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Chamber Formation and Morphogenesis in the Developing Mammalian Heart

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In this study we challenge the generally accepted view that cardiac chambers form from an array of segmental primordia arranged along the anteroposterior axis of the linear and looping heart tube. We traced the spatial pattern of expression of genes encoding atrial natriuretic factor, sarcoplasmic reticulum calcium ATPase, Chisel, Irx5, Irx4, myosin light chain 2v, and β-myosin heavy chain and related these to morphogenesis. Based on the patterns we propose a two-step model for chamber formation in the embryonic heart. First, a linear heart forms, which is composed of "primary" myocardium that nonetheless shows polarity in phenotype and gene expression along its anteroposterior and dorsoventral axes. Second, specialized ventricular chamber myocardium is specified at the ventral surface of the linear heart tube, while distinct left and right atrial myocardium forms more caudally on laterodorsal surfaces. The process of looping aligns these primordial chambers such that they face the outer curvature. Myocardium of the inner curvature, as well as that of inflow tract, atrioventricular canal, and outflow tract, retains the molecular signature originally found in linear heart tube myocardium. Evidence for distinct transcriptional programs which govern compartmentalization in the forming heart is seen in the patterns of expression of *Hand1* for the dorsoventral axis, *Irx4* and *Tbx5* for the anteroposterior axis, and *Irx5* for the distinction between primary and chamber myocardium.

Key Words: **cardiogenesis; cardiac development; chamber myocardium; mammalian heart; inner curvature.**

INTRODUCTION

The heart is the first functional embryonic organ. During development, the cardiogenic mesodermal sheets form a crescent that folds toward the ventral midline to form a linear heart tube (Manasek, 1968; van Mierop, 1979). This embryonic heart initiates rhythmic contractions at about the eight- to nine-somite stage (Goss, 1938) (mouse embryonic day 8 (E8)) and begins to function as a peristaltic pump, despite lacking valves and conduction system (de Jong *et al.*, 1992; Moorman *et al.*, 1994). In a complex morphogenetic progression, the linear tube becomes gradually transformed into the synchronously contracting four-chambered heart, in which atrial and ventricular chamber myocardium, a conduction system, and physically separate systemic and pulmonary blood streams have formed (de Jong *et al.*, 1997). A rapidly growing number of factors have been shown to be involved in these processes. So far, however, a model which lucidly describes the process of chamber formation has not been articulated.

During looping of the linear heart an array of distinct compartments becomes visible, which are dubbed outflow tract (OFT), embryonic right ventricle (RV), embryonic left ventricle (LV), atria, and sinus venosus (Anderson *et al.*, 1978; Moorman *et al.*, 1994). These compartments, typically indicated as segments of the heart tube, represent key structures in the developing heart, since they roughly correspond to their functional counterparts in the formed heart. Fate map studies showed that the anteroposterior

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(A/P) order of compartments within the tubular heart corresponds to their A/P position within the heart-forming region (Garcia-Martinez *et al.*, 1993). Several cardiac genes have been reported to display an apparent segmental expression pattern from the moment distinct atrial and ventricular structures are visible (Franco *et al.*, 1998). The relevance of segments therefore seems clear, since they are associated with functionally distinct compartments and distinct transcriptional domains. These observations have led to the classical and commonly held concept of the developing heart as a linear array of segments (Anderson *et al.*, 1978; De la Cruz *et al.*, 1989; Stalsberg, 1969).

Several observations need to be incorporated in the currently used segmental concept for cardiac development (Fig. 7E). First, the entire linear heart is characterized by a low density of gap-junction proteins and by slow and uniformly spreading conduction velocities of action potentials. Only after looping does the forming chamber myocardium of the atria and ventricles become characterized by much higher conduction velocities and density of gap junctions (de Jong *et al.*, 1992; Moorman *et al.*, 1998). Second, during the transformation of the linear heart into a four-chambered structure, the right atrium becomes directly connected to the right ventricle and the left ventricle to the OFT. This remodeling would require *trans*-differentiation of the left-ventricular segment (see Fig. 7). Third, segments are seen only at the outer curvature of the looped heart tube and not at the inner curvature (O'Rahilly *et al.*, 1987; de Jong *et al.*, 1997). Thus, trabeculations within the formed ventricle are found at the outer curvature only, whereas the inner curvature remains smooth walled. Finally, the endocardial cushions and ridges are not limited to the classic atrioventricular canal (AVC) and OFT as often assumed, but are almost contiguous along the entire heart at the inner curvature (Netter, 1969; Steding *et al.*, 1990; Mjaatvedt *et al.*, 1999). They are not found in the trabeculated atrial and ventricular compartments at the outer curvature.

The above-mentioned considerations led us to reexamine the patterns of expression of "chamber-specific" genes encoding atrial natriuretic factor (ANF), sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a), Chisel, myosin light chain (MLC) 2v, β-myosin heavy chain (MHC), and transcription factors Irx4, Irx5, Hand1, and Tbx5 and their relation to the formation of chambers in the linear heart through early looping stages. Although the developmental patterns of expression of most of these genes have been described (Lyons *et al.*, 1990; Moorman *et al.*, 1995; Lyons, 1994; Franco *et al.*, 1998; Zeller *et al.*, 1987; O'Brien *et al.*, 1993; Biben *et al.*, 1997; Bruneau *et al.*, 1999; Chapman *et al.*, 1996; Bao *et al.*, 1999; Thomas *et al.*, 1998; Bruneau *et al.*, 2000), emphasis in previous studies was placed on expression in the already formed embryonic atrial and ventricular myocardium (e.g., Lyons, 1994; O'Brien *et al.*, 1993), which constitutes the bulk of the mass of the fetal and adult heart. Expression in the inflow tract (IFT), AVC, and OFT, and especially the inner curvature, has been severely underemphasized, even though these structures form major components of the early embryonic heart and are of profound importance to the proper alignment of the cardiac chambers.

In this study we show that the onset of the transcriptional program that is associated with the formation of ventricular and atrial chamber myocardium is restricted to specific sites of the linear heart that are at the outer curvature of the looped heart. The inner curvature does not participate in the initiation of the ventricular and atrial transcriptional programs. Based on our data we formulate a two-step model for the formation of chamber myocardium.

METHODS

Embryos

Wistar rat embryos were obtained from timed-pregnant rats (9 to 13 embryonic days). FVB mouse embryos were obtained from timed-pregnant mice; noon of detection of the vaginal plug was considered E0.5. Samples were fixed for 4 h to overnight in 4% freshly prepared formaldehyde in phosphate-buffered saline at room temperature. The embryos were dehydrated in a graded series of ethanol and embedded in paraplast. Serial sections were cut at 7 μm thickness and mounted on RNase-free 3-aminopropyltriethoxysilane-coated slides for *in situ* hybridization

In Situ Hybridization on Sections

RNA probes complementary to rat SERCA2 (Moorman *et al.*, 1995), α and β-MHC (Boheler *et al.*, 1992), MLC2v (O'Brien *et al.*, 1993), Chisel (S. J. Palmer, C. Biben, and R. P. Harvey, manuscript in preparation), Cx43 (van Kempen *et al.*, 1996), ANF (Zeller *et al.*, 1987), and ATP binding site (Habets *et al.*, 1999) mRNA were labeled with [^{35}S]UTP and hybridized to 7-μm-thick serial sections of 4% formaldehyde-fixed embryos as detailed elsewhere (Moorman *et al.*, 1999). The signal was kept within the dynamic range of detection by adjusting probe concentration and exposure time (Moorman *et al.*, 1999). Images were taken using a Photometrix camera coupled to a Zeiss Axiophot microscope and digitized files were recorded. Digital images were colorized with NIH Image software (image processing software package Version 1.61. 1997, available from http://rsb.info.nih.gov/nih-image).

