Trabeculated Right Ventricular Free Wall in the Chicken Heart Forms by Ventricularization of the Myocardium Initially Forming the Outflow Tract

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Abstract—Recent molecular lineage analyses in mouse have demonstrated that the right ventricle is recruited from anterior mesoderm in later stages of cardiac development. This is in contrast to current views of development in the chicken heart, which suggest that the initial heart tube contains a subset of right ventricular precursors. We investigated the fate of the outflow tract myocardium using immunofluorescent staining of the myocardium, and lineage tracer, as well as cell death experiments. These analyses showed that the outflow tract is initially myocardial in its entirety, increasing in length up to HH24. The outflow tract myocardium, subsequently, shortens as a result of ventricularization, contributing to the trabeculated free wall, as well as the infundibulum, of the right ventricle. During this shortening, the overall length of the outflow tract is maintained because of the formation of a nonmyocardial portion between the distal myocardial border and the pericardial reflections. Cell death and transdifferentiation were found to play a more limited contribution to the initial shortening than is generally appreciated, if they play any part at all. Cell death, nonetheless, plays an important role in the disappearance of the myocardial collar that continues to invest the aorta and pulmonary trunk around HH30, and in the separation of the intrapericardial arterial vessels. Taken together, we show, as opposed to some current beliefs, the development of the arterial pole is similar in mammals and birds. (Circ Res. 2007;100:0-0.)

Key Words: cardiac development ■ outflow tract ■ differentiation ■ cell death

It is now generally accepted that, in addition to the primary heart field, a second source of cardiomyocytes contributes to formation of the definitive heart.1–3 In mouse, this second source is located cranial to the heart tube, and provides material for the right ventricle and outflow tract (OFT)4,5. In avian hearts, however, it is held, on the basis of marking experiments in young embryos, that the linear heart itself contains a subset of presursors of the right ventricle (re-viewed in7–9 Figure 1). Deposits of Indian ink placed on the ventromedial fusion line of the heart-forming regions at HH9 have purportedly been found in the developing right ventricle at HH12, with subsequent experiments suggesting that this region of the developing ventricle contributes to the ventric-ular septum.6 Indian ink labels placed at the cranial side of the forming heart tube at around HH9 also marked the right ventricular component of the looping heart tube at HH12.11 Labeling the heart-forming regions at HH5 using retroviruses have also been said to mark the apical trabeculated region of the right ventricle at HH15.7 Those making these interpretations, however, seem to have ignored the fact that the definitive ventricles can be identified in unambiguous fashion only after the appearance of the ventricular septum, which does not begin until HH17.10,12

Since these initial experiments, 2 independent studies have identified mesodermal cells added to the chicken heart from extracardiac sources between HH12 and 22/24. Mjaatvedt and coworkers13 identified cells in the pharyngeal arches, which they called the anterior heart field, and showed how they contributed to the proximal (conus) and distal (truncus) parts of the outflow tract. Waldo and coworkers14,15 identified cells ventral to the pharynx, and caudal to the developing outflow tract, which they called the secondary heart field, and showed them to be the source of the distal myocardial part of the outflow tract, as well as the nonmyocardial intrapericardial arterial trunks. These workers had previously demonstrated the contribution from the cardiac neural crest to these nonmyocardial components.16 Thompson and coworkers, using radioactive tattoos, had also shown that cells initially located, at HH16, in the outflow tract at the level of the pericardial reflections eventually were found in the subpulmonary infundibular myocardium of the right ventricle.17 None of these studies in avian hearts, however, addressed explicitly the developmental origin of the right ventricular trabecular free wall.

In mouse and human, therefore, it is the proximal outflow tract that is generally presumed to give rise to the right

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ventricle, whereas the primordium of this structure in avian hearts is currently believed to be present in the straight heart tube. We have now studied the developmental origin of the right ventricular free wall in chicken, using DiI to follow the fate of the cells initially belonging to the outflow tract. Our findings show that, in chicken as in mouse, part of the right ventricular free wall is derived from myocardium initially belonging to the outflow tract. We submit, therefore, that the developmental origin of the right ventricle is similar, rather than different, in birds and mammals.

