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# Patterning of the heart field in the chick

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ABSTRACT

In human development, it is postulated based on histological sections, that the cardiogenic mesoderm rotates 180° with the pericardial cavity. This is also thought to be the case in mouse development where gene expression data suggests that the progenitors of the right ventricle and outflow tract invert their position with respect to the progenitors of the atria and left ventricle. However, the inversion in both cases is inferred and has never been shown directly. We have used 3D reconstructions and cell tracing in chick embryos to show that the cardiogenic mesoderm is organized such that the lateralmost cells are incorporated into the cardiac inflow (atria and left ventricle) while medially placed cells are incorporated into the cardiac outflow (right ventricle and outflow tract). This happens because the cardiogenic mesoderm is inverted. The inversion is concomitant with movement of the anterior intestinal portal which rolls caudally to form the foregut pocket. The bilateral cranial cardiogenic fields fold medially and ventrally and fuse. After heart looping the seam made by ventral fusion will become the greater curvature of the heart loop. The caudal border of the cardiogenic mesoderm which ends up dorsally coincides with the inner curvature. Physical ablation of selected areas of the cardiogenic mesoderm based on this new fate map confirmed these results and, in addition, showed that the right and left atria arise from the right and left heart fields. The inversion and the new fate map account for several unexplained observations and provide a unified concept of heart fields and heart tube formation for avians and mammals.



## Introduction

During gastrulation in chick, myocardial progenitors ingress in the cranial 2/3 of the primitive streak at (Hamilton–Hamburger, 1951, HH) stage 3 (Garcia-Martinez and Schoenwolf, 1993). These cells migrate craniolaterally in a semicircular trajectory with little or no cellular mixing (Yang et al., 2002; Rosenquist, 1970) and by HH6 organize bilaterally as a sheet of anterior lateral plate mesoderm (Rawles, 1936; Rawles, 1943; Rudnick, 1938; DeHaan, 1965; Rosenquist and DeHaan, 1966; Rosenquist, 1970; Garcia-Martinez and Schoenwolf, 1993). When the lateral plate mesoderm splits into two layers: the splanchnic and somatic mesoderm, the myocardial progenitors are restricted to the splanchnic mesoderm layer and constitute the cardiogenic mesoderm or heart fields (Linask, 1992). These bilateral fields of cardiogenic mesoderm move medially and fuse at the ventral midline to form the heart tube starting at HH9 (Stalsberg and DeHaan, 1969). Interestingly, the craniocaudal order of cells in the primitive streak reflects their craniocaudal position in the heart tube. However, it is not understood how this colinearity is achieved.

We now know that the chick heart fields do not form a cardiac crescent, although this has remained a point of confusion for many years. An early report claimed that the fusion of the progenitors took place as early as HH6 (DeHaan, 1963a); however, this was later corrected to HH9 (9 somites, Stalsberg and DeHaan, 1969), but unfortunately, the correction was not picked up (see for example Bruneau et al., 1999; Jiang et al., 1998; Yamada et al., 2000; Redkar et al., 2002) except by a few investigators (Colas et al., 2000). The misconception was reinforced when the well known heart marker Nkx2.5 showed up as a crescent at stages HH5, 6, 7, and 8 (Schultheiss et al., 1995). It has since been shown that Nkx2.5 appears in a crescent because of endodermal expression across the midline between the bilateral heart fields starting at HH5 (Alsan and Schultheiss, 2002).

By contrast the cardiogenic precursors in mouse, and probably human, join at the midline cranial to the buccopharyngeal membrane forming a cardiogenic crescent rather than two separate heart fields (Tam et al., 1997; Davis, 1927; DeRuiter et al., 1992). In addition, recent studies in mouse point to a craniocaudal inversion of the crescent-shaped cardiogenic mesoderm based on molecular expression patterns of the cardiomyocyte progenitor marker, Isl1, and the cardiomyocyte marker, MLC2a (Cai et al., 2003). The initially lateral and cranial MLC2a expressing cells are inverted with the initially medial, caudal progenitors marked by Isl1. The MLC2a expressing cells then form the initial heart tube while the Isl1-positive progenitors are added to the heart tube progressively over a longer period of time. The

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same inversion may be true in human development as Davis (1927) argued for 180° inversion of the cardiogenic mesoderm along with the coelom during foregut pocket formation by the anterior intestinal portal in early human heart development. However, this observation was made from the analysis of median sagittal sections of human embryos during the 4th week of gestation and has never been tested. Even so, this point was adopted in almost all human embryology textbooks (Moore, 2003).

Another issue brought about by differential expression patterns in the mouse cardiogenic mesoderm along with clonal analysis of early and late populations of myocardial cells (Meilhac et al., 2003) has suggested the presence of multiple "heart fields" in the mouse (Buckingham et al., 2005). However, unlike the mouse, all of the cardiogenic progenitors in the bilateral heart fields of the chick are Isl1 positive (Yuan and Schoenwolf, 2000; Abu-Issa and Kirby, unpublished observation) precluding any observation of an inversion using molecular expression patterns and suggesting a single heart field prior to any differentiation. While it is possible to mark cells to observe an inversion, this has previously not been done. The difference in Isl1 expression and lack of a cardiogenic crescent in chick has led to the recent assumption that the development of the heart from the heart fields is dramatically different from that of mammalian development.

In order to construct a model that conciliates the information available and to understand if the formation of the heart tube in avians and mammals follows a comparable pattern of development, we have systematically analyzed chick heart field/tube formation by 3D reconstruction, fate mapping by single and double labeling and physical ablation of selected areas of the cardiogenic mesoderm. Our data indicate that there is likely a single, albeit bilateral heart field, in the chick. Various parts of the heart field move dynamically in relation to each other and are added to the heart tube based on their position within the heart field. This suggests that there are subdivisions of cells that will be added to the heart tube earlier versus later within the heart field. Fate mapping shows that the cardiogenic mesoderm is organized roughly in craniocaudal stripes with the atria and left ventricle represented most laterally and the right ventricle and outflow tract represented most medially. The heart field along with the coelom inverts 120–130° and then bends ventrally to reestablish the craniocaudal polarity recognized in the primitive streak. Ablation of selected areas of the heart field confirms the fate mapping studies. These observations allow us to present a model of heart field/tube formation and to propose that the major difference between chick and mammalian heart development appears to be the timing of fusion of the heart fields across the midline: the mouse has a cardiac crescent while the chick does not. Recent data in the mouse suggest that all of the heart progenitors also express Isl1 (Sun et al., 2007) as seen in the chick. Otherwise, the formation of the heart tube is similar between these two classes.

## Materials and methods

### *Animal preparation*

Fertilized fresh Ross Hubbard eggs were obtained from the Goldkist Hatchery, Siler City, NC. The eggs were incubated at 37 °C in 100% humidity until they reached appropriate stages. For in ovo experiments windows were made in the shell. After the experimental procedure the windows were sealed with cellophane tape. For whole embryo culture we used a modification of the original New whole embryo culture technique (New, 1955; Chapman et al., 2001). Embryos grown in this manner develop relatively normally to HH12.

### *3D reconstructions*

Amira 3.1 software was used to generate 3D reconstructions from digitally rendered histological sections using the manufacturer's instructions: http://www.tgs.com/support/amira_doc/index.htm?ReleaseNotes/real_notes.htm~framedown.

### *Fate mapping*

Embryos were injected with a mixture of 5-carboxytetramethylrhodamine, succinimidyl ester (CRSE, Molecular Probes, Inc. Eugene, Oregon) and 1,1′-dioctadecyl-3,3,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, Inc.) and/or 5-carboxytetramethylfluorescein (CFSE) and 3,3′-dioctadecyloxacarbocyanine, perchlorate (DiO, Molecular Probes, Inc.) as described previously (Darnell et al., 2001; Kirby et al., 2003). The dye solution was injected using a Picospritzer II (General Valve Corp., Fairfield, NJ) and a micromanipulator. Images of the embryos were captured by video microscopy immediately after injection and at 3–12 h intervals after the injection using an inverted Nikon microscope equipped with a Nikon digital camera. To confirm the placement of the label initially and the endpoint of the label selected embryos were fixed in formalin, cryosectioned and the relevant sections imaged.