Whole-Mount in Situ Hybridization

RNA probes complementary to MLC2v, ANF, *Irx4* (Bruneau *et al.*, 2000), eHand, and Tbx5 (Chapman *et al.*, 1996) mRNA were labeled with digoxigenin–UTP and hybridized to embryos fixed in 4% formalin overnight at 4°C as detailed in Franco *et al.* (1999). Whole-mount *in situ* hybridization was performed essentially as described by Riddle *et al.* (1993) and Franco *et al.* (1999) with some modifications. After isolation of E9–10 embryos, the brain was perforated to enable accessibility. Bleaching with hydrogen peroxide was omitted and hydration was directly followed by proteinase K treatment (15 μg/ml, 20 min for E8–8.5 or 30 min for older stages). The embryos were incubated in prehybridization mix (50% formamide, 5× SSC, pH 7, 10 μg/ml blocking powder (Boehringer Mannheim), 1 mg/ml total yeast RNA, 0.1 mg/ml heparin (Becton–Dickinson), 0.1% Tween 20, 0.1% Chaps (Sigma), and 5 mM EDTA). After 1 h denatured digoxigenin-labeled riboprobe was

added (1 μg/ml). Washing solution 1 contained 0.1% Tween 20 instead of SDS. All further washings were performed in phosphate-buffered saline containing 0.1% Tween 20. After 3 washes for 10 min in NTM (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), alkaline phosphatase activity was demonstrated using purple AP reagent (Boehringer Mannheim). Images of dissected embryos were taken on a thin layer of 1% agarose with a Photometrix camera or Nikon color camera coupled to a Zeiss Axiophot microscope and digitized files were recorded.

RESULTS

ANF, Chisel, Connexin 43, and Irx5 Are Markers for Formation of Chamber Myocardium

Our model for chamber formation in the mammalian heart distinguishes primary myocardium of the linear heart tube from chamber myocardium of the ventricles and atrial appendages, which arise from the primary myocardium. The formation of the smooth-walled atrial myocardium that develops relatively late in development from caval and pulmonary myocardium has not been addressed because the complex morphology requires a separate study (D. Franco *et al.*, in preparation). We describe in this section four genes that are expressed exclusively in the myocardium of the chamber components of the developing heart tube and not in primary myocardium (see Introduction) nor in regions of the heart that derive from it. Studies were performed on mouse and/or rat hearts as indicated.

ANF. The gene encoding atrial natriuretic factor was not found to be expressed in the very early linear heart tube. Expression was initiated in E8.25 mouse embryos specifically on the ventral side of the linear to early looping heart (Fig. 1). This finding was confirmed by dissection of stained embryos of comparable stages and is consistent with *in situ* hybridization data shown by Zeller *et al.* (1987). Fate mapping in the chick has shown that the ventral surface of the linear heart tube gives rise to the outer curvature of the looped tube (De la Cruz *et al.*, 1999). From E9–9.25 onward, expression of *ANF* was seen strongly in the outer curvature of the left ventricle and more weakly in the outer curvature of the right ventricle. Around the same time, expression was initiated in the presumptive primordia of the atrial appendages (auricles) at the laterodorsal side of the posterior pole of the looping heart tube. The configuration of the heart tube at this stage of development is such that the atrial myocardium is also positioned at the outer curvature. The inner curvature, corresponding to the original dorsal side of the heart tube at the ventricular level, and the ventral/caudal side at the atrial level form a continuity of myocardium with the IFT, AVC, and OFT that does not express the *ANF* gene (Figs. 1F, 1G, and 3A). Between E9 and E11 *ANF*-positive regions expanded (ballooned) and the posterior atrial regions became positioned anterior to the ventricular region of the tube. The expression of *ANF* then decreased in the right ventricle and became confined to the auricles of the atria and left ventricular trabeculations. Taken together, we find that the *ANF* gene is expressed solely in the outer curvature of the atrial and ventricular compartments of the early heart tube and not in the IFT, AVC, OFT, and inner curvature. Therefore, this gene qualifies as an excellent marker for studying formation of chamber myocardium.

Chisel. The *Chisel* gene is a potential downstream target of the cardiac homeodomain factor Nkx2-5 (S. J. Palmer, C. Biben, and R. P. Harvey, manuscript in preparation). It is first expressed in mice from the beginning of heart tube formation (about E8.25), restricted to the outer curvature of the ventricles and auricles of the atria (data not shown). In looped hearts (E9–9.5), expression was confined to the atrial and ventricular myocardium but was clearly absent from the AVC, inner curvature, and OFT (Fig. 2), a pattern resembling that of *ANF* at this stage. In contrast, a probe specific for the ATP binding domain of MHC highlights the complete myocardium (Fig. 2, right side). *Chisel* expression was further increased at E10.5 but remained confined to the myocardium expanding from the outer curvature. The expression of *Chisel* in the ventricles shows a transmural gradient, with lower expression in trabeculations.

Connexin 43. The pattern of *Chisel* and *ANF* expression in the looped mouse heart is similar to that reported for *Connexin 43* (*Cx43*) in E13 rat hearts (van Kempen *et al.*, 1996). Analysis of serial sections of E13 rat hearts confirmed this pattern and, in particular, the absence of *Cx43* mRNA in the inner curvature (not shown).

Irx5. The spatial and temporal expression profile of a novel mouse member of the *Iroquois*-related homeobox gene family in the heart (*Irx5;* manuscript in preparation) resembled that of *ANF* and *Chisel*, although it appeared slightly later (E9) in ventricular myocardium at the outer curvature. Thereafter (E9.5), the pattern resembled that of *ANF*, being confined to the atrial and ventricular myocardium (Fig. 3). No expression was observed in the inner curvature. The pattern was examined until E13 at which it continued to resembled that of *ANF*, except that expression was retained in the trabeculated myocardium of the right ventricle.

Other Genes Expressed in a Regional Fashion in the Linear and Looped Heart

SERCA2a. The *SERCA2a* gene encodes the sarcoplasmic reticulum calcium pump, a key component of cardiac excitation–contraction coupling (Fabiato *et al.*, 1979; Bers, 1991; Moorman *et al.*, 1995). The gene was first expressed in the cardiac crescent. In the linear and looping heart (rat E10–11, mouse E8–9), we found using quantitative *in situ* hybridization to tissue sections that expression was graded along the A/P axis, decreasing from the IFT toward the OFT (Figs. 4A and 4B). No dorsoventral (D/V) differences were observed at this stage. In E11 and E13 rat hearts (comparable to stages E9–11 in the mouse), expression was upregulated in ventricular myocardium at the outer curvature and in forming atrial myocardium (Figs. 4C and 4D). In the atrial

region, expression was high in the forming auricles, but at the ventral side of the atrial region (corresponding to the inner curvature), which is connected to the body wall, expression was lower (Fig. 4D, arrowhead). Thus, in the AVC, inner curvature, and OFT, expression remained low, similar in level to that found in the anterior region of the linear E10 heart tube (Figs. 4C and 4D).

MLC2v/Irx4. Expression of *MLC2v* has been detected in bilateral cell clusters in the cardiogenic mesoderm of the mouse from E7.5–E8 onward (Lyons *et al.*, 1995; Ross *et al.*, 1996). Using radioactive probes on tissue sections, a technique which is less sensitive than the whole-mount method, we and O'Brien *et al.* (1993) observed earliest expression in the linear hearts of E10 rat and E8.5 mouse embryos, respectively, suggesting that expression in the cardiac crescent is only very weak. Like *MLC2v*, the *Iroquois*-related homeobox gene *Irx4* is expressed in the cardiac crescent of mouse E8 embryos (Bruneau *et al.*, 2000). Subsequently, both *MLC2v* and *Irx4* genes were expressed in the linear heart tube at higher levels and in a bilateral gradient along the A/P axis, centered in the middle ventricular region and encompassing both the ventral and the dorsal sides (Figs. 4E, 4F, and 5A). In mouse E9–9.5 hearts, expression was retained in the AVC, inner curvature, and proximal OFT, but also upregulated in the ventricular myocardium at the outer curvature. At this time, these genes were not expressed in *ANF*-positive domains within atrial myocardium (Figs. 4G, 4H, 3C, and 3D). However, the AVC region extending into the atrial wall did express *MLC2v* and *Irx4* (Figs. 4G, 3C, and 3D). This AVC portion of the primary tube (*ANF*-negative, see above) becomes incorporated into the body of the common atrial chamber with further development (Wessels *et al.*, 1996). These results highlight the fact that expression of *MLC2v* and *Irx4* is not restricted just to future ventricular myocardium in the looping heart. Within formed ventricles, expression of *MLC2v* and *Irx4* both showed a transmural gradient, with lower levels in trabeculations.

β-MHC. The *β-MHC* gene represents a group of genes that are expressed in the entire linear heart in a graded manner along the A/P axis. This group includes genes encoding α-MHC, MLC2a, cTnI, and SERCA2a that are expressed in gradients opposite to that of *β-MHC* (Franco *et al.*, 1998). *β-MHC*, however, is expressed in the entire linear heart tube of rat E10 (Figs. 4I–4L) and mouse E8–8.5 embryos (not shown) in an A/P gradient. With formation of ventricular and atrial myocardium (rat E10–13, mouse E8.5–E11.5), this pattern is essentially unchanged, resulting in high levels of expression in the OFT and ventricular myocardium, including the inner curvature, and lower expression in the AVC, atria, and IFT.