Materials and Methods

Tracing Groups of Cells by DiI Labeling

Fertilized chicken eggs (Drost BV, Nieuw Loosdrecht, The Netherlands) were incubated, windowed and staged. Having exposed the surface, small groups of cells were labeled with the lipophilic tracer DiI (1,1-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, D3911). The coated needle used for introducing the label was pricked into the tissue for approximately 15 seconds, allowing the dye to transfer to the surrounding cells. The eggs were sealed and reincubated. After various incubations, the embryos were harvested, staged, and fixed in 4% (wt/vol) paraformaldehyde (PFA) dissolved in PBS (10 mmol/L H2NaPO4/HNa2PO4, 150 mmol/L NaCl, pH 7.6). Fluorescence was visualized using Confocal Laser Scanning Microscopy (CLSM, Bio-Rad MRC 1024).

Visualization of the Myocardial Component of the Heart

Embryos were isolated, staged, and fixed in DMSO/methanol (1:4) for 4 hours. Embryos were hydrated in graded series of methanol-PBS and permeabilized by incubation in PBST (0.25% Triton-X100 in PBS). To reduce background staining, they were incubated in PBS-A (1% BSA in PBS) with 5% goat serum. The myocytes were stained using a monoclonal antibody against sarcomeric myosin heavy chain (MF20; Developmental Studies Hybridoma Bank, Iowa, USA) and goat-anti-mouse-Alexa-488 (Molecular Probes). Following extensive washing in PBS-A, analysis was done with CLSM. All images are shown as brightest point projections. The length of the outflow tract was determined using NIH-image (version 1.62).

Visualization of Regions of Increased Cell Death

Hearts were isolated in Earle’s Balanced Salt Solutions (EBSS, ICN) and transferred to M199 culture medium (Life Technologies) supplemented with the vital dye Lysotracker Red (Molecular Probes). After a 30 minute incubation at 37°C, the hearts were washed in EBSS, and fixed in 4% PFA in PBS for 1 hour. After extensive washing in PBS fluorescence was visualized using CLSM.

Results

With the start of looping at HH11, trabeculations appear at the outer curvature of the heart tube, identifying the ventricle as distinct from the more cranial OFT. The OFT as thus defined has a smooth-walled myocardial mantle, and exhibits extensive internal ridges. Its distal border is located where the OFT leaves the pericardial cavity, draining via the extrapericardial aortic sac into the pharyngeal arch arteries. This border between OFT and the aortic sac is described as the pericardial reflections. The proximal border of the OFT can be identified externally as a distinctive groove, whereas within the heart, the distal walls are smooth, lacking ridges and trabeculations. This groove is the distal ventricular groove, although others have termed it the arterioventricular or bulboventricular groove. The presence of trabeculations identifies the distal ventricular part of the tube, this is generally considered to represent the right ventricle (Figure 1). Differentiation of left and right ventricles at this stage, however, is difficult, because the ventricular septum has still to form. Previous histological and tissue tagging studies had shown that, with time, the walls of the OFT itself change from being exclusively myocardial into a tube with distal nonmyocardial and proximal myocardial portions. The nomenclature of these components is also currently confusing, so we describe them simply as the nonmyocardial and myocardial components. With ongoing development, the intrapericardial portions of the aorta and pulmonary trunk are formed at the site initially occupied by the nonmyocardial component.

As a first step to elucidate the absolute changes occurring in the OFT, we visualized the myocardium, measuring its...
myocardial and nonmyocardial components. At HH12 (n = 3), the OFT is myocardial to the pericardial reflections (Figure 2A), and measures 290 ± 25 μm (Figure 3). The myocardial border remains at the level of the pericardial reflections until HH22 (Figure 2A–D), during which time the OFT increases more than 4-fold in length, to 1265 ± 147 μm (n = 5) (Figure 3). Subsequently, the overall OFT elongates still further, reaching its maximal length at HH26, when it measures 1677 ± 137 μm (n = 36) (Figure 3). This corresponds with the appearance of the nonmyocardial component between the distal myocardial border and the pericardial reflection (Figure 2E and F). Between HH26 and HH34, the latest stage analyzed, the nonmyocardial component also elongates (Figure 2G–I). Overall lengthening, however, ceases beyond HH26, with elongation of the nonmyocardial component compensated by shortening of the myocardial component (Figure 3) of more than five-fold between HH26 and HH31 (Figure 3). Within this developmental period, the OFT also widens gradually up to HH28 (Figure 3), after which its width remains relatively constant, at 876 ± 93 (n = 24). The mechanism of shortening of the myocardial component remains contentious. Some have suggested apoptosis (see review2), others transdifferentiation, 24 or absorption into the right ventricle.18,25

