used to calculate the percentage of Ki67-positive nuclei within the regions of the myocardium and coelomic wall. A nucleus was depicted as Ki67-positive when its staining intensity was above the 1 standard deviation of the average local background staining variation. The calculated percentage was then converted to the colour map, which was projected onto the morphological model. The PCNA labelling index was calculated from systematic samples of serial sections from embryos of stages 10, 11 and 12, stained for troponin I, NKX2-5 and PCNA. The positivity of PCNA was determined using the same technique as used for the Ki67 reconstruction. PCNA expression level peaks around early S-phase and is low during other phases. We defined the basal fluorescence intensity as a symmetric distribution around the modal PCNA staining intensity. All nuclei with PCNA staining intensities above two times the modal value were thus counted as positive. Labelling indices within the myocardium were obtained by dividing the observed number of PCNA positive cells by the number of cells stained by NKX2.5.
The 3D data from the Amira viewer were exported into the Adobe Acrobat 9 Pro Extended (Adobe Systems Inc., www.adobe.com) to generate the file in interactive 3D portable document format.10

**Supplemental References**