Tbx5. We examined the expression of the T-box transcription factor gene, *Tbx5*, in E8, E8.5, E9.5 (Fig. 5), and E11.5 mouse embryos and confirmed the posteroanterior gradient of expression previously reported (Chapman *et al.*, 1996; Bruneau *et al.*, 1999). In linear hearts, expression was high posteriorly and gradually declined to low levels in the OFT (Fig. 5C). Similarly, at E9.5, expression was seen in a posterior-high gradient over the looping tube (Fig. 5D): we detected expression across the entire looped tube and not restricted to the posterior portion as previously reported (Bruneau *et al.*, 1999). The contrasting observations can easily be explained by differences in sensitivity of respective *in situ* hybridization procedures. At E11.5, expression was weak in the right ventricle and absent from the OFT, indicating that *Tbx5* progressively withdraws from the anterior portion of the heart. We did not observe a clear difference between inner and outer curvatures in our experiments. We conclude that *Tbx5* retains its posteroanterior gradient during development in the fully looped heart and is not specifically upregulated in the forming chamber myocardium.

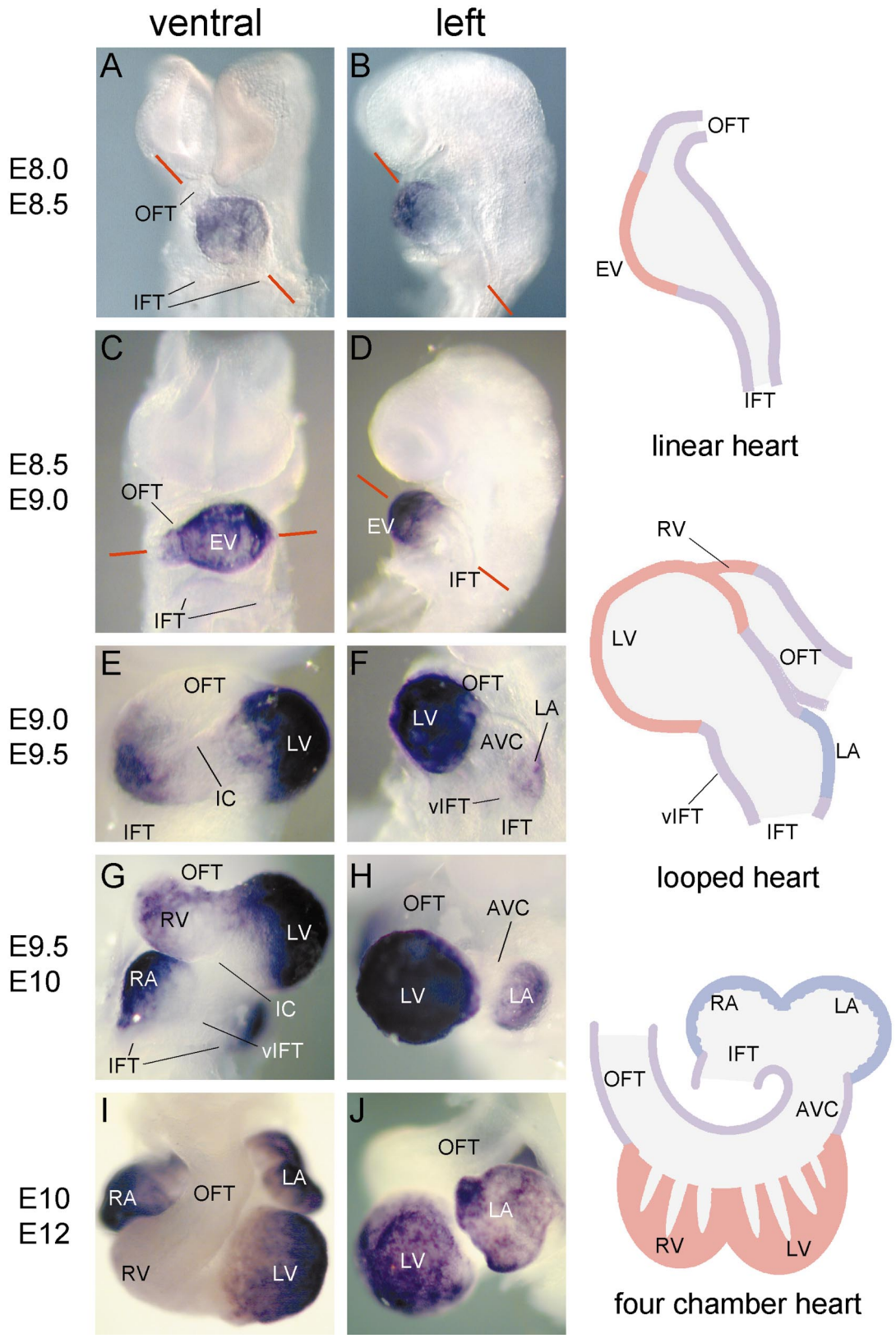
Hand1. The expression of the bHLH transcription factor gene *eHand/Hand1* is heterogeneously distributed along the A/P and D/V axes of the tubular heart (Biben *et al.*, 1997; Thomas *et al.*, 1998). Expression occurs specifically at the ventral side in the caudal half of the linear heart tube of E8.5 mouse embryos (Fig. 5F). The pattern of *Hand1* expression in the linear heart, along with that of *ANF* and other genes such as *Chisel* and *Cx43*, clearly demonstrates the presence of D/V patterning in the heart tube. In E9.5 mouse hearts the gene is expressed in the outer curvature (original ventral side; De la Cruz *et al.*, 1999) of the AVC and LV and weakly in the outer curvature of the RV/OFT and is absent from the atria and inner curvature (Fig. 5G).

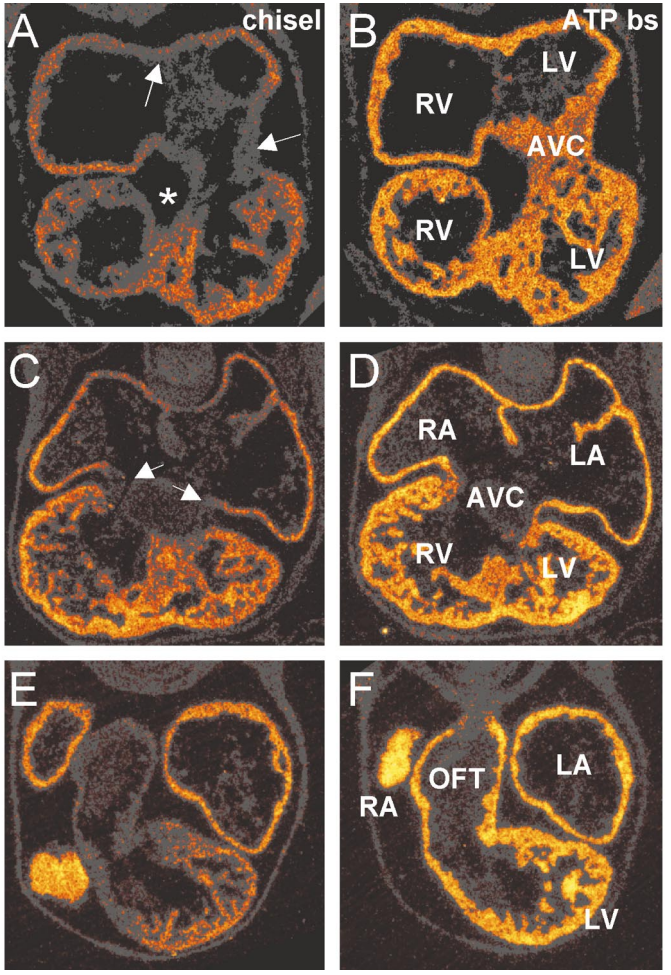
DISCUSSION

Patterns of Gene Expression and the Ballooning Heart Concept

This study demonstrates that the onset of a transcriptional program involving *ANF*, *Chisel*, *Irx5*, and *SERCA2a*,

FIG. 1. The spatial and developmental expression profile of *ANF* analyzed by whole-mount *in situ* hybridization. The red lines in A–D mark the changing anteroposterior axis. During looping, the anteroposterior alignment at the ventricular level is converted into a right/left alignment, the anteroposterior alignment of the atria and left ventricle is converted into a dorsoventral alignment. The right-hand column shows cartoons of a left view of the near-linear heart, a left view of the typical looped heart, and a ventral view of the preseptational four-chambered heart. The outflow tract has been hinged to the right. The *ANF*-expressing chamber myocardium is shown in blue (atria) and red (ventricles). AVC, atrioventricular canal; IFT, inflow tract; EV, embryonic ventricle; OFT, outflow tract; IC, inner curvature; vIFT, ventral side of IFT; LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium.