### *In situ hybridization*

A 221 bp fragment of chick Nkx2.5 (Accession number X91838) was made using RT-PCR with primers and protocol as described previously (Schultheiss et al., 1995; Waldo et al., 2001). In situ hybridizations were done with digoxigenin-labeled riboprobes generated from cDNA fragments cloned into pCRII (Invitrogen) using one promoter primer and one of the gene specific primers, as reported previously (Waldo et al., 2001). The protocol followed Wilkinson (Wilkinson, 1992). After examination and documentation of whole mount staining, the embryos were embedded in paraffin, sectioned transversely at 8 μm, mounted, and documented by digital photomicroscopy using an Olympus microscope equipped with a Nikon digital camera.

## Results

### *Identification of the cardiogenic mesoderm using Nkx2.5 expression*

To identify an appropriate marker of cardiogenic mesoderm, we examined expression of Nkx2.5 and several other cardiac markers in transverse serial sections spanning the whole chick embryo at several stages of development (data not shown). We used the following criteria for selecting cell populations for 3D reconstruction: the cells expressed Nkx2.5, were cylindrically shaped, located in the splanchnic mesoderm lining the intraembryonic coelom, or were directly continuous with it. Nkx2.5 was also expressed in the ventral pharyngeal endoderm and ventral ectoderm but these layers were excluded from the analysis. Subsequent marking and ablation experiments showed that all of the Nkx2.5 expressing splanchnic mesoderm was incorporated into structures located inside the pericardial cavity.

We chose HH8 for our initial analysis because this was the first stage when the splanchnic and somatic mesoderm layers were separated by a well-formed coelomic cavity. The coelom extended from the buccopharyngeal membrane cranially to the first somite caudally, and from the paraxial mesoderm medially to the extraembryonic mesoderm laterally (DeRuiter et al., 1993).

Digital pictures were taken of cardiogenic mesoderm extending from the buccopharyngeal membrane cranially to the first somite caudally in transverse serial sections (Fig. 1). As we followed Nkx2.5 expression craniocaudally, the position of the Nkx2.5 expressing cells changed in relation to the coelom: cranially the cells formed the mediodorsal wall of the coelom while caudally they formed the ventral wall of the coelom (Fig. 1, compare C and G). This shift in the position of the Nkx2.5 expressing splanchnic mesodermal cells coincided with the anterior intestinal portal (Fig. 1E). We found that Nkx2.5 was also expressed in some cylindrically shaped mesodermal cells that were located between the splanchnic and paraxial mesoderm in the position of the mesodermal core of the nascent pharyngeal arch 1 (Fig. 1D). These cells were included in the analysis. Caudal to the first somite, the splanchnic mesoderm was negative for Nkx2.5 expression, coinciding with the end of the coelom (Fig. 1H).

### *Formation of the heart tube*

Serial images of the cardiogenic cells selected using the criteria discussed above (Nkx2.5 expression in cylindrically shaped cells) were aligned using the Align module of Amira software (Soufan et al., 2003). The cells were manually traced with the Amira segmentation module. At HH8, the field spanned 44 sections, 8 μm each. The 3D structure was generated using the surfaceGen module (Fig. 2). Movies of this stage and other stages are provided in the online Supplementary data (Movies 1–5). This 3D reconstruction provoked several observations. The

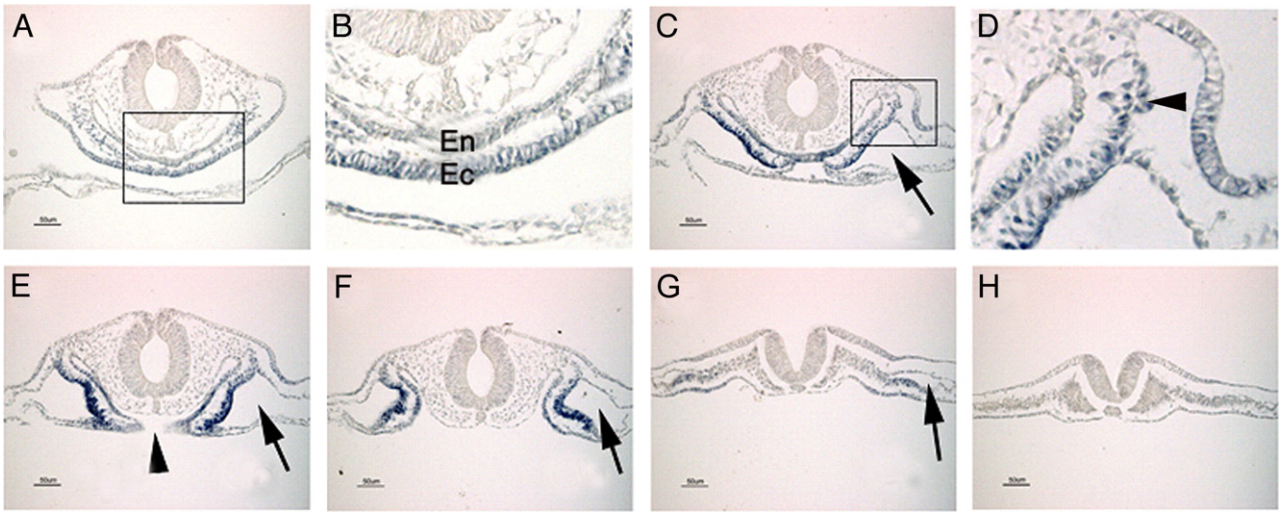


**Fig. 1.** Nkx2.5 expression pattern in transverse sections of stage HH8 chick embryo. The sections are oriented with dorsal up and ventral down. The sections span from the buccopharyngeal membrane cranially (A) to the first somite caudally (H). (B) Higher magnification of the box in panel A showing Nkx2.5 expression in the ventral endoderm (En) and ventral ectoderm (Ec) at the midline and the mesoderm laterally. (C) Mesoderm expressing Nkx2.5 in the dorsomedial wall of the coelom (coelom indicated by arrow in panels D–G). The Nkx2.5 expressing mesodermal cells extend medially but are separated by a gap at the midline. (D) Higher magnification of the box in panel C. Nkx2.5 expression in the splanchnic mesoderm continues partially into the paraxial mesoderm (arrowhead). (E, F) Strong expression of Nkx2.5 in the splanchnic mesoderm at the anterior intestinal portal (arrowhead). Nkx2.5 expressing cells are located mediodorsal to the coelom in panel E and mediodorsally and medioventrally in panel F. (G) Just cranial to somite 1, the splanchnic mesoderm can be seen as a small area of cells expressing Nkx2.5 that are positioned ventral to the coelom (arrow). (H) Nkx2.5 and the coelom both disappear at the first somite.

cardiogenic mesoderm changed its orientation along with the coelom concomitant with the formation of the foregut pocket by the anterior intestinal portal (Fig. 2A). To simplify the description we will call the region of cardiogenic mesoderm cranial to the anterior intestinal portal "wings" and the region caudal to the anterior intestinal portal "legs". The term "wings" is also consistent with the paper of Moreno-Rodrigo et al. (2006). The wings of cardiogenic mesoderm approached each other in the ventral midline but remained separate. The smallest distance at the midline occurred at an intermediate level just cranial to the anterior intestinal portal (Fig. 2A′). The wings of the cardiogenic mesoderm bent ventrally from the dorsal wall of the coelom (Fig. 2A′).

3D reconstruction of the cardiogenic mesoderm was also performed for HH9–12. At HH9, the overall structure of the cardiogenic mesoderm was maintained but with some important changes. The two wings of the bilateral cardiogenic mesoderm met at the midline at an intermediate level but had not fused, forming instead two ventral bulges (Fig. 3A, two arrows). The area of the two wings increased dramatically in both the craniocaudal and mediolateral dimensions. At the same time that the wings were increasing in area the legs were reduced. The distance between the two legs of the cardiogenic mesoderm decreased corresponding to the narrowing of the foregut and anterior intestinal portal (Fig. 3A and Movie 2).