To address the issue of shortening, we labeled small groups of OFT cells using DiI at HH16 (Figure 4) and HH22 (Figure 5) in ovo. At both these stages the myocardial OFT extends to the pericardial reflections (Figure 1A–D), still possesses the capacity to lengthen, (Figure 1J), and is not covered with epicardium.26,27 Labels were placed adjacent to the pericardial reflection (position 1), half way along its length (position 2), adjacent to the distal ventricular groove (position 3), and in the ventricle adjacent to distal ventricular groove (position 4). To confirm reliability and reproducibility of labeling, we marked at least ten embryos for each position and developmental stage. After 3 hours of reincubation, we reanalyzed the hearts, all labels remaining in their initial positions (Figure 4 and 5).

Subsequently, labeled hearts were reincubated for longer periods. When considering those labeled at HH16 (Figure 4),
cells labeled in the most distal portion of the OFT (Figure 4A position 1, and Figure 4B red dots) were identified in the middle of the OFT after both 24 and 48 hours of re-incubation, but had reached the proximal OFT after 72 hours. After 96 hours of re-incubation, 70% of labeled cells were found in the proximal OFT, and 30% in the ventricle. Cells labeled in the middle portion (Figure 4A position 2, and Figure 4B blue dots) had moved to the proximal OFT after 48 hours of re-incubation, and had reached the ventricle after 72 hours. Of the cells labeled in the proximal OFT (Figure 4A position 3, and Figure 4B green dots), 30% had already reached the ventricle after 24 hours of re-incubation. After longer re-incubations, all the labeled cells had moved to the ventricular portion of the heart, specifically at the right ventricular side of the developing ventricular septum. In line with previous studies in the chicken17 and mouse,28 the labeled cells had not only moved upstream, but had also shifted to the right, showing that myocardium initially forming the OFT had become incorporated into the developing right ventricle.

When considering those cells labeled at HH21, cells marked adjacent to the pericardial reflections remained at the distal myocardial border of the OFT at all time points analyzed (Figure 5A position 1 and Figure 5B red dots). Considering cells labeled at the other positions (Figure 5, position 2 and 3), the results paralleled those obtained after labeling at HH16 (Figure 4). Cells labeled proximally (position 3) at HH21 had migrated to the ventricle after 24 hours of re-incubation. After 72 hours of re-incubation, the labeled cells were found in the ventricle in decreasing numbers of embryos, and after 120 hours of re-incubation (data not shown) none of the labeled cells could be traced back. The labeled cells could have disappeared for several reasons. The label could have become undetectable because of dilution by

Figure 3. Graph showing the changing dimensions of the developing outflow tract. The blue squares indicate the length of the overall outflow tract, being the combination of the myocardial (green triangles) and nonmyocardial components. The red dots indicated the width of the OFT.

Figure 4. Tracing the fate of cells within the outflow tract labeled at HH16. We labeled the outflow tract in at least 10 HH16/17 hearts with DiI at the level of the pericardial reflections (position 1), half way between the distal ventricular groove and the reflections (position 2), adjacent to distal ventricular groove (position 3), or in the ventricle itself (position 4). Panel A shows representative examples of the ultimate location of the marked cells (arrow) after various periods of reincubation. Panel B shows a summary of all the individual labeling experiments. The different positions are color coded, and each dot represents an individual experiment. The white dotted line indicates the pericardial reflections, the green one the distal myocardial border, the blue one the distal ventricular groove, and the red one the ventricular groove. Scale bar=500 μm.
proliferation of the myocardial cells, the labeled cells might no longer be detectable at the ventricular surface, or the labeled cells might have died. The first option seems unlikely, previous studies having shown that the myocardium of the OFT proliferates very slowly during development.\textsuperscript{24,29,30} Moreover, if dilution did underlie their disappearance, we would expect the size of the initially labeled group of cells to increase in diameter, with a concomitant decrease in fluorescence. Neither of the two phenomena was observed.

To assess whether the labeled cells had become displaced interiorly, we cleaved the hearts and analyzed them from the endocardial surface, also permitting us to establish the position of the labeled cells relative to the forming ventricular septum. We found that, rather being at the endocardial surface, the marked cells had entered the ventricular trabeculations (Figure 4), as previously shown.\textsuperscript{31} Moreover, the cells initially labeled in the ventricle adjacent to the distal ventricular groove at HH16 or HH21, were found in the right, rather than the left, ventricular trabeculations (Figure 4, 5, and 6A–C). To investigate further the origin of the ventricular septum and the left ventricle, we labeled cells half way between the distal ventricular groove and the atrioventricular canal. These labeled cells were all found in the left ventricular trabeculations (Figure 6D–F). Cells labeled midway between these two positions were found in the ventricular septum (Figure 6G–I).