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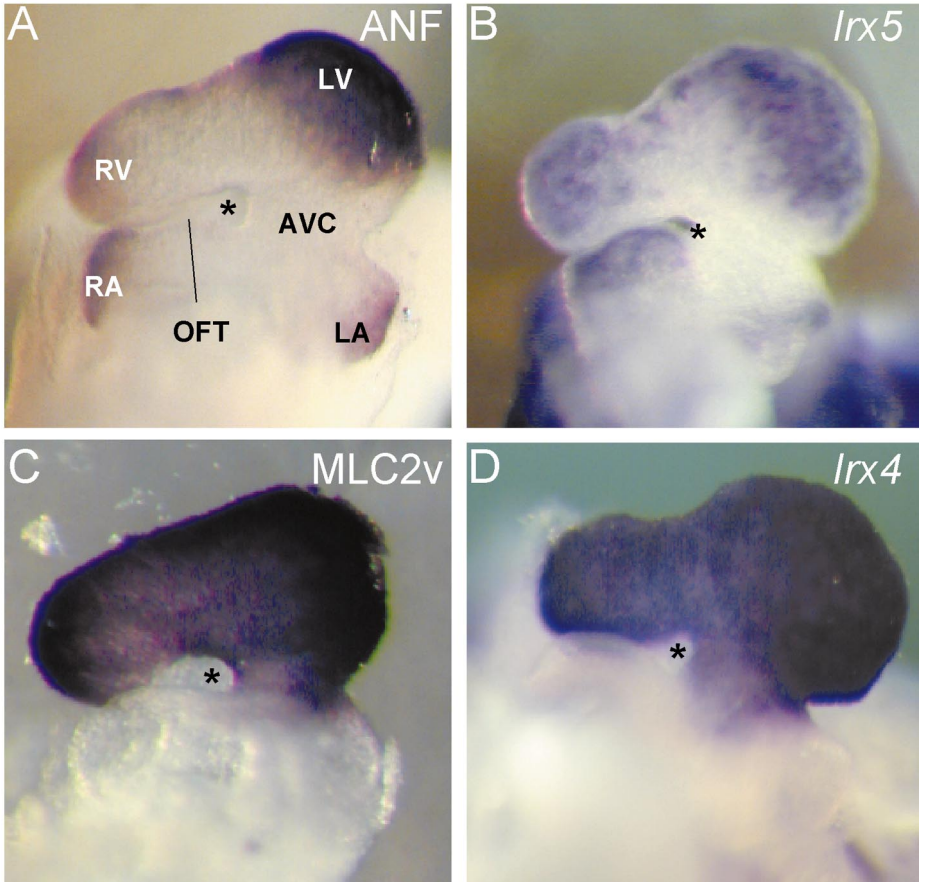

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FIG. 2. The expression of *Chisel* in the developing mouse heart analyzed by *in situ* hybridization on sections. (A) At E9.5 the mRNA for *Chisel* is confined to the myocardium of the atria and ventricles, whereas the atrioventricular canal (arrow) and myocardium facing the inner curvature (*) do not express the gene. (C and E) At E10.5 *Chisel* is expressed in the atria and ventricles but not in the atrioventricular canal and outflow tract. In the ventricles the expression shows a transmural gradient toward the lumen. (B, D, and F) All the myocardium is shown by the expression of myosins using a probe for the conserved ATP binding site (ATP bs). For abbreviations see Fig. 1.

FIG. 3. The expression of *ANF, Irx5, MLC2v,* and *Irx4* in the heart of E9.5 mouse embryos. The images show hearts of comparable stages from the caudal side, exposing the inner curvature (marked *) and the original ventral side of the atrial region. The outflow tract that does not express *ANF* is just visible behind the atria (A). Note the expression of *MLC2v* (C) and *Irx4* (D) radiating into the "bottom" of the atria. For abbreviations, see Fig. 1.

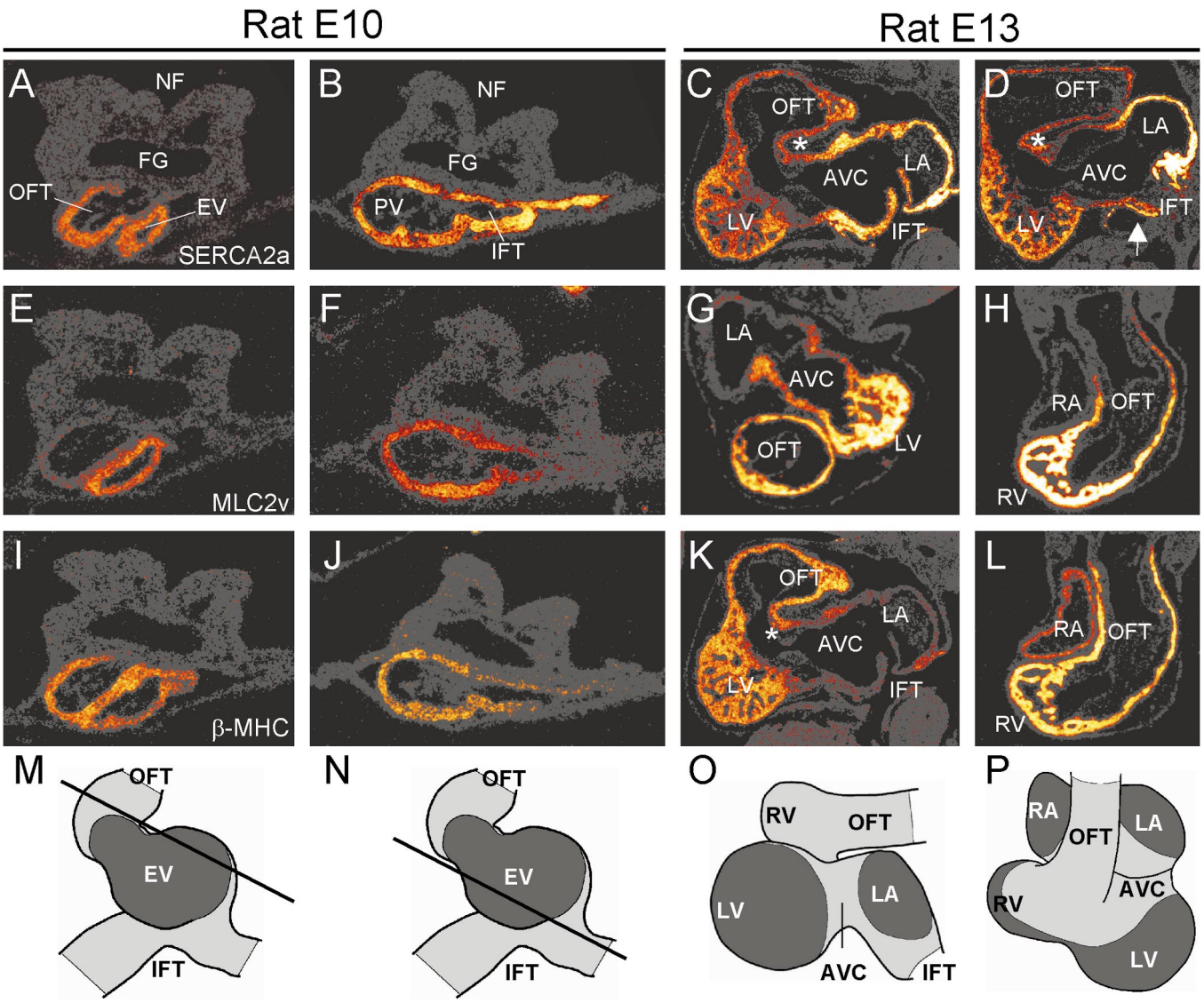

FIG. 4. Expression of *SERCA2a*, *MLC2v*, and *β-MHC* during cardiogenesis in the rat. The left images show serial sections of E10 rat embryos (comparable to E8.5 mouse embryos), the right shows hearts of E12.5 embryos (comparable to E11 mouse embryos). (A and B) *SERCA2a* in a linear, early looping heart, (C and D) sagittal sections of the looped heart. C shows a section lateral from that shown in D. Myocardium at the ventral side of the inflow tract (arrow) shows less expression than the surrounding atrial myocardium. This myocardium also does not express *ANF* or *Irx5*. (E–H) *MLC2v*. Compared to the other structures the level of expression has substantially increased in the ventricular myocardium of the E12.5 heart. (I–L) *β-MHC*. The *in situ* of H was only shortly exposed to allow the visualization of the gradient in expression. The bottom (M–P) shows diagrams for orientation. The dark gray regions depict the *ANF*-expressing myocardium. The lines in M and N depict the plane of sectioning. The * marks the inner curvature. See Fig. 1 for abbreviations; EV, embryonic ventricle; NF, neural fold; FG, foregut.

specifically associated with formation of chamber myocardium, is initially restricted to the ventral side of the linear heart and the outer curvature of the looped heart. These data provide a transcriptional basis for the morphological finding that segmental primordia and derivative chambers are formed at the outer curvature of the heart only (de Jong *et al.*, 1997), where proliferation rates are highest (Rumyantsev, 1977; Thompson *et al.*, 1990). Owing to this high proliferative activity and/or changes in cell shape (Manasek *et al.*, 1972), the chambers are seen to balloon out from the primary heart tube. Therefore, we have dubbed this model the ballooning model for chamber formation.