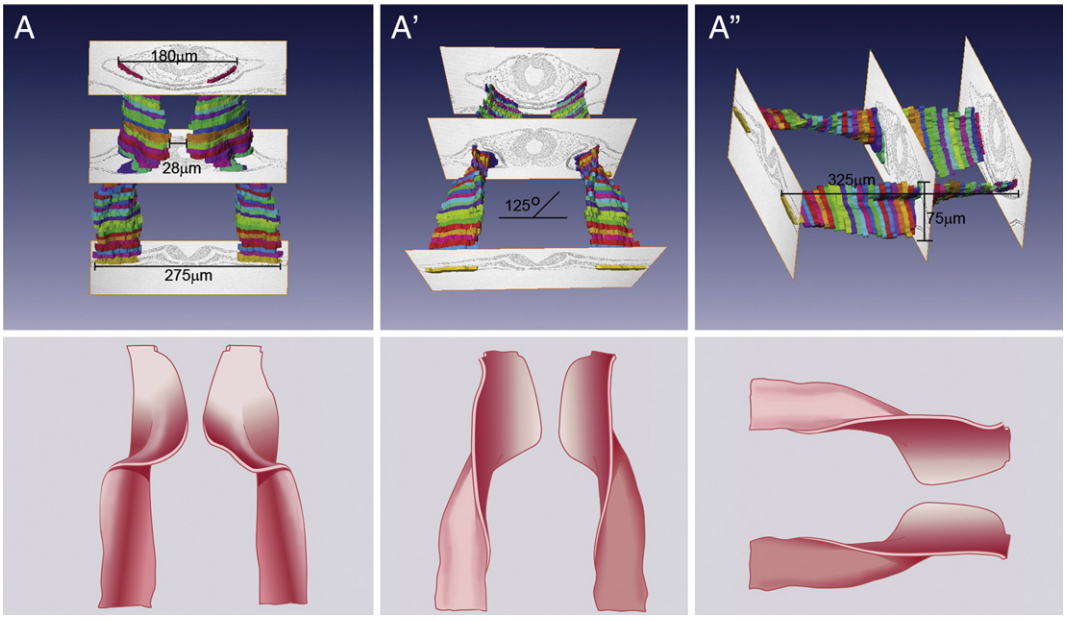


**Fig. 2.** Three different orientations of the 3D reconstructions of stage HH8 heart field. Each is shown embedded in three representative histological sections. Below each panel is an artist's rendering of the 3D image. The histological sections are at the level of the buccopharyngeal membrane, anterior intestinal portal, and first somite from cranial to caudal. The colors represent the 44 sections incorporated into the 3D reconstruction. (A) Ventral view shows the proximity of the unfused cardiogenic mesoderm across the midline at an intermediate level within the wings of the heart field. The length of the left–right axis cranially and caudally is also shown. (A′) Dorsal view shows the 120–130° inversion of the cardiogenic mesoderm which coincides with the anterior intestinal portal. (A″) Lateral view shows the dorsoventral axis of the cardiogenic mesoderm, and the craniocaudal axis.

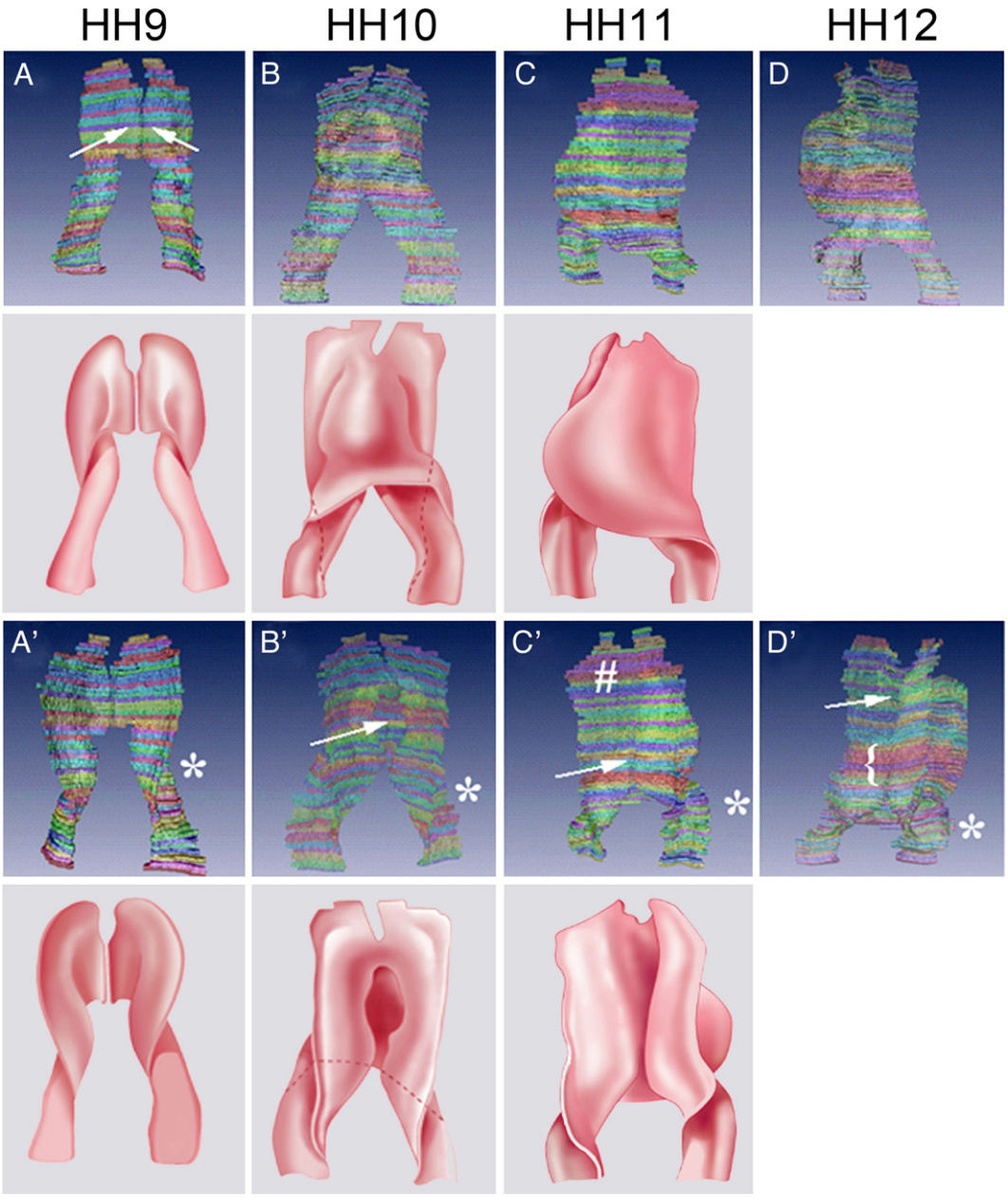


**Fig. 3.** 3D reconstruction of the cardiogenic mesoderm at stages HH9–12. Below the 3D images for stages HH9–11 is an artist's rendering of the 3D image. (A–D) Ventral view. (A′–D′) Dorsal view. (A, A′) Stage HH9 cardiogenic mesoderm from right and left approximate at the midline but are not fused. They form two bulges (arrows) separated by the ventral mesocardium. (B, B′) Stage HH10, the cardiogenic mesoderm fuses ventrally at the midline and forms a myocardial trough which is open dorsally (arrow). (C, C′) At stage HH11, the myocardial trough begins looping but is still open dorsally. The caudal portion of the myocardial trough just cranial to the anterior intestinal portal is closing (arrow). (D, D′) Stage HH12 shows a looped myocardial tube with dorsal fusion in progress (bracket). Asterisk (*) indicates the position of the mediolateral inversion, which moves caudally with the anterior intestinal portal during development.

Ventral fusion of the heart fields to form a semitubular heart (open to the endoderm dorsally and referred to as the "myocardial trough" by de la Cruz and Markwald, (1998)) could be seen in the 3D reconstruction of HH10 embryos (Fig. 3B). The wings continued to grow larger while the legs were progressively reduced in size. The dorsal side of the semitubular heart was open and was continuous with the wings on both sides (Fig. 3B′, arrow). The two legs of the cardiogenic mesoderm continued to draw near the midline as the anterior intestinal portal narrowed (Fig. 3B and Movie 3).