These observations, in contrast to the general contention that, in the chicken, apoptosis underlies shortening of the myocardial OFT,\textsuperscript{2} exclude the possibility that labeled cells die during prolonged periods of re-incubation. With this in mind, we re-evaluated the role of cell death in the OFT using Lysotracker Red, which becomes strongly fluorescent when taken up into lysosomes that are enriched in cells processing cellular debris.\textsuperscript{21} As controls, we included in each analysis a limb of an HH33 chicken embryo, in which the tissue webs are being removed by apoptosis (Figure 5J).\textsuperscript{32} Very strong staining was observed in the nonmyocardial OFT between HH30 and HH36 (Figure 7E–H), suggesting an important role for apoptosis in the formation of the intrapericardial portions of the aorta and pulmonary trunk, and the left and right brachiocephalic arteries (Figure 7F–I). In line with earlier reports (reviewed in\textsuperscript{2}) the myocardial investment of pulmonary trunk showed intense staining before its disappearance—(Figure 7E–G). Examination of earlier stages showed more
stained cells in the OFT between HH24 and HH27 (Figure 7A–C) than in the remainder of the heart. A slightly higher density of stained cells was observed at the border between the myocardial and nonmyocardial components, and immediately adjacent to the distal ventricular groove. Although the initial appearance of Lysotracker Red positive cells corresponds with the onset of the shortening of the myocardial OFT, it does not correlate with ongoing shortening of the myocardial OFT (Figure 3), suggesting a limited contribution of apoptosis to this initial shortening.

Discussion

The 2 fields of cardiac progenitors are now recognized as the primary, and secondary or anterior, heart fields. It is the primary heart field that produces the straight heart tube. With looping of the heart tube, the ventricular trabeculations start to form at the outer curvature, permitting identification of the cranial part of the tube as the OFT. In mouse, there is firm evidence that the primary heart field gives rise to the left ventricle, with the secondary field forming both the right ventricle and the outflow tract. It has also been suggested that the proximal part of the OFT is incorporated in human hearts into the ventricles as the subarterial outlets. In vitro and in vivo analyses in both chicken and mouse have shown that these intracardiac portions of the outlets are initially mesenchymal, but become muscularized during subsequent development.

The developmental origin of the trabecular myocardium of the free wall of the right ventricle, however, has never been studied in detail. In vivo and in vitro studies have shown that in chicken, there is an additional contribution to the OFT from cells located cranially. Experiments using Indian ink marks, iron particles, electro-cautery, or radioactive marks, has been interpreted to suggest that the right ventricular trabeculated portion is derived from the primary linear heart tube, with only the smooth-walled infundibulum originat-
Thus, it is currently presumed that the trabeculated part of the chicken right ventricle is derived from the primary heart field (see review). If true, then this part of the right ventricle has a different developmental origin in mammals and birds (see reviews), and might raise issues with respect to the direct extrapolation of avian findings to mammalian cardiac development. The majority of congenital cardiac malformations in humans, however, involve aberrant formation of the arterial pole. It was to explore further this issue that we performed these experiments.

It is well accepted that the initial OFT extends proximally from the ventricular trabeculations, marked externally by the distal ventricular groove, to its distal margin at the pericardial reflections. This part of the heart increases almost 6-fold in length between HH12 and 26 (Figure 1). At HH26, when at its maximal length, it has an extensive myocardial and a small nonmyocardial component, the latter derived from cells of the cardiac neural crest and secondary heart field. With ongoing development, the myocardial part gradually disappears so that, apart from the myocardial investment of the pulmonary trunk, the outflow tracts are exclusively nonmyocardial, remodelling into the arterial valves and their supporting sinuses, the intrapericardial portions of the aorta and pulmonary trunk, and the left and right brachiocephalic arteries. The myocardial component is held to disappear because of apoptosis (for review), transdifferentiation, and/or absorption into the right ventricle.

To assess the latter two options, we labeled small groups of cells with DiI at HH16 and HH21 in ovo, following them during subsequent development. This approach differs in several ways from earlier studies. We labeled the cells using a vital dye, marking them at various levels in ovo, and determined their subsequent location shortly after labeling, and subsequently every 24 hours. We selected HH16 and HH21 because, at these stages, the OFT is not yet covered with epicardium, and is myocardial throughout its length...