The temporal and spatial expression patterns of the genes described in this study, as well as those of most other

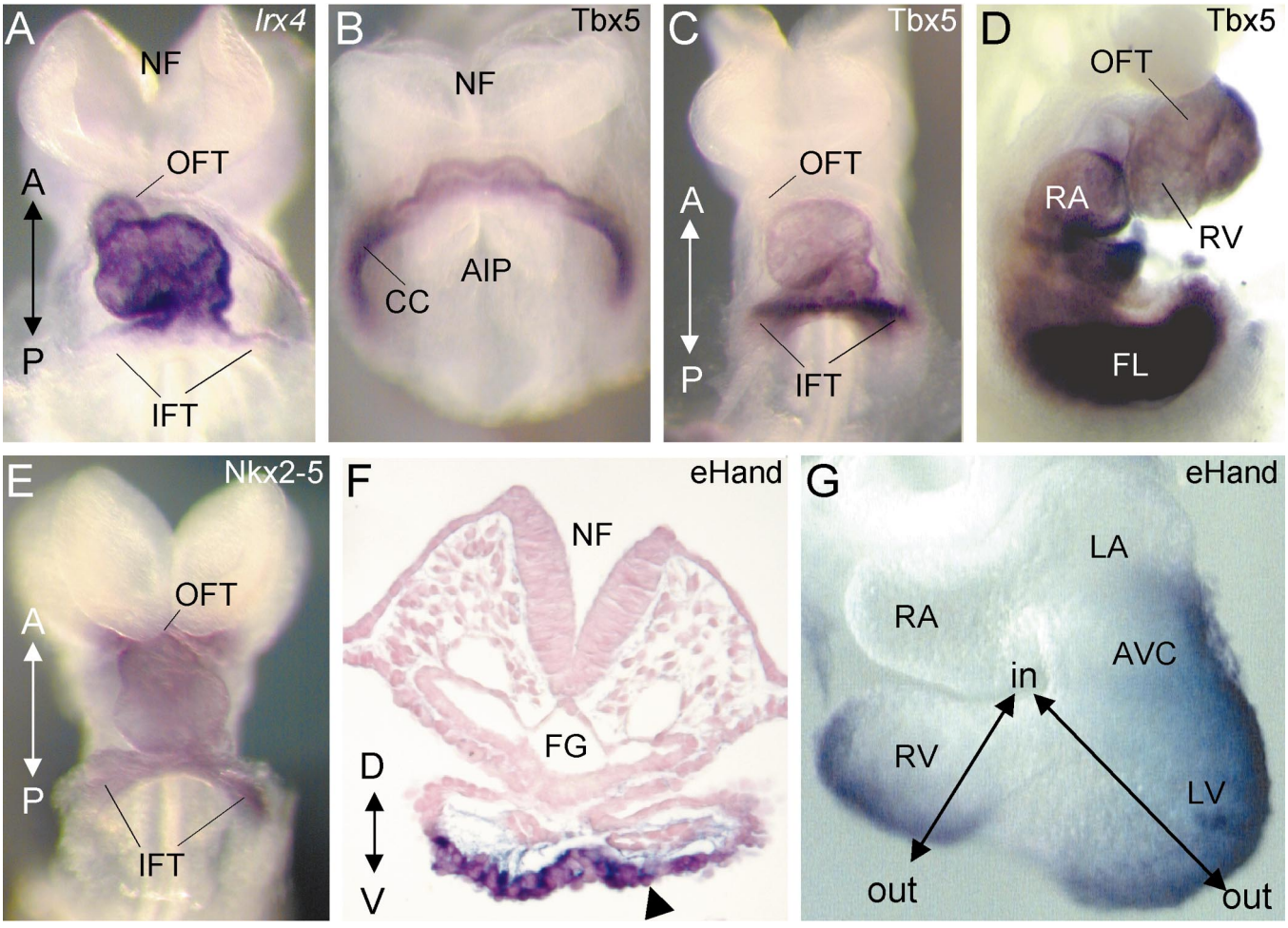

FIG. 5. Pattern of expression of *Irx4, Tbx5, Nkx2-5,* and *eHand* in the mouse heart analyzed by whole-mount *in situ* hybridization. (A) *Irx4,* expressed in the middle portion or "segment" of the linear heart of E8.5 embryos, is an example of an anteroposterior patterned factor. *Nkx2-5* (E) is expressed in a larger region encompassing all myocardium (control). (B–D) The gene for *Tbx5* is expressed in the cardiac crescent of E8 (B) and in the linear heart of E8.5 (C) embryos, in an anteroposterior gradient. This gradient is essentially unchanged in E9.5 embryos (D). (F) A transverse section of an E8.5 embryo hybridized with a probe for *eHand.* Note the clear dorsoventral pattern (arrow). At E9.5 (G) the expression of *eHand* is confined to the outer curvature of the ventricles and atrioventricular canal. For abbreviations see Fig. 1. A, anterior; P, posterior; D, dorsal; V, ventral; in, inner curvature; out, outer curvature; CC, cardiac crescent; FL, forelimb.

cardiac genes, all differ from each other. Yet general trends or "rules of behavior" can be formulated. Our analysis shows that the formation of chamber myocardium is a two-step process: (1) the establishment of the "primary" transcriptional program within the cardiac crescent and linear heart tube, followed by (2) the development of the "secondary" transcriptional program of the myocardium of heart chambers (Fig. 6). The pattern formed within the linear heart is maintained during subsequent cardiogenesis in the IFT, AVC, OFT, and inner curvature and for a number of genes also in the forming chambers (e.g., *α-MHC, β-MHC, Tbx5*). This pattern can therefore be regarded as a primary or default pattern. Chamber myocardium acquires an additional and presumably more evolutionarily advanced transcriptional program as is evident from the initiation of *ANF, Chisel,* and *Irx5* expression and upregulation of *SERCA2a, MLC2v,* and *Irx4.* This program develops in specific regions of the linear heart tube, which are then found at the outer curvature of the looping heart tube. What determines the specificity of these spatial events is unknown.

In chicken, the ventral side of the linear heart tube was shown to form the outer curvature of the looped heart (De la Cruz *et al.,* 1999). Whether the *ANF*-positive myocardium of the linear and early looped heart (E8.25–) represents the same lineage as the *ANF*-positive myocardium of the E9.5 heart formally requires a lineage study in mouse that, however, is technically difficult to achieve.