At HH11, even though the semitubular heart had elongated and started looping, the dorsal wall was still not fused to form a tube (Fig. 3C′, arrow). Ventrally the semitubular heart remained flattened cranially (Fig. 3C′) and the two wings of cardiogenic mesoderm were still present cranially wrapped around the forming pharynx.

HH12 was the last stage analyzed by 3D reconstruction. At this stage dorsal fusion to form the heart tube had progressed through all of the apposing myocardium (Fig. 3D′, bracket), but the tube was still open more cranially as had been observed during ventral fusion. The cranial heart tube was extended by smaller wings of cardiogenic mesoderm. Looping was more advanced at this stage. The legs were very much reduced (Fig. 3D and Movie 5).

The 3D reconstructions of HH8–12 suggest that change in orientation of the cardiogenic mesoderm as the anterior intestinal portal moved caudally and prior to fusion might represent an inversion point. Ventral fusion of the cardiogenic mesoderm occurred between HH9 and 10 just above the anterior intestinal portal. Dorsal fusion to form the heart tube began between HH11 and 12 also just above the anterior intestinal portal as reported previously by Linask et

al. (2005). The heart tube appeared to lengthen as the wings and legs of cardiogenic mesoderm diminished. Because these observations were made from static 3D reconstructions, we undertook labeling and cell tracing to obtain a more dynamic image of the movement of cardiogenic mesoderm.

*The cranial cardiogenic mesoderm contributes to the outer curvature of the heart tube*

To determine what portion of the heart fields are brought to the ventral midline to form the ventral seam, DiI/rhodamine (right side) and DiO/fluorescein (left side) was placed at the cranial extent of the cardiogenic mesoderm in embryos with 1–3 somites (HH 7) in New culture ($n$=12). Placement of the fluorescent dye was based on myocardin expression in the flat blastodisc (Warkman et al., 2008). As the anterior intestinal portal moved caudally, the dyes could be seen approaching each other in the ventral midline (Figs. 4A–C). As the heart began to loop the red and green dyes could be seen at the outer curvature and both dyes maintained a sharp boundary with no mixing (Figs. 4D–F). The green dyes were carried in slightly more DMSO so there was more spreading but the red dye maintained its position very well and in paraffin sections could be seen as a sharply distinguished group of cells in the ventral midline of the caudal heart tube (Fig. 4G) and gradually moving rightward in the more cranial and more rightward looped part of the heart tube (Figs. 4H and I). These results showing that the cranialmost region of the flat heart field gives rist to the ventral midline/outer curvature of the heart tube confirm a similar observation of Moreno-Rodriguez et al (2006).

*The cardiogenic mesoderm inverts 120–130° concomitant with the anterior intestinal portal*

To determine whether medial and lateral positions in the cardiogenic mesoderm inverted at the anterior intestinal portal, embryos were placed in modified New culture for double labeling (Chapman et al., 2001). DiO (green) and DiI (red) were injected into the legs of the cardiogenic mesoderm located just caudal to the anterior intestinal portal where the splanchnic mesoderm is still flat and located on the ventral side of the coelom at HH8 ($n$=7) (Figs. 5A and B). The labeled cells were followed at 3 h intervals for the first 12 h and at longer intervals afterward. In all of the descriptions that follow, the term "moved" is used to indicate relative movement within the embryo with no allegation about the mechanism for the rearrangement of cells. Over the first 3 h, the anterior intestinal portal moved caudally and narrowed (Fig. 5C). The medial cells (green) appeared to remain stationary, while the lateral cells (red) moved medially with the narrowing of the foregut pocket. This positioned the red cells underneath the green cells as confirmed in section through similarly labeled embryos (see below). The formerly laterally placed cardiogenic mesoderm was now located in the ventromedial wall of the coelom while the initially medial cardiogenic mesoderm was now in the dorsomedial wall of the coelom (Figs. 5I and J).

At 6 h after the labeling the anterior intestinal portal moved caudally and narrowed further (Fig. 5D). The green cells remained stationary while the red cells moved medially crossing under the green cells. This effected a complete inversion of the two cell populations. After the inversion, the red cells moved caudally away from

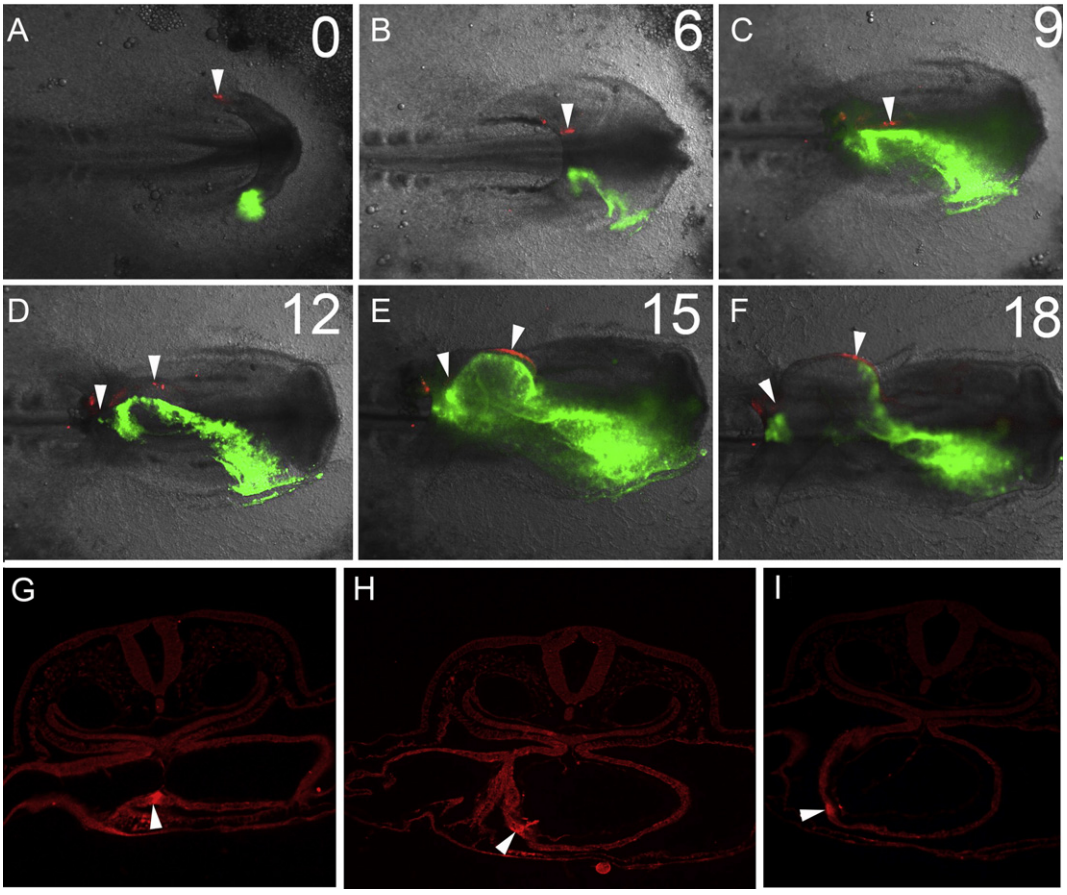


**Fig. 4.** Stage 7/8 chick embryo with 3+ somites labeled at the cranial end of the heart field with DiI/rhodamine on the right and DiO/fluorescein on the left. Panels A–F show the embryo at the 0 timepoint and at 3 h intervals thereafter. The green dye contained a higher concentration of DMSO and diffused more widely while the red dye was more limited and better represents the cranial heart field cells. However, both dyes show labeling in the outer curvature with red limited to the outer curvature. Panels G–I show a caudocranial series of sections of the same embryo. In the most caudal section nearest to the anterior intestinal portal (G) the labeling is in the ventral midline. In more cranial sections the label can be seen on the right as the outer curvature develops and the heart loops to the right side.