**Figure 7.** Pattern of cell death during remodelling. We visualize the regions of cell death using LysoTracker Red. Positive cells were first detected in the heart at HH24 (panel A), with one region of dying cells found at the level of the distal ventricular groove (panels A and B, white arrow), and another at the distal border of the myocardial OFT (panels A and B, red arrow). The white dotted line indicates the pericardial reflection. By HH27 (panel C) and HH28 (panel D), the only remaining region of cell death is at the level of the distal ventricular groove. During septation of the OFT, increased levels of cell death are seen at the margins of separation of the forming arterial trunks (panels E through H). Between HH30 and HH36 (panels E through H), cell death is observed in the persisting myocardial cuff around the developing pulmonary root. At HH37, no focuses of cell death are visible within the heart apart from a few scattered cells (panel I). To validate the specificity of the staining, and to calibrate the confocal laser scanning microscopy, we also processed the lower limb of chicken embryo at HH33, in which it is well established that the interdigital webs are being removed by apoptosis (panel J). Scale bar = 500 μm.
(Figure 2), allowing unambiguous labeling of myocardial cells. Our findings revealed that cells labeled adjacent to the pericardial reflection (Figure 4 and 5) move away from it and displace to the right, finishing close to the distal ventricular groove, or even crossing the groove to contribute to the smooth-walled right ventricular infundibular myocardium. In none of our experiments could we find labeled cells in the nonmyocardial OFT, indicating that transdifferentiation does not contribute substantially to the shortening of the myocardial OFT. These findings are consistent with earlier autoradiographic tattoo analyses.17 The fact that, during re-incubation, the label does not disappear indicates a limited role for apoptosis,2 a conclusion further underscored by our results using Lysotracker Red (Figure 7). Apoptosis does underlie the separation of the nonmyocardial OFT into the discrete intrapericardial arterial vessels, and the disappearance of the myocardial investments of the aorta and pulmonary trunk around HH30, as previously reported.21,47

When tracing them more proximally in the OFT (Figure 4 to 6), we showed that the marked cells finish in the developing right ventricle. Earlier investigators have analyzed the marked cells before the formation of the ventricular septum,12 invalidating unambiguous ventricular identification. We showed that cardiomyocytes labeled in the region of the distal ventricular groove (Figure 4 and 5) never passed the ventricular septum, but rather entered the right ventricular trabeculations (Figure 6A–C). Only myocytes marked half way between the distal ventricular groove and the atrioventricular canal could be traced to the left ventricle (Figure 6D–F). Cells tagged in between these positions were found in the ventricular septum (Figure 6G–I). This latter finding was also shown by de la Cruz and coworkers,10 although the contribution of more distal myocardium of the HH16 heart to the trabeculated right ventricular free wall has not yet been reported. The finding that ventricular cells labeled immediately adjacent to the distal ventricular groove at HH16 contribute to the trabeculations of the right ventricle adjacent to the ventricular septum (Figure 6A–C) suggests that not the entire, but a large part of, the trabeculated right ventricular free wall is derived from OFT myocardium.

Our present data, therefore, are in agreement with murine studies (for review), but in contrast to previous avian studies.10,11,17,48 This discrepancy might be because of the use previously of superficial extracellular deposits.48 Although it is thought that these marks displace along with the surrounding tissue, they could become trapped in the epicardial layer, and hence not be displaced along with the underlying OFT myocardium. The epicardial layer covering the myocardium is derived from the proepicardium, whereas that of the nonmyocardial component is from a cephalic source.27 If the superficial marks are trapped into the epicardial covering, these marks will never reach the ventricular component of the heart.

Taken together, our labeling studies suggest that part of the trabeculated free wall of the right ventricle is derived from myocardium initially belonging to the outflow tract. Since earlier studies have shown that such myocardium is recruited from mesodermal cells located at the cranial side of the heart tube, we propose that this contribution is also derived from the anterior, or secondary, heart field. In keeping with this finding, we submit that the component ballooning from the initial linear heart tube is destined to become the left ventricle, theventricular septum, and the adjacent trabeculations. Most importantly, our study shows that the right ventricle, in essence, has comparable origins in mammals and birds, allowing direct extrapolation of findings in birds to mammalian cardiac development.

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Disclosures
None.

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