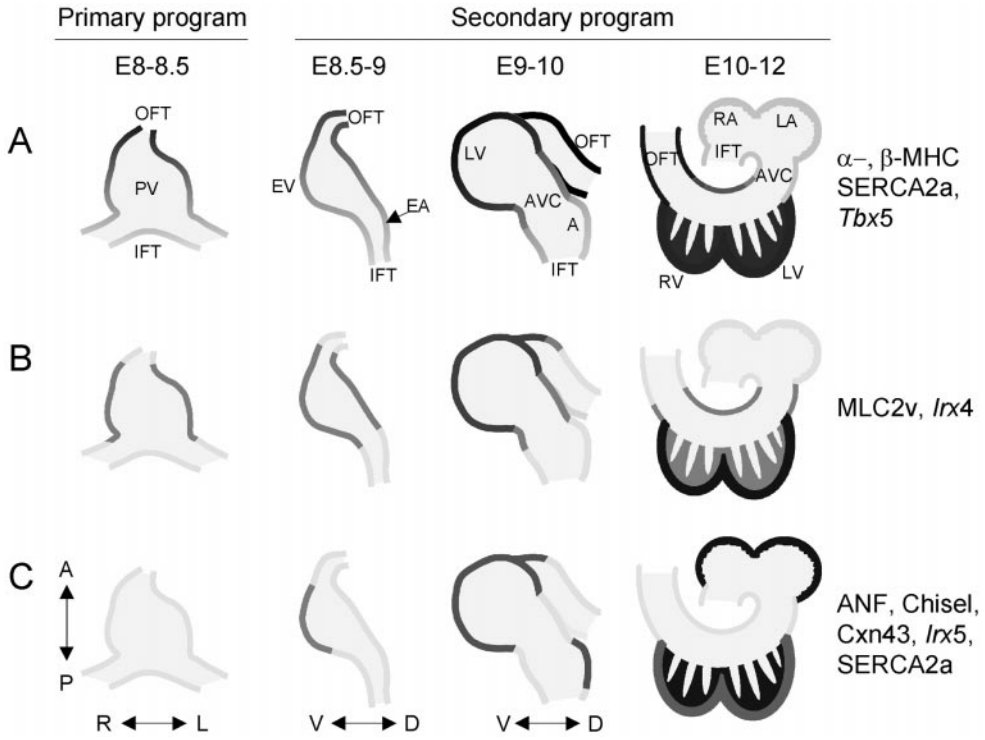

FIG. 6. An overview of the expression patterns of genes during chamber formation. The primary program of expression is found in the linear heart (first column). Several genes are either abundantly expressed or expressed in a gradient (row A) toward the inflow tract (e.g., *β-MHC*) or outflow tract (e.g., *SERCA2a, α-MHC, Tbx5*). *MLC2v* and *Irx4* are expressed in a bilateral gradient resulting in an expressing segment within the tube (row B). Genes for ANF, Chisel, Cx43, and Irx5 are not expressed in the linear heart (row C). At E8.5–9 the secondary program is initiated. Initiation or upregulation of expression of several genes is observed specifically at the ventral side of the linear heart, at the level of the prospective ventricle, and at E9–9.5 at the dorsolateral side at the level of the prospective atria (row C). During these stages, the genes already expressed in the linear heart essentially do not alter their original pattern along the A/P axis (rows A and B). From E10 onward a clear expansion of chamber myocardium is seen that is accompanied by upregulation of specific genes (e.g., *SERCA2a, Irx4*) and formation of transmural gradients in the ventricles. Since the chamber myocardium is growing rapidly compared to the persisting primary myocardium, genes in rows A and B (e.g., *MLC2v, Irx4, α-MHC,* and *β-MHC*) become increasingly chamber-specific. For abbreviations see Fig. 1.

Site-Specific Formation of Chamber Myocardium

The linear heart tube is essentially polarized along the A/P axis, as is evident from physiological and molecular data (van Mierop, 1967; Satin *et al.*, 1988; Kamino *et al.*, 1981; Yutzey *et al.*, 1994, 1995; Garcia-Martinez *et al.*, 1993; Lyons, 1994; Moorman *et al.*, 1994, 1995; Franco *et al.*, 1998). The number of genes and signals showing a heterogeneous distribution along the A/P axis is steadily growing and includes *Tbx5* (Bruneau *et al.*, 1999), *dHand* (Biben *et al.*, 1997; Srivastava *et al.*, 1997), *GATA-4* (Molkentin *et al.*, 1997), and *HRT1* and *-2* (Nakagawa *et al.*, 1999). The A/P polarity in transcriptional potential likely reflects or gives rise to the A/P organization of the forming heart. Formation of atrial and ventricular compartments in their correct A/P and D/V positions in the linear heart presumably requires positional information encoded by A/P and D/V signals, and this may involve secreted peptide factors and transcription factors. An additional candidate for an A/P polarity-defining pathway is the retinoic acid signaling system, which has been shown to be dynamically involved in the specification of posterior (sinoatrial) structures and the regulation of gene expression along the A/P axis (Xavier-Neto *et al.*, 1999). The *Irx4* expression pattern may demarcate the limits of the A/P region in the linear heart within which the ventricular chamber myocardium is to be formed. This does not necessarily imply that the entire *Irx4*-expressing segment becomes ventricular chamber myocardium. Our analysis shows that only the ventral side/outer curvature of the *Irx4*-positive "ventricular segment" initiates the expression of *ANF, Chisel,* and *Irx5*. This implies that the combinatorial action of an A/P signal such as retinoic acid and/or *Irx4,* and a second signal along the D/V axis, defines the sites of chamber myocardium within the

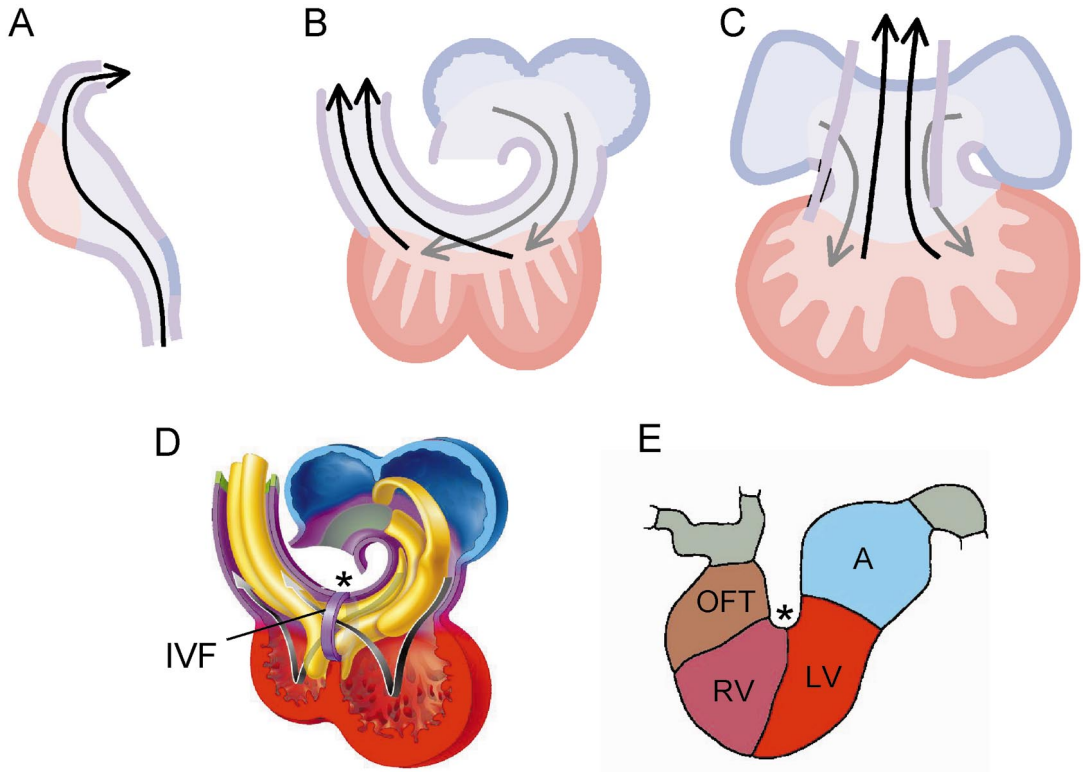

FIG. 7. Making the four-chambered heart: ballooning versus segmentation. (A) A linear heart in which ventricular (red) and atrial (blue) myocardium is being formed. This heart functions as a peristaltic pump propelling a laminar flow. During looping and chamber formation (B) the heart is gradually being transformed into a synchronous contracting pump with a flow into the ventricles during relaxation (gray arrows) and a flow into the outflow tract during contraction (black arrows). During the transformation toward a four-chambered heart, the right atrium becomes directly connected to the right ventricle and the left ventricle directly connected to the outflow tract (C). (D) A heart similar to that of B, with cushions and the OFT hinged toward the right side. The cardiac cushions are separating the flows. Note that the flow from the right atrium into the right ventricle never passes through the left ventricular chamber myocardium and that the flow from the left ventricle to the outflow tract never passes through the right ventricular chamber myocardium. This is principally impossible in the segmented tubular heart (E). The atrioventricular canal is essentially expanded rightward relative to the ventricles to connect both ventricles with both atria. The outflow tract is essentially expanded leftward (and ventral to the atrioventricular canal) to become connected to both ventricles (C). This is achieved by remodeling the inner curvature. This sequence of events enables the gradual transition from a single laminar into two separate blood flows and at the same time explains the transition from a tubular heart with anteroposteriorly aligned compartments into the properly connected four-chambered heart.

linear and looping heart. Based on its pattern of expression, *Hand1* is a potential candidate for mediating D/V specification (see Fig. 5F).