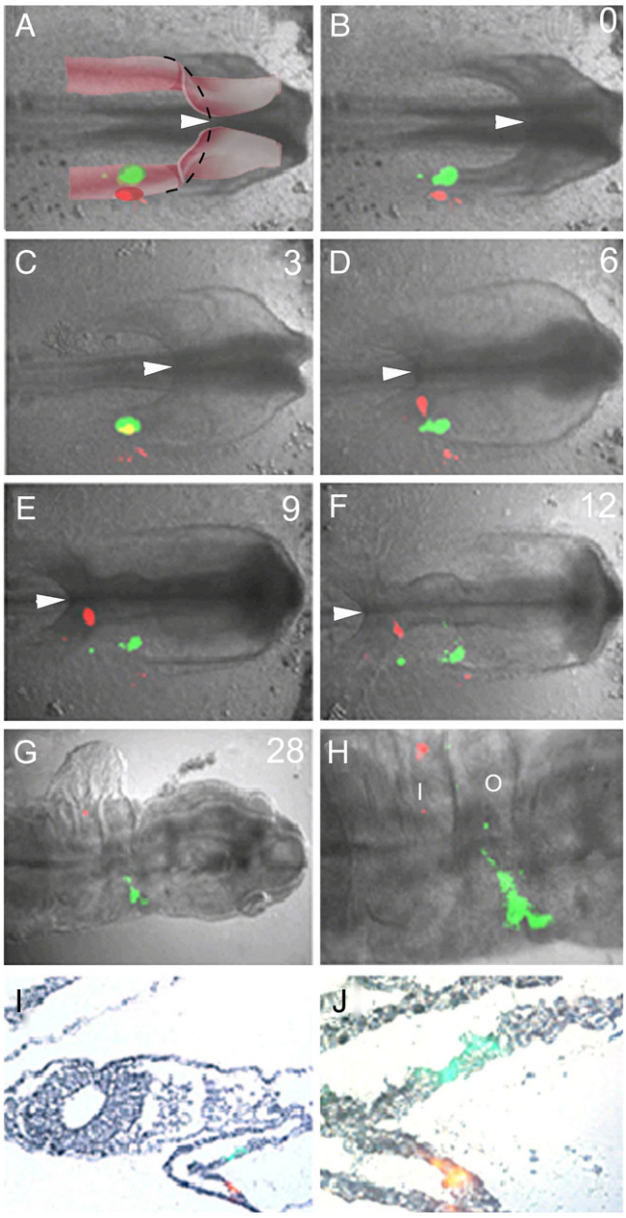


**Fig. 5.** Double labeling of the cardiogenic mesoderm of a stage HH8 chick embryo. (A) Position of the DiI (red) and DiO (green) labels in the cardiogenic mesoderm based on the 3D reconstructions. The 3D reconstruction of the same stage is overlaid on the embryo. (B–H) Ventral views of the same embryo at successive times from 0–28 h after labeling. (B–D) From 0 time to 6 h, the two labeled populations (green medially and red laterally) are at the caudal edge of the anterior intestinal portal (white arrowhead). (C–F) The red labeled cells move toward the midline, inverting their position completely with the green labeled cells. (D, E) The red labeled cells also begin to move caudally along with the anterior intestinal portal away from the green cells, which are stationary (D). (E–G) From 9 to 28 h, the heart tube forms and loops. The red labeled cells located in the heart tube continue their movement caudally along the anterior intestinal portal giving the impression that the green population is moving cranially. Red labeled cells are incorporated into the inflow (I) myocardium. The green population approaches the outflow (O) tract and is incorporated into it. (H) Higher magnification of the heart in panel G. (I, J) Transverse cryosections 5 h after double labeling of a stage HH8 embryo. (J) Higher magnification of the labeled cells in panel I. Note that both the red and green labeled cells are in the cardiogenic mesoderm.

the green cells (Fig. 4E). Some red and green cells that were spuriously marked were displaced away from the embryo into the extraembryonic tissue. That the cells were extraembryonic was verified when the extraembryonic tissues were removed during analysis.

At 9 h, the heart tube formed, and the anterior intestinal portal continued its caudal movement. The red cells moved medially and caudally creating a significant craniocaudal separation between the two labeled populations (Fig. 5E).

At 12 h the heart tube initiated rightward looping and the anterior intestinal portal reached the level of the first somite. The initially lateral red cells began to be incorporated into the inflow limb of the heart tube while the initially medial green cells began to be incorporated into the outflow limb (Fig. 5F). At 28 h the red cells were clearly located deep in the inner curvature of the inflow limb (Fig. 5G). A few green cells moved medially and were closer to the outflow tract. Embryos processed for histological analysis showed the green cells entering the outflow myocardium (not shown).

To confirm that the initial labeling was in the splanchnic mesoderm and that the movement observed was of the splanchnic mesoderm, additional embryos ($n$=4) were labeled, fixed after 5 h, and cryosectioned to check the position of the labeled cells. Indeed both labels were in the splanchnic mesoderm as described above (Figs. 5I and J).

### *Evaluating the movements of labeled cells in the cardiogenic mesoderm between HH8 and 11*

Because cells in the cardiogenic mesoderm located at the same craniocaudal location ended up in the outflow and inflow based on their mediolateral location, we designed a series of experiments to further examine the relative movements of cells within the cardiogenic mesoderm to determine how the field is organized with respect to the heart tube. We used two approaches. First we labeled embryos in modified New culture where visualization of label placement and movement of cells are most accurately achieved. However, the disadvantage of this method was that the embryos did not remain healthy and develop normally enough to evaluate the latest populations added to the outflow tract between HH14 and 18 (Waldo et al., 2005). Thus, the second approach was to label cells in ovo. This technique allowed less accuracy in placing the labels but the embryos could develop to later stages. The in ovo embryos were analyzed at HH20–22 when all of the myocardial progenitors have been added to the outflow tract (Waldo et al., 2005).

#### *Cells in the cranial cardiogenic mesoderm form the outer curvature*

When DiI was injected at the craniolateral border of the wing of the cardiogenic mesoderm (Figs. 6A and B) and followed at 3 h intervals for the next 36 h, the labeled cells moved medially and reached the outflow limb after 9 h. They were incorporated into the outer curvature of the outflow myocardium over the next 25 h which showed that marking the cranialmost part of the heart field in the chick led to labeled cells in the outer curvature of the outflow limb of the heart tube. The results suggest that cells in the heart field move to the cranialmost position in the wing before they are incorporated into the outer curvature of the outflow limb.

#### *Cells at the inversion point are incorporated into both the outflow and inflow myocardium*

In a second series of studies, a single injection of DiI was placed across the inversion point of the cardiogenic mesoderm (Figs. 6F and G). After 2 h of culture the labeled cells split into two populations. Over the next 29 h, the more craniolateral population was added to the outer curvature of the outflow myocardium while the caudomedial population moved caudally with the anterior intestinal portal and was incorporated into the outer curvature of the inflow myocardium (Figs. 6H–J). This shows that inflow and outflow myocardial progenitors are located adjacent to each other at the inversion point.

#### *Cells in the caudal cardiogenic mesoderm form the inner curvature*

In order to analyze the movement of the cells caudal to the anterior intestinal portal relative to those cranial to the anterior intestinal portal, double labeling was employed. DiI (red) was injected in the caudomedial leg and DiO (green) in the craniolateral wing of the heart