Identity of Chamber Myocardium

As discussed above, expression of a number of cardiac genes is specifically initiated (*ANF, Chisel, Cx43, Irx5*) or upregulated (*SERCA2a, Irx4*) in defined regions of the linear and looping heart. Since expression of these genes is found in the ventricular chambers and atrial appendages of the fetal and adult heart, we assume that they mark the forming chamber myocardium. The chamber myocardium therefore acquires additional transcriptional and phenotypic properties over that evident in primary myocardium of the linear tube. This is in line with the observation that forming chamber myocardium of the atria and ventricles acquires much higher conduction velocities and density of gap junctions than the linear heart tube. Our findings are also in line with observations that the myocardium of flanking "segments" of the heart, i.e., IFT, AVC, and OFT, maintains a poor electrical coupling and a long contraction duration that is characteristic of the linear heart tube (de Jong *et al.*, 1992; Moorman *et al.*, 2000). The precise border between primary and chamber myocardium is not strictly defined, and the pattern may be evolving. Comparison with the patterns of *Chisel, Irx5,* and *SERCA2a* indicates that the region of chamber myocardium is larger than the *ANF*-positive regions indicate. The downregulation of *ANF* in

the RV and its restriction to the auricles of the atria show that *ANF* is not by definition marking all chamber myocardium in a prolonged manner.

Expression patterns of several regionalized genes are not restricted to morphologically identifiable chambers (e.g., cardiac transcription factor genes such as *Irx4* and *Tbx5* and several sarcomere genes). Thus, we can anticipate that the final A/P and D/V regions in which cardiac chambers are specified evolve progressively from broader patterns. Understanding which signals interact and how they interact to form cardiac chambers is a major question in cardiac biology.

Implications of the Ballooning Model of Chamber Formation

The inner curvature is a very crucial structure within the developing heart. Forming the continuity between IFT, AVC, and OFT, it is essential in the process of septation of the systemic and pulmonary circulations (Fig. 7). It has been shown that the myocardium of the inner curvature upstream of the interventricular foramen will be incorporated into the right atrium, whereas that downstream of the interventricular foramen will contribute to the outlet of the left ventricle (Wessels *et al.*, 1992, 1996; Lamers *et al.*, 1992). According to the segmental concept, this myocardium would display the left and right ventricular phenotype, respectively (Fig. 7E), meaning that a "phenotype switch" from ventricular to atrial, or from ventricular to OFT, would be required. We now show that the myocardium of the inner curvature is not (yet) differentiated into ventricular chamber myocardium prior to septation, thereby offering a solution to this longstanding issue.

The postulation of chamber myocardium formation as an event occurring from within the linear heart tube in response to patterning information provides a possible explanation for the phenotype of the *Mef2C* and *dHand/Hand2* mutant mice. In addition to other phenotypic characteristics, these mice were reported to fail in formation of the right ventricular segment (Lin *et al.*, 1997; Srivastava *et al.*, 1997). However, the entire tubular heart is formed, including the part which is designated the right ventricle in the segmental model. Therefore, we hypothesize that these factors are not essential for the formation and specification of regions of the linear hear, but are essential for the proliferation, survival, or differentiation of chamber myocardium at the position of the prospective right ventricle. In *Nkx2-5* mutant mice, a linear heart tube is formed (Lyons *et al.*, 1995) but looping is perturbed and a set of genes which are expressed or upregulated in the ballooning chamber myocardium (e.g., *ANF, Chisel, Hand1, MLC2v*) is specifically downregulated in the mutant background (Lyons *et al.*, 1995; Tanaka *et al.*, 1999). The mutant phenotype indicates that a primary myocardium can form, but the ability to generate chamber myocardium is lost, and therefore the hearts are blocked at the primary heart tube stage (Harvey, 1999).

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REFERENCES

Anderson, R. H., Wilkinson, J. L., and Becker, A. E. (1978). The bulbus cordis—A misunderstood region of the developing human heart: Its significance to the classification of congenital cardiac malformations. *In* "Birth Defects: Original Article Series," Vol. 14, No. 7, pp. 1–28.

Bao, Z. Z., Bruneau, B. G., Seidman, J. G., Seidman, C. E., and Cepko, C. L. (1999). Regulation of chamber-specific gene expression in the developing heart by *Irx4*. *Science* **283,** 1161–1164.

Bers, D. M. (1991). "Excitation–Contraction Coupling and Cardiac Contractile Force." Kluwer Academic, Dordrecht.

Biben, C., and Harvey, R. P. (1997). Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene *eHand* during murine heart development. *Genes Dev.* **11,** 1357–1369.

Boheler, K. R., Chassagne, C., Martin, X., Wisnewsky, C., and Schwartz, K. (1992). Cardiac expressions of α- and β-myosin heavy chains and sarcomeric α-actins are regulated through transcriptional mechanisms. *J. Biol. Chem.* **267,** 12979–12985.

Bruneau, B. G., Bao, Z. Z., Tanaka, M., Schott, J.-J., Izumo, S., Cepko, C. L., Seidman, J. G., and Seidman, C. E. (2000). Cardiac expression of the ventricle-specific homeobox gene Irx4 is modulated by Nkx2-5 and dHand. *Dev. Biol.* **217,** 266–277.

Bruneau, B. G., Logan, M., Davis, N., Levi, T., Tabin, C. J., Seidman, J. G., and Seidman, C. E. (1999). Chamber-specific cardiac expression of *Tbx5* and heart defects in Holt–Oram syndrome. *Dev. Biol* **211,** 100–108.

Chapman, D. L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S. I., Gibson-Brown, J. J., Cebra-Thomas, J., Bollag, R. J., Silver, L. M., and Papaioannou, V. E. (1996). Expression of the T-box family genes, *Tbx1–Tbx5*, during early mouse development. *Dev. Dyn.* **206,** 379–390.

de Jong, F., Opthof, T., Wilde, A. A. M., Janse, M. J., Charles, R., Lamers, W. H., and Moorman, A. F. M. (1992). Persisting zones of slow impulse conduction in developing chicken hearts. *Circ. Res.* **71,** 240–250.

de Jong, F., Virágh, S., and Moorman, A. F. M. (1997). Cardiac development: A morphologically integrated molecular approach. *Cardiol. Young* **7,** 131–146.

De la Cruz, M. V., and Sanchez-Gomez, C. (1999). Straight tube heart. Primitive cardiac cavities vs. primitive cardiac segments. *In* "Living Morphogenesis of the Heart" (M. De la Cruz and R. R. Markwald, Eds.), Chap. 3, pp. 85–98. Birkhäuser, Boston.

De la Cruz, M. V., Sánchez-Gómez, C., and Palomino, M. (1989). The primitive cardiac regions in the straight tube heart (stage 9) and their anatomical expression in the mature heart: An experimental study in the chick embryo. *J. Anat.* **165,** 121–131.

Fabiato, A., and Fabiato, F. (1979). Calcium and cardiac excitation–contraction coupling. *Annu. Rev. Physiol.* **41,** 473–484.

Franco, D., Lamers, W. H., and Moorman, A. F. M. (1998). Patterns of gene expression in the developing myocardium: Towards a

morphologically integrated transcriptional model. *Cardiovasc. Res.* **38,** 25–53.

Franco, D., Markman, M. W. M., Wagenaar, G. T. M., Ya, J., Lamers, W. H., and Moorman, A. F. M. (1999). Myosin light chain 2A and 2V identifies the embryonic outflow tract myocardium in the developing rodent heart. *Anat. Rec.* **254,** 135–146.

Garcia-Martinez, V., and Schoenwold, G. C. (1993). Primitive streak origin of the cardiovascular system in avian embryos. *Dev. Dyn.* **159,** 706–719.

Goss, C. M. (1938). The first contractions of the heart in rat embryos. *Anat. Rec.* **70,** 505–524.

Habets, P. E. M. H., Franco, D., Ruijter, J. M., Sargeant, A. J., Sant'Ana Pereira, J. A. A., and Moorman, A. F. M. (1999). RNA content differs in slow and fast muscle fibres: Implications for the interpretations of changes in muscle gene expression. *J. Histochem. Cytochem.* **47,** 995–1004.

Harvey, R. P. (1999). Seeking a regulatory roadmap for heart morphogenesis. *Semin. Cell Dev. Biol.* **10,** 99–107.

Kamino, K., Hirota, A., and Fujii, S. (1981). Localization of pacemaking activity in early embryonic heart monitored using voltage-sensitive dye. *Nature* **290,** 595–597.

Lamers, W. H., Wessels, A., Verbeek, F. J., Moorman, A. F. M., Virágh, S., Wenink, A. C. G., Gittenberger-de Groot, A. C., and Anderson, R. H. (1992). New findings concerning ventricular septation in the human heart—Their implications for maldevelopment. *Circulation* **86,** 1194–1205.

Lin, Q., Schwarz, J., Bucana, C., and Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276,** 1404–1407.

Lyons, G. E. (1994). In situ analysis of the cardiac muscle gene program during embryogenesis. *Trends Cardiovasc. Med.* **4,** 70–77.

Lyons, G. E., Schiaffino, S., Sassoon, D., Barton, P., and Buckingham, M. E. (1990). Developmental regulation of myosin expression in mouse cardiac muscle. *J. Cell Biol.* **111,** 2427–2437.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L., and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev.* **9,** 1654–1666.

Manasek, F. J. (1968). Embryonic development of the heart. I. A light and electron microscopic study of myocardial development in the early chick embryo. *J. Morphol.* **125,** 329–366.

Manasek, F. J., Burnside, M. B., and Waterman, R. E. (1972). Myocardial cell shape change as a mechanism of embryonic heart looping. *Dev. Biol.* **29,** 349–371.

Mjaatvedt, C. H., Yamamura, H., Wessels, A., Ramdell, A., Turner, D., and Markwald, R. R. (1999). Mechanisms of segmentation, septation, and remodeling of the tubular heart: Endocrinal cushion fate and cardiac looping. *In* "Heart Development" (R. P. Harvey and N. Rosenthal, Eds.), pp. 195–208. Academic Press, San Diego.

Molkentin, J. D., Lin, Q., Duncan, S. A., and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11,** 1061–1072.

Moorman, A. F. M., and Lamers, W. H. (1994). Molecular anatomy of the developing heart. *Trends Cardiovasc. Med.* **4,** 257–264.

Moorman, A. F. M., Vermeulen, J. L. M., Koban, M. U., Schwartz, K., Lamers, W. H., and Boheler, K. R. (1995). Patterns of expression of sarcoplasmic reticulum Ca^{2+}ATPase and phospholamban mRNAs during rat heart development. *Circ. Res.* **76,** 616–625.

Moorman, A. F. M., de Jong, F., Denyn, M. M. F. J., and Lamers, W. H. (1998). Development of the cardiac conduction system. *Circ. Res.* **82,** 629–644.

Moorman, A. F. M., de Boer, P. A. J., Ruijter, J. M., Hagoort, J., Franco, D., and Lamers, W. H. (1999). Radio-isotopic in situ hybridization on tissue sections: Practical aspects and quantification. *In* "Methods in Molecular Biology," Vol 137, "Developmental Biology Protocols" (R. S. Tuan and C. W. Lo, Eds.), Vol. III, Chap. 11, pp. 97–115. Humana Press, Totowa, NJ.

Moorman, A. F. M., Schumacher, C. A., de Boer, P. A. J., Hagoort, J., Bezstarosti, K., van den Hoff, M. J. B., Wagenaar, G. T. M., Lamers, J. M. J., Wuytack, F., Christoffels, V. M., and Fiolet, J. W. T. (2000). Presence of functional sarcoplasmic reticulum in the developing heart and its confinement to chamber myocardium. *Dev. Biol.* **223,** 279–290.

Nakagawa, M., Richardson, J. A., Olson, E. N., and Srivastava, D. (1999). HRT1, HRT2, and HRT3: A new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. *Dev. Biol.* **216,** 72–84.

Netter, F. H. (1969). Embryology. *In* "The CIBA Collection of Medical Illustrations," Vol. 5, "The Heart" (F. F. Yonkman, Ed.), pp. 112–126. Colorpress, New York.

O'Brien, T. X., Lee, K. J., and Chien, K. R. (1993). Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube. *Proc. Natl. Acad. Sci. USA* **90,** 5157–5161.

O'Rahilly, R., and Müller, F. (1987). "Developmental Stages in Human Embryos." Carnegie Inst., Washington.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75,** 1401–1416.

Ross, R. S., Navankasattusas, S., Harvey, R. P., and Chien, K. R. (1996). An HF-1a/HF-1b/MEF-2 combinatorial element confers cardiac ventricular specificity and establishes an anterior–posterior gradient of expression. *Development* **122,** 1799–1809.

Rumyantsev, P. P. (1977). Interrelations of the proliferation and differentiation processes during cardiac myogenesis and regeneration. *Int. Rev. Cytol.* **51,** 187–273.

Satin, J., Fujii, S., and de Haan, R. L. (1988). Development of cardiac heartbeat in early chick embryos is regulated by regional cues. *Dev. Biol.* **129,** 103–113.

Srivastava, D., Thomas, T., Lin, Q., Kirby, M. L., Brown, D., and Olson, E. N. (1997). Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. *Nat. Genet.* **16,** 154–160.

Stalsberg, H. (1969). The origin of heart asymmetry: Right and left contributions to the early chick embryo heart. *Dev. Biol.* **19,** 109–127.

Steding, G., and Seidl, W. (1990). Cardio-vaskulares System. *In* "Humanembryologie: Lehrbuch und Atlas der Vorgeburtlichen Entwickelung des Menschen" (K. V. Hinrichsen, Ed.), pp. 205–294. Springer-Verlag, Berlin.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N., and Izumo, S. (1999). The cardiac homeobox gene *Csx/Nkx2.5* lies genetically upstream of multiple genes essential for heart development. *Development* **126,** 1269–1280.

Thomas, T., Yamagishi, H., Overbeek, P. A., Olson, E. N., and Srivastava, D. (1998). The bHLH factors, dHAND and eHAND, specify pulmonary and systemic cardiac ventricles independent of left–right sidedness. *Dev. Biol* **196,** 228–236.

Thompson, R. P., Lindroth, J. R., and Wong, Y. M. M. (1990). Regional differences in DNA-synthetic activity in the presepta-

tion myocardium of the chick. *In* "Developmental Cardiology: Morphogenesis and Function" (E. B. Clark and A. Takao, Eds.), pp. 219–234. Futura, Mount Kisco, NY.

van Kempen, M. J. A., Vermeulen, J. L. M., Moorman, A. F. M., Gros, D. B., Paul, D. L., and Lamers, W. H. (1996). Developmental changes of connexin40 and connexin43 mRNA-distribution patterns in the rat heart. *Cardiovasc. Res.* **32,** 886–900.

van Mierop, L. H. S. (1967). Localization of pacemaker in chick embryo heart at the time of initiation of heartbeat. *Am. J. Physiol.* **212,** 407–415.

van Mierop, L. H. S. (1979). Morphological development of the heart. *In* "Handbook of Physiology: The Cardiovascular System." Vol. I, "The Heart" (R. M. Berne, Ed.), pp. 1–28. Am. Physiol. Soc., Bethesda, MD.

Wessels, A., Markman, M. W. M., Vermeulen, J. L. M., Anderson, R. H., Moorman, A. F. M., and Lamers, W. H. (1996). The development of the atrioventricular junction in the human heart. *Circ. Res.* **78,** 110–117.

Wessels, A., Vermeulen, J. L. M., Verbeek, F. J., Virágh, S., Kálmán, F., Lamers, W. H., and Moorman, A. F. M. (1992). Spatial distribution of "tissue-specific" antigens in the developing human heart and skeletal muscle. III. An immunohistochemical analysis of the distribution of the neural tissue antigen G1N2 in the embryonic heart: Implications for the development of the atrioventricular conduction system. *Anat. Rec.* **232,** 97–111.

Xavier-Neto, J., Neville, C. M., Shapiro, M. D., Houghton, L., Wang, G. F., Nikovits, W., Stockdale, F. E., and Rosenthal, N. (1999). A retinoic acid-inducible transgenic marker of sino-atrial development in the mouse heart. *Development* **126,** 2677–2687.

Yutzey, K. E., Gannon, M., and Bader, D. (1995). Diversification of cardiomyogenic cell lineages *in vitro*. *Dev. Biol.* **170,** 531–541.

Yutzey, K. E., Rhee, J. T., and Bader, D. (1994). Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart. *Development* **120,** 871–883.

Zeller, R., Bloch, K. D., Williams, B. S., Arceci, R. J., and Seidman, C. E. (1987). Localized expression of the atrial natriuretic factor gene during cardiac embryogenesis. *Genes Dev.* **1,** 693–698.

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