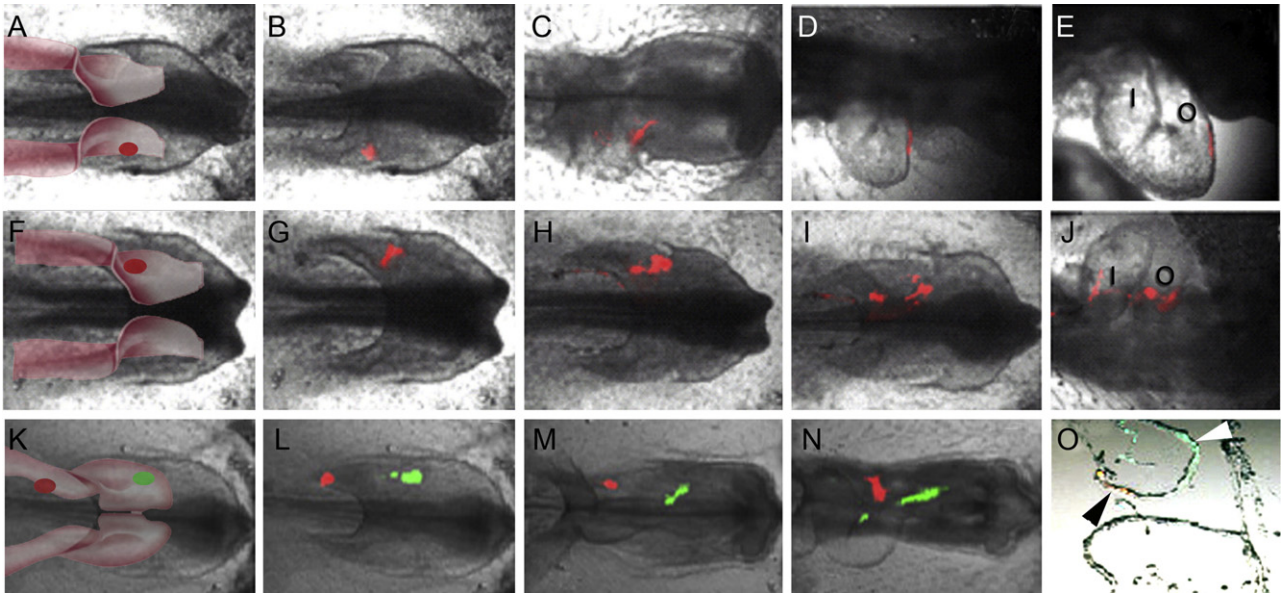


**Fig. 6.** Mapping the cardiogenic mesoderm in cultured stage HH8–9 embryos. 3D reconstructions are overlaid on embryos of the same stages in the far left panels (A, F, K). (A–E) Labeling of the craniolateral cardiogenic mesoderm. The same embryo is shown from a ventral view from the time of labeling (0 h) to 45 h after the label was placed. The red labeled cells appeared to move toward the midline and were incorporated into the myocardium of the outer curvature of the outflow tract (O). I, inflow tract. (F–J) Labeling the mid-wing region of the cardiogenic mesoderm. The same embryo is seen from the ventral side at the times indicated after injection of the label. At 0 time, the labeled cells (red) were at the center of the inversion point of the heart field. The labeled cells appeared to move toward the midline and split into two populations. The more lateral population appeared to move cranially to the outer curvature of the outflow (O) myocardium while the more medial population appeared to move caudally to the outer curvature of the inflow (I) myocardium. (K–O) Double labeling of the cranial and caudal extremities of the cardiogenic mesoderm. Whole embryos are seen from the ventral side at the times indicated. At 0 time, the two labeled populations (green cranially and red caudally) in the medial part of the cardiogenic mesoderm. The labels both move toward the outflow tract with the cranial labeled cells (green) populating the outer curvature while the caudal labeled cells (red) populate the inner curvature. Panel O is a section through the outflow tract of the same embryo showing red (black arrowhead) and green (white arrowhead) labeled cells on opposite sides of the outflow myocardium.

field in HH8 or 9 embryos (Figs. 6K and L). The prediction was that even though the labeled cells are initially far away from each other, the destination of both should be the outflow myocardium. The cranial red labeled cells were closer to the caudal green labeled cells after the anterior intestinal portal moved caudally and then both populations moved into the outflow myocardium (Figs. 6M and N). To verify the myocardial position of the labeled cells, the embryos were cryosectioned. Indeed red and green labeled cells were in the outer and inner curvatures of the outflow myocardium, respectively (Fig. 6O).

To resolve the destination of the caudalmost portion of the cardiogenic mesoderm which is added last to the heart, we labeled in ovo to allow the embryo to develop to later stages. To prevent dye dilution we implanted small DiI/DiO crystals or NeuroTrace green (Molecular Probes) rather than injecting solutions of dyes which dilute

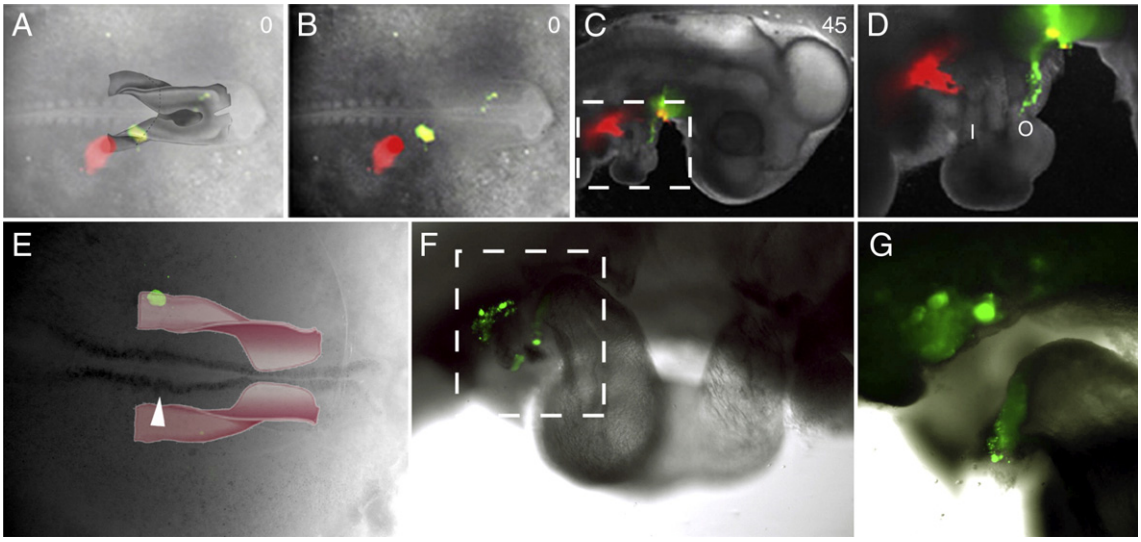


**Fig. 7.** Labeling in ovo to observe location of labeled cells after addition of cardiogenic mesoderm to the outflow myocardium and the atrium. (A) The position of the labels in the cardiogenic mesoderm shown in a dorsal view of the 3D reconstruction of an HH11 embryo. Double labeling with crystals of DiI (red) and DiO (green). The green label is implanted into inverting region while the red label is in the lateral leg of the cardiogenic mesoderm, of a stage HH11 embryo. The labeling was followed for 45 h. (B) Position of the crystals immediately after they were placed in the embryo. (C, D) Labeling in the outflow (O, green) and inflow (I, red) after 45 h. Panel D shows higher magnification of the boxed region in panel C. (E) Embryo labeled at stage 8 with NeuroTrace green placed in the caudal lateral cardiogenic mesoderm. White arrowhead indicates somite 1. (F, G) show the same embryo at 45 h after the labeling. Panel G shows an enlargement of the box in panel F. Green cells can be seen in the wall of the left side of the atrium.

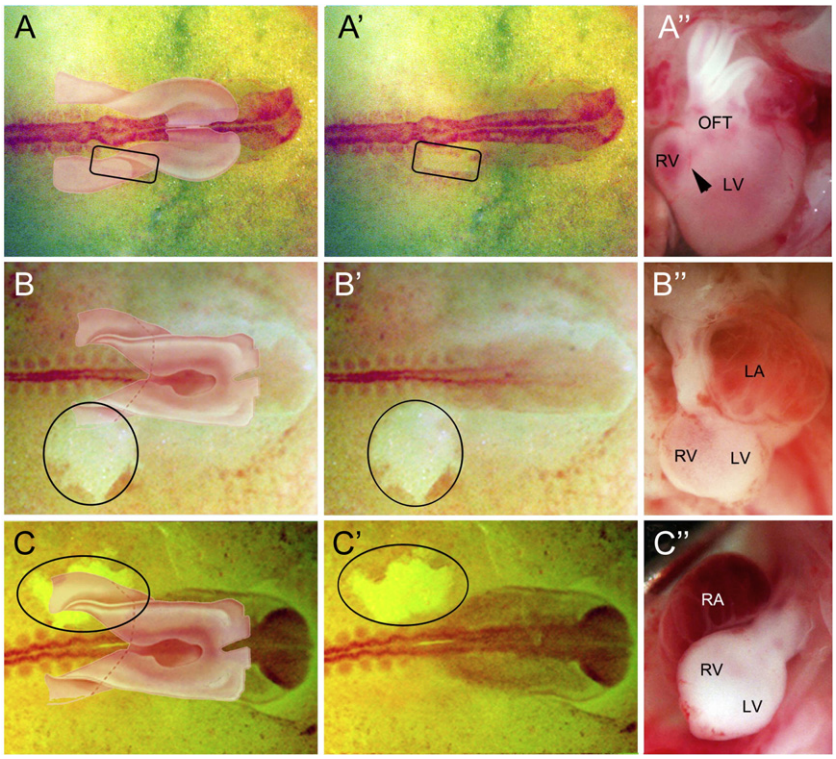


**Fig. 8.** Ablations of selected regions of the heart field and the resulting heart phenotype. The far left panels show 3D reconstructions overlaid on the embryos to indicate what part of the heart field was ablated. (A–A″) Unilateral right ablation of the medial leg and region of inversion (square, A and A′) at stage HH9 caused reduction of the right ventricle (RV) and outflow tract (OFT) myocardium which resulted in double outlet right ventricle (A″). Arrowhead indicates the ventricular septum; LV, left ventricle. (B–B″) Ablation of the right leg of cardiogenic mesoderm at stage HH10 (circled, B and B′) resulted in the absence of the right atrium (LA) and reduction of the left ventricle while the left atrium (LA) is developing well (B″). (C–C″) Ablation of the left leg of cardiogenic mesoderm (circled, C and C′) resulted in the absence of the left atrium and reduction of the ventricles while the right atrium (RA) is developing well (D″).

too rapidly for long term tracing. Labeling in ovo requires that the embryo be approached dorsally. This would involve damage to the head and labeling non-cardiogenic mesoderm when marking the cranial cardiogenic mesoderm. Thus these experiments were limited to the caudal portion of the cardiogenic mesoderm which is more accessible.

DiI and DiO crystals were inserted into the caudal portion of the cardiogenic mesoderm into lateral and medial locations, respectively (Figs. 7A and B). The labeling was followed for 45 h and as predicted the red DiI labeled cells were found in the inflow while green DiO labeled cells were in the outflow (Figs. 7C and D).

In a separate series of experiments NeuroTrace green was placed in the caudal and lateral portion of the left or right heart field. After 45 h the embryos were examined and green cells could be seen in the forming atrium on the same side as the dye was placed (Figs. 7E–G).

Collectively the results from these and the previous experiments indicate that the cardiogenic mesoderm cranial to the anterior intestinal portal, i.e. the "wings" is inverted while the cardiogenic mesoderm caudal to the anterior intestinal portal, i.e. the "legs" is not inverted. Furthermore, cells contributing to the atria are located in the caudal lateral part of the heart field and are added relatively late to the heart tube.

### *Testing the fate mapping by ablation*

To further confirm the mapping, we ablated specific regions of the cardiogenic mesoderm. The fate map predicts that outflow precursor cells occupy the most lateral part of the cardiogenic mesoderm in the wing cranial to the foregut pocket while they are located medially in the leg caudal to the foregut pocket. Conversely, the inflow precursor cells occupy the most medial part of the wing of cardiogenic mesoderm cranial to the foregut pocket and laterally in the leg caudal to the foregut pocket. This makes the ablation quite difficult because outflow and inflow precursor cells are adjacent to each other within the cardiogenic mesoderm.

HH9–10 embryos, were ablated unilaterally or bilaterally at different locations in the cardiogenic mesoderm caudal to the anterior intestinal portal. Following the ablation the embryos were incubated (3 to 9 days) until they showed signs of heart failure or until complete outflow septation was achieved. Only embryos with normal circulation were analyzed.

Ablation of caudal, medial and newly inverted lateral cells resulted in dramatic reduction in the right ventricle and abnormal outflow development (Fig. 8A). When the ablation of the most caudolateral part of the leg was performed unilaterally, the atrium of the ablated side was compromised giving rise to a single dilated atrium of the opposite side. This was true for both right and left atria (Figs. 8C and D) suggesting that when the atria are added to the heart tube, the cardiogenic mesoderm from each side gives rise to its respective atrium. This observation supports several recently published studies (Galli et al., 2008; Ramsdell et al., 2006).

## Discussion

Our data show the organization of the heart tube progenitors in the chick heart field and unexpected relative movements of cell populations within the field. It is clear from these studies that the craniocaudal polarity of the chick heart tube is represented mediolaterally in the heart field similar to the pattern suggested by mouse molecular expression studies (Cai et al., 2003; Kelly et al., 2001). This means that the inflow precursors are located in the most lateral part of the heart field while the outflow precursors are located in the most medial part of the heart field prior to formation of the foregut pocket. In this regard the inflow limb of the looped cardiac tube is usually

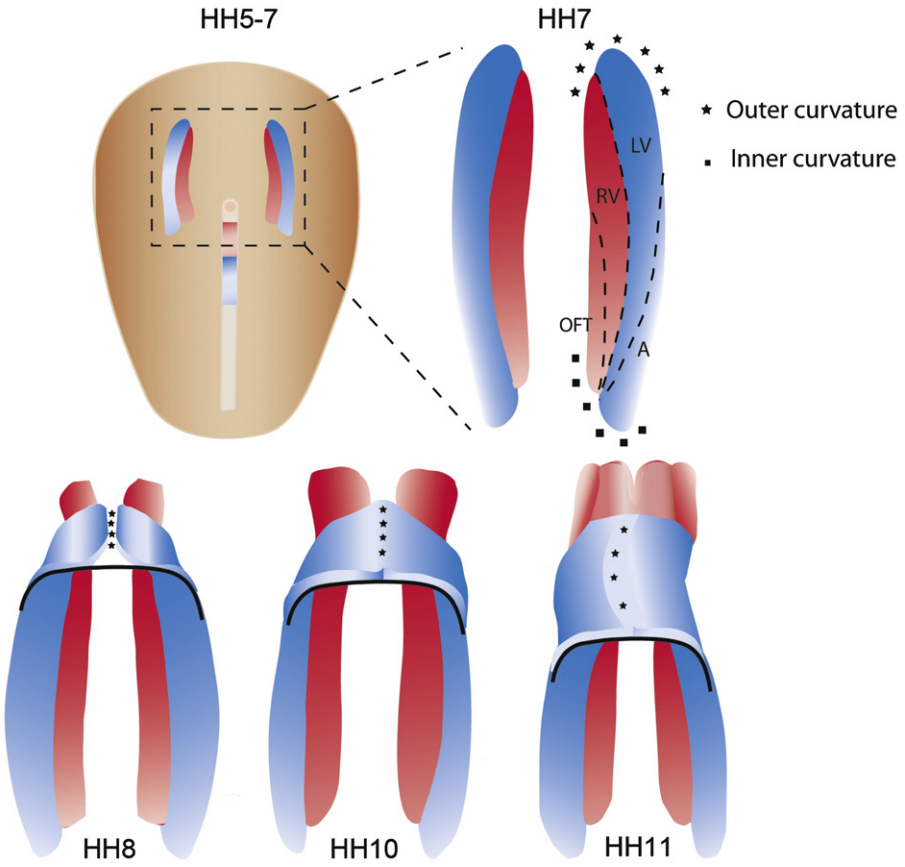


**Fig. 9.** Model of cardiogenic mesoderm and heart tube formation. Between stages HH3 and 7, cells in the epiblast ingress through the primitive streak and become localized in the anterolateral lateral plate mesoderm. In the epiblast and primitive streak the cells are located in the same craniocaudal position as they will be in the looped heart tube. During gastrulation however there is a 90° shift such that inflow precursors (blue) are located laterally in the cardiogenic mesoderm and outflow precursors (red) are located medially in the flat cardiogenic mesoderm at stage HH7. Formation of the foregut pocket by the anterior intestinal portal inverts the cardiogenic mesoderm 120–130° which returns the position of the inflow and outflow progenitors to the original craniocaudal orientation. The cranial parts of the heart fields are brought to the ventral midline where they will form the outer curvature after fusion at stage HH10. Note that even at stage HH11 when the heart tube has started to loop, there is still a cardiogenic mesoderm that will be added to both the inflow and outflow myocardium.

understood to represent the atria and left ventricle while the outflow limb represents the right ventricle and outflow myocardium.

To effect the change in orientation from mediolateral to craniocaudal, the cells in the heart field undergo a 120–130° inversion as the anterior intestinal portal moves caudally to form the foregut pocket. The inversion actually has two outcomes: it effects realignment of mediolateral to craniocaudal polarity and it brings the newly inverted cranial part of the heart fields (wings) toward the ventral midline where the first fusion occurs (Fig. 9). This initial fusion forms the ventral myocardial seam which will, after looping, become the greater curvature of the looped tube. Closure of the ventral myocardial seam creates a hemitube or "trough" that is not closed dorsally (de la Cruz and Sanchez-Gomez, 1999). The dorsal myocardial wall is closed later to form the tubular heart (HH11–12). The tubular heart then becomes detached from the pharynx as the dorsal mesocardium breaks down (HH13, Linask et al., 2005).

After the heart tube is detached from the pharynx, cells can no longer be added along the length of the tube even though many cardiogenic precursors remain in the splanchnic mesoderm. These are added over a prolonged period of time via the inflow and outflow poles of the tube that remain attached to the pharynx (HH14–18). The dynamic movements within the heart field allow heart tube formation with continuing addition of progenitor cells at the inflow and outflow poles over a prolonged period of time.

Our data suggest that the cardiogenic mesoderm in the chick forms as a single bilateral heart field from which progenitors differentiate as myocardium over an extended period of time. The initial progenitors that differentiate are continuously reinforced by cells added from the heart fields that have not differentiated in the first wave.

HH5–18 represent about 20–72 h of incubation, a much longer period than has been appreciated in the past and this explains many of the conflicting results in heart field analysis obtained previously.

The addition of myocardium to the inflow pole in the chick has not been examined thoroughly in this study but much myocardium is added to the inflow pole in the mouse (Christoffels et al., 2006) including non-Nkx2.5 expressing cells. It is currently not known if these exist in chick. If they do then our 3D reconstructions based on Nkx2.5 probably underrepresent the myocardial progenitors for the inflow limb.

Our data show for the first time relative movements in the heart fields that have not been appreciated in previous studies. However, two other studies support the conclusions of these studies. Ehrman and Yutzey (1999) showed that medially placed DiI crystals migrated cranially even though the DiI marking was done in the endoderm at HH4–5, 3 stages prior to our marking (Ehrman and Yutzey, 1999). That paper also showed that removal of the anterior medial mesoderm at HH5 had no effect on heart development at HH12 as shown by vmhc1 expression. Current data suggests that the embryos in these studies were not followed long enough to determine whether the outflow myocardium developed, i.e. HH18. On the other hand removal of lateral precursors resulted in loss of vmhc1 positive inflow heart tissue on the operated side of the embryo. These data suggest that the medial (outflow) and lateral (inflow) position of the precursors are in place already at HH5. Our results marking the cranial boundary of the heart field support recent marking studies that showed head mesenchyme

contributed to the myocardium of the outflow tract (Tirosh-Finkel et al., 2006) although it has been known for some time that outflow endocardium is contributed by head mesenchyme (Noden, 1991).

Our results differ from those of Redkar et al. (2001) who marked potential cardiogenic cells on a grid at HH5–8. These studies were done in cultured embryos to obtain precise marking and the results showed no particular organization in the cardiogenic mesoderm. However, the disadvantage of this in vitro technique is that the embryos do not survive much past HH12 and so predictions must be made of where cells in the HH12 heart tube will actually be in the fully formed heart. The designations of sinus venosus, ventricle, atria and bulbus cordis were made in the belief that all of these chamber progenitors were present in the heart tube by HH12 which has been shown by numerous recent studies including this one to not be the case. Making the prediction of what chamber a cell in the heart tube will finally reside in is made even more difficult by the dramatic changes in relative positions of cells once they are in the heart tube (Rana et al., 2007).

After the discovery that the craniocaudal polarity of cells in the primitive streak is maintained in the heart tube (Rosenquist and DeHaan, 1966; Garcia-Martinez and Schoenwolf, 1993; Lopez-Sanchez et al., 2001), no mechanism was proposed to explain how the heart fields might be organized to account for this. Building on previous data showing that mesodermal cells exiting the primitive streak more cranially are situated in the more medial mesoderm while cells exiting more caudally are located in the more lateral mesoderm (Yang et al., 2002; Zamir et al., 2006), we propose that the craniocaudal heart tube progenitors are initially oriented mediolaterally in the heart field. The mediolaterally organized heart tube progenitors regain their craniocaudal orientation by rolling with the caudally moving anterior intestinal portal (120–130°). This effectively inverts their position cranial to the anterior intestinal portal. The cranial borders of the heart fields are also brought to the ventral midline which reestablishes the same craniocaudal polarity in the heart tube that exists in the primitive streak (Fig. 9). This new model also explains the rotation of the cardiogenic plate deduced from histological sections of early human embryos (Davis, 1927). It also correlates with the dynamic expression pattern of Isl1, Fgf10, and Fgf8 in mouse (Cai et al., 2003; Kelly et al., 2001; Ilagan et al., 2006).

### *New fate map of the heart field*

Our data lead us to propose a model conciliating our new observations with data available from previous studies in chick and recent studies in mouse (Fig. 9). The actual shape of the heart fields draws heavily on a recently published study of myocardin expression in the early chick which seems to correlate well with the tracing data in the present study (Warkman et al., 2008). The new model consists of four major elements: 1) the splanchnic mesoderm undergoes an inversion; 2) the heart field is organized in craniocaudal stripes prior to rotation with the most medial cardiogenic mesoderm contributing to the most cranial portion of the heart tube (outflow) and the most lateral part of the cardiogenic mesoderm contributing to the caudal heart tube (inflow); 3) the cranial and caudal extremes of the cardiogenic mesoderm contribute to the outer and inner curvatures, respectively; and 4) the right and left atria arise from right and left cardiogenic fields of mesoderm in contrast to the ventricles which are a mixture of progenitors from the right and left fields.

### *Signals that establish arterial versus venous poles of the cardiogenic mesoderm*

The current model for cardiac induction involves two different pathways. Inhibition of Wnt signaling by Dkk1 induces Hex in the endoderm underlying the anterolateral mesoderm. Hex in turn induces a diffusible cardiac inducing factor (Foley and Mercola, 2005). The second pathway involves Nodal signaling via Alk4/Cripto to induce Cerberus in the endoderm underlying the anterolateral mesoderm and this in turn promotes cardiac differentiation (Foley et al., 2007). Cerberus in the chick underlies the cardiogenic mesoderm (Chapman et al., 2002). Other diffusible factors that are known to affect cardiac differentiation include BMP2 which is expressed in chick at HH4–6 lateral to the heart field. BMP4 which is expressed more medially may coincide with the heart fields (Chapman et al., 2002). FGF2 and 4 have been shown in cultured mesoderm to interact with BMP in myocardial induction of non-cardiogenic mesoderm (Barron et al., 2000). However, only FGF8 appears to express in a pattern that could involve it in induction (Alsan and Schultheiss, 2002). Graded expression of FGF receptors (FGFR)1–4 has been described. FGFR3 appears to be most medial and caudal, 1 and 2 overlap each other and the heart fields and 4 is expressed most cranially (Lunn DB 2007). As the heart begins to form at HH7–8 only FGFR2 is lightly expressed by cardiogenic mesoderm while FGFR4 is expressed craniomedially in a pattern that might coincide with outflow limb progenitors. Interestingly, phosphorylated Erk1/2 and downstream FGF8 targets Pea3 and Mkp3 are expressed in undifferentiated progenitors as the cardiac mesoderm is brought to midline (Lunn et al., 2007).

While these observations may bring us somewhat closer to understanding heart induction, the absence of a precise map such as the one presented here, has not allowed correlation of gene expression with various regions of the heart field. Given the current new model, these correlations should now be possible.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.04.014